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The Chemokine SDF-1/CXCL12 Contributes to the 'Homing' of Umbilical Cord Blood Cells to a Hypoxic-Ischemic Lesion in the Rat Brain

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Previous studies have shown that transplanted human umbilical cord blood (hUCB)-derived mononuclear cells exert therapeutic effects in various animal models of CNS impairments, including those of perinatal hypoxic-ischemic brain injury. However, the mechanisms of how transplanted cells exert their beneficial effects on the damaged tissue are still unclear. As detection of hUCB cells at the lesion site coincides with the therapeutic effects observed in our model, we investigated the role of the chemokine stromal derived factor (SDF)-1 (CXCL12) as a possible candidate for chemotaxis-mediated 'homing' of transplanted hUCB cells to a hypoxic-ischemic lesion in the perinatal rat brain. Following the hypoxic-ischemic insult expression of SDF-1 significantly increased in lesioned brain hemispheres and was mainly associated with astrocytes. Transplanted hUCB cells expressing the SDF-1 receptor CXCR4 migrated to the lesion site within one day. Inhibition of SDF-1 by application of neutralizing antibodies in vivo resulted in a significantly reduced number of hUCB cells at the lesioned area. The increase in glial SDF-1 expression shortly after induction of the lesion and hUCB cells expressing the corresponding receptor makes SDF-1 a potential chemotactic factor for hUCB cell migration. The reduction of hUCB cells present at the lesion site upon functional inhibition of SDF-1 strengthens the view that the SDF-1/CXCR4 axis is of major importance for cell 'homing'.

Key words: cell transplantation; chemotaxis; hypoxic-ischemic brain injury; migration; umbilical cord blood

The therapeutic potential of human umbilical cord blood (hUCB) cell transplantation in diseases of the nervous system has been demonstrated in many experimental animal models, including those of stroke (Chen et al., 2001), perinatal hypoxic-ischemic brain injury (Meier et al., 2006; Pimentel-Coelho et al., 2009), amyotrophic lateral sclerosis (Garbuzova-Davis et al., 2003), and spinal cord injury (Saporta et al., 2003). 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 (Chen et al., 2001; Meier et al., 2006; Yasuhara et al., 2009).

The mechanisms how transplanted hUCB cells fulfill their beneficial effects on the damaged tissue are still unknown. As cell replacement seems to be a rare event, research now focuses on possible indirect mechanisms following hUCB cell transplantation. Whether this indirect mode of action depends on the presence of cells at the lesion site is still under discussion. Some studies report that despite the observation of therapeutic effects none or only few hUCB cells were present in the lesioned brain (Borlongan et al., 2004; Yasuhara et al., 2009), other data demonstrate the existence of transplanted cells at the lesion site (Chen et al., 2001). The correlation of hUCB cells present in the lesioned brain and the beneficial effects observed is strengthened by studies pointing to the relevance of spatial proximity for neuroprotective effects of hUCB cells in vitro (Hau et al., 2008; Reich et al., 2008). As we have previously detected hUCB cells at the lesion site (Meier et al., 2006), we now focus on the mechanisms responsible for the cell migration in our model. This data will contribute to the utilization of hUCB cells in therapies of perinatal hypoxic-ischemic brain lesions.

The precise 'homing' from the application site to the areas of pathology requires active navigation over

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long distances, possibly mediated by generation of strong signals at the lesion.

However, it is still unclear, which factors contribute to the interaction between lesion site and transplanted cells. Potential cues that would be able to induce directed migration include components of the extracellular matrix or adhesion molecules on the one hand and chemotactic factors on the other.

One candidate of chemotactic factors is the CXC chemokine stromal derived factor (SDF)-1 (CXCL12), which was previously shown to be a potent chemoattractant for lymphocytes (Bleul et al., 1996), monocytes (Bleul et al., 1996), endothelial cells (Gupta et al., 1998), hematopoietic precursor cells (Aiuti et al., 1997), tumor cells (Muller et al., 2001), and $CD34^+$ hematopoietic stem cells from cord blood (Christopherson et al., 2002; Ohno et al., 2008). SDF-1 has also been suggested to provide the chemotactic signal for 'homing' of transplanted cells in other systems, including models of myocardial infarction (Zhang et al., 2007), multiple sclerosis (Krumbholz et al., 2006), and glioma (Tabatabai et al., 2005).

We investigated the role of SDF-1 in mediating the directed migration of transplanted hUCB cells after hypoxic-ischemic brain lesion. In this model, we have previously shown that transplanted hUCB cells migrate from the transplantation site, i.e. the intraperitoneal cavity, to lesioned brain areas within three days (Meier et al., 2006). Thus, if SDF-1 was involved in this process of cell 'homing', its expression should be detectable at the lesion site.

We therefore analyzed the expression of SDF-1 in the brain after induction of a hypoxic-ischemic damage at two different time points. Two and fourteen days after lesion, SDF-1 expression occurred specifically at the lesion site, with astrocytes being the cellular source of the SDF-1 protein. HUCB cells, which were shown to express the CXC chemokine receptor (CXCR) 4, mainly assembled in SDF-1 enriched regions. The functional relationship between SDF-1 expression and hUCB cell migration was demonstrated by application of SDF-1 inhibitory antibodies, which diminished 'homing' of hUCB cells, thus, providing evidence for the functional importance of the SDF-1/CXCR4 axis in migration of hUCB cells in vivo.

MATERIALS AND METHODS

Preparation of hUCB-Derived Mononuclear Cells for Transplantation

Blood from umbilical cord and placenta was obtained from the Depts. of Gynecology and Obstetrics (St. Elisabeth-Hospital and Ruhr-University, Bochum, Germany), after receiving the mother's 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 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.

TABLE I. Timeline of Experimental Procedures (Age of Animals)

Day 0 (P7)	Seven-day old rats exposed to hypoxic-ischemic brain injury
Day 1 (P8)	Transplantation of hUCB cells, vehicle, neutralizing SDF-1 antibodies, IgG antibodies or combinations
Day 2 (P9)	Morphological and immunohistochemical analysis
Day 14 (P21)	Morphological and immunohistochemical analysis

Animals and Hypoxic-Ischemic Injury Surgical Procedure

All surgical and experimental protocols were approved by the appropriate institutional review committee (Bezirksregierung Arnsberg, Germany) and met the guidelines of the German animal protection law. A timeline of the surgical procedure and treatment of animals is shown in Table I.

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 4% isoflurane and maintained with 1.5% isoflurane in 50% N_2O / 50% O_2. 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.

Transplantation of hUCB-derived mononuclear cells (1×10^7 / 500 µl 0.9% NaCl), vehicle (NaCl) or antibodies was performed by intraperitoneal injection 24 hr after the insult as established in previous studies (Meier et al., 2006) without any further use of anesthesia or immunosuppressant. At P9, and 21, rats were anesthetized and decapitated. Brains were dissected and brain injury was assessed immediately (Bona et al., 1997). All further animal testing and quantification were performed blinded.

Neutralization of SDF-1 In Vivo

Neutralizing antibodies to SDF-1 (R&D Systems, Wiesbaden, Germany) or control antibodies (Immunoglobulin (Ig) G; Millipore, Schwalbach, Germany) were applied at a concentration of 30 µg per animal by intraperitoneal injection simultaneously with the injection of hUCB-derived mononuclear cells 24 hr after hypoxic-ischemic lesion. The experiment was terminated 24 hr later (at P9), and brains were cryopreserved for subsequent analysis.

Immunohistochemistry and Histological Analysis

Brains were covered in tissue freezing medium (Leica, Nussloch, Germany) and cryopreserved. Histology and immunohistochemistry were performed on cryosections of 12 µm thickness that had been mounted onto Superfrost Plus slides

(Menzel, Braunschweig, Germany) and dried for 30 min at 40°C prior to fixation. 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 before (Meier et al., 2006). Briefly, brain cryosections were fixed in -20°C cold ethanol, rinsed in PBS and pre-incubated in blocking buffer (BB; 10% normal goat serum and 0.1% TritonX-100 in PBS) for 2 hr. Primary antibodies were diluted in BB and sections were incubated at 18°C overnight. Samples were rinsed in PBS, and incubated in 0.2% BSA in PBS (PBSA). Incubation with secondary antibodies was performed in PBSA at 18°C for two hr. Sections were mounted using the ProLong Antifade Kit (Invitrogen, Karlsruhe, Germany). Fluorescence was documented using conventional fluorescence microscopy (Zeiss 200M inverted microscope including the Apotome device). Data were exported as TIFF files into Adobe Photoshop CS2 (Adobe Imaging Systems Inc., USA). Nuclear labeling was performed by applying the nuclear dye Hoechst 33258 (Invitrogen, Karlsruhe, Germany) at a concentration of 2 μg/ml for one min. For all immunohistochemical analysis controls omitting the primary antibody were performed.

Primary antibodies were used as described before (Meier et al., 2006; Neuhoff et al., 2007). In addition CD45 (1:100; BD Biosciences, Heidelberg, Germany), CD68 (ED1; 1:80; AbD Serotec, Düsseldorf, Germany), glial fibrillary acidic protein (GFAP; monoclonal: 1:400; polyclonal: 1:1000), CXCR4 (1:100) (all Millipore, Schwalbach, Germany), zonula occludens protein-1 (ZO-1; 1:200; Zytomed, Berlin, Germany), and pericytic aminopeptidase N (PapN; 1:1) (Krause et al., 1991) were used. To detect SDF-1, rabbit anti-mouse antibodies (1:50; Acris, Hiddenhausen, Germany) were employed, which, according to the manufacturer, recognize mouse and rat SDF-1, and, as established by immunoblot analysis of the recombinant protein, also human SDF-1 (data not shown). Antibodies are expected to recognize both splice variants of SDF-1, i.e. SDF-1α and SDF-1β (Shirozu et al., 1995). Secondary antibodies were Alexa Fluor 488 or 546 conjugated goat-anti-rabbit or goat-anti-mouse (1:3000; Invitrogen, Karlsruhe, Germany).

Quantification of SDF-1 Expression

Randomly selected animals were assigned to three different experimental groups and investigated at two different time points: sham animals (no lesion; P9: n = 3; P21: n = 3), lesion without transplantation (lesion; P9: n = 6; P21: n = 5), and lesion followed by intraperitoneal transplantation of 1×10^7 mononuclear cells (lesion + hUCB; P9: n = 5; P21: n = 5). At P9, the lesion group consisted of three animals that had received an intraperitoneal sham injection of 500 μl 0.9% NaCl, and three animals without injection. As analysis did not reveal any obvious microscopic or macroscopic differences, these two groups were subsequently combined. Quantification of SDF-1 immunoreactivity was performed on cryosections at a level corresponding to Bregma -2.9 ± 0.5 in the adult brain by determining expression in eight (P9) or eleven (P21) brain regions per hemisphere. Photographs of immunohistochemical staining of these regions were adjusted using the threshold mode in the imageJ program (NIH, http://rsb.info.nih.gov/ij/), i.e. any fluorescent signal, irrespective of its intensity, was assigned black color, and any area devoid of immunostaining was attributed white. The area covered by the fluorescence signal (black) was expressed as percent background (white). For statistical analysis of SDF-1 expression, the mean of all regions investigated per hemisphere was compared.

Quantification of Human Leukocyte Antigen (HLA)-DR Positive Cells

For quantification of transplanted hUCB cells immunohistochemistry for the HLA-DR antigen was carried out as described elsewhere (Meier et al., 2006). For quantification of hUCB cells present at the lesion in correlation to inhibition of SDF-1, analysis was performed on animals that had sustained hypoxic-ischemic lesion plus transplantation of hUCB cells, and were subsequently randomly assigned to three different experimental groups: no further treatment (lesion + hUCB; n = 5), application of neutralizing SDF-1-antibodies (n = 4), or application of IgGs (n = 4). At P9, the number of HLA-DR positive cells was counted in eleven selected areas per brain section at a level corresponding to Bregma -2.9 ± 0.5 in the adult brain. The mean value of the eleven regions was used for statistical analysis.

Reverse Transcriptase Polymerase Chain Reaction for Detecting Human DNA

For detection of hUCB cells in the brain, reverse transcriptase polymerase chain reaction was carried out. RNA was extracted from a 400 μm thick brain cryosection, containing part of the lesioned brain (lesion + hUCB, n = 3). RNA extracted from human umbilical cord blood was used as positive control (n = 3). To rule out false positive results, RNA extracted from brains of rats that did not receive any human umbilical cord blood (n = 3) served as negative control. The RNA was reversed transcribed, followed by cDNA synthesis. Primers specific for human growth hormone 1 (GH1) (Ende et al., 2000) were as follows: 5′- tgccttcccaaccattccctta - 3′ and 5′- ctctaggttggatttctgttgtgtttc - 3′. Cycling was performed using the following protocol: 2 min at 95°C were followed by 45 cycles of 15 sec at 95°C, 15 sec at 45°C, and 15 sec at 72°C.

PCR products were run on agarose gels (1.0%) (Biozym, Oldendorf, Germany) on a horizontal electrophoresis apparatus and visualized with ethidium bromide.

Fluorescence-Activated Cell Analysis

Cell analysis was performed on six samples of human cord blood, investigating expression of cell surface marker proteins before and after isolation of the mononuclear fraction using a FACS Calibur System (BD Biosciences, Heidelberg, Germany). Labeling was performed as described before (Neuhoff et al., 2007) using a phycoerythrin (PE)-conjugated mouse anti-human CD184 (CXCR4) monoclonal antibody and phycoerythrin-conjugated Immunoglobulin (Ig) G2a as its isotype control.

Cytospin Preparation

Cytospins (12.4 mm diameter) were prepared from 2×10^6 hUCB-derived mononuclear cells centrifuged at 2000 rpm (ROTOFIX 32, Hettichlab, Tuttlingen, Germany) for 10 min. The cytospins were heat dried at 40°C prior to fixation. For CXCR4-immunohistochemistry, cytospins were fixed in 100% ethanol at -20°C for 10 min, rinsed in PBS, and, to block non-specific binding sites pre-incubated in blocking buffer (20% normal porcine serum, 0.1% TritonX-100 in PBS) for 2 hr. Subsequent steps for immunohistochemistry are described above.

Statistical Analysis

Initial analysis of variance (ANOVA) was followed by t-test or Tukey HSD post hoc test, using the SPSS 13 statistic program. A probability of error less than 0.05 ($P < 0.05$) was considered statistically significant. In diagrams, $P < 0.05$ is indicated by ⋆, and $P < 0.001$ by ⋆⋆⋆. All data are expressed as mean ± standard error of the mean.

RESULTS

SDF-1 is Present at Hypoxic-Ischemic Lesion Sites

To identify hypoxic-ischemic lesions in the hemisphere ipsilateral to the carotid artery occlusion, brains were inspected macroscopically. At postnatal day (P) 9, i.e. two days after the insult, lesioned hemispheres displayed a characteristic white opaque area with few blood vessels. At P21, i.e. fourteen days after the insult, lesioned hemispheres revealed a cystic infarction on the brain surface. In addition, immunohistochemistry with markers of apoptosis (cleaved-caspase-3) and inflammation (CD68) was employed on brain cryosections to locate the lesioned area, which is immunopositive for both marker proteins (Meier et al., 2006) (data not shown).

To evaluate the expression pattern of endogenous rat SDF-1 in control and lesioned brains, immunohistochemical analysis was carried out and quantified. At P9, minimal SDF-1 immunoreactivity was observed in sham-operated animals (Fig. 1A). In contrast, lesioned hemispheres expressed high amounts of SDF-1 specifically located at the lesion site (Fig. 1B). In detail, SDF-1 was expressed in regions of the hippocampus, corpus callosum, and periventricular areas of lesioned hemispheres. To quantify SDF-1 expression at P9, we determined the immunoreaction of eight regions within and around the lesioned area (Fig. 1D). Statistical evaluation of the mean intensity of these regions exhibited a significant increase of SDF-1 in lesioned brain hemispheres as compared to the corresponding hemispheres of sham-treated animals ($P < 0.001$; Fig. 1E).

Distribution and intensity of SDF-1 immunoreactivity was further investigated fourteen days after the insult (P21). At this time point, the lateral ventricle was enlarged due to the hypoxic-ischemic lesion, resulting in destruction of most of the morphology of the lesioned hemisphere. SDF-1 protein was expressed around the lesion, i.e. in remaining parts of the hippocampus, corpus callosum, basal ganglia, and cortex. Quantification of SDF-1 in eleven regions around the lesion (Fig. 1F) also revealed a significant higher expression in lesioned hemispheres compared to non-lesioned brains ($P < 0.001$; Fig. 1G).

At P9 and P21, right hemispheres of all groups showed minimal SDF-1 immunoreactivity, which was considered the basal level of SDF-1 protein expression. In general, quantification of SDF-1 expression in right hemispheres did not show any significant differences between experimental groups, except that at P21 expression of SDF-1 was significantly higher in right hemispheres of lesioned animals that had received hUCB cells than in those of sham-treated or lesioned animals ($P < 0.05$). In support of this data, direct comparison of left and right hemispheres revealed significant differences upon lesion with and without hUCB-transplantation.

With respect to the damage, SDF-1 immunoreactivity was detected close to the lesion site, predominantly located in the area also containing CD68 immunopositive cells (Fig. 2). Interestingly, the intensity of SDF-1 immunolabeling seemed to reflect the amount of CD68 immunopositive cells. Furthermore, expression of SDF-1 protein in lesioned hemispheres at P21 appeared to be higher as compared to P9 (Fig. 2), which also correlated with an increase in CD68 immunoreactivity.

Using RNA isolated from whole brain hemispheres, we were able to detect endogenous rat SDF-1 mRNA in P9 and P21 brains using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Quantification of SDF-1 expression in lesioned versus non-lesioned hemispheres using real time PCR, however, revealed no significant differences on mRNA level (data not shown).

Astrocytes are the Cellular Source of SDF-1 Protein

To analyze the cellular origin of SDF-1 expression, double-immunostaining was performed with antibodies directed to neuronal, astrocytic, microglial and endothelial antigens. SDF-1 immunolabeling did neither co-localize with the neuronal marker proteins neuronal nuclei protein (NeuN; Fig. 3A), neurofilament (NF) 200, and β3-tubulin (data not shown) nor with the CD68 protein, which labels activated microglia and macrophages (Fig. 3B). Vascular endothelial cells, labeled by antibodies to the tight junction protein zonula occludens (ZO-1; Fig. 3C), and pericytes, visualized by pericytic aminopeptidase (PapN; Fig. 3D) immunoreactivity, showed no association with SDF-1 either. In contrast, immunostaining for the glial fibrillary acidic protein (GFAP) labeling astrocytes co-localized with SDF-1 immunoreactivity at P9 and P21 (Fig. 3E,F). Thus, reactive astrocytes at the lesion site are likely to be the main source of SDF-1 protein.

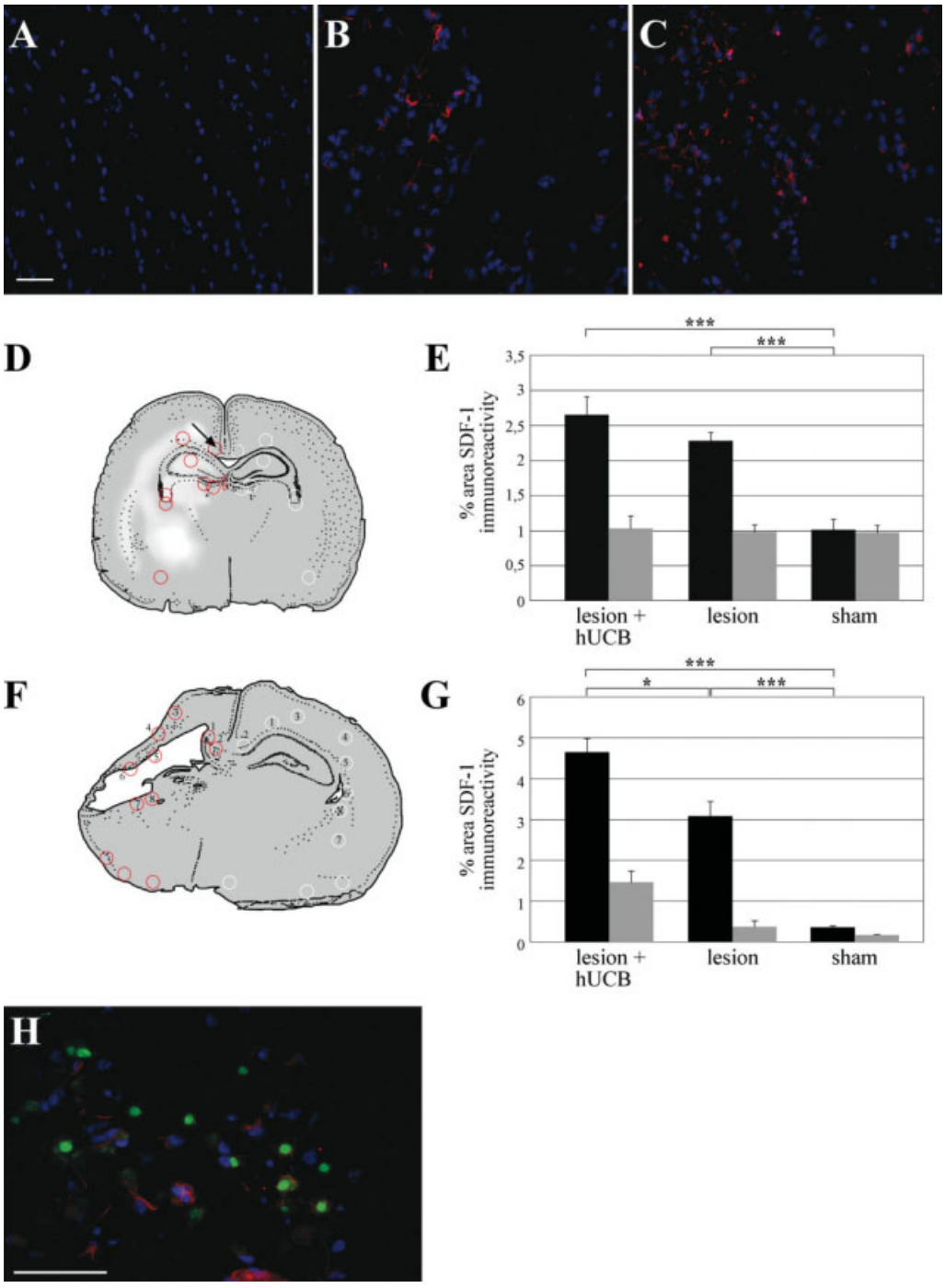

Fig. 1. Expression of SDF-1 in non-lesioned and lesioned brain hemispheres. Immunolabeling of SDF-1 (red) of a postnatal day (P) 9 rat brain without lesion (sham-operated; **A**), with hypoxic-ischemic lesion (**B**), or with hypoxic-ischemic lesion followed by transplantation of hUCB cells (**C**). Schematic representation of the regions chosen for quantification of SDF-1 expression in P9 (**D**) and P21 (**F**) rat brains with hypoxic-ischemic lesion. Arrow in D indicates the region shown in A-C. Quantification of SDF-1 immunoreactivity (sum of all regions investigated) in left (black columns) and right (grey columns) hemispheres at P9 (**E**) and P21 (**G**). Significant differences of $p < 0.05$ are indicated by ⋆, and $p < 0.001$ by ⋆⋆⋆. (**H**) Double-immunostaining of SDF-1 (red) and HLA-DR (green) in a hypoxic-ischemic P9 rat brain with transplantation of hUCB cells. Nuclei are labeled in blue (Hoechst staining, A-C, H). Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

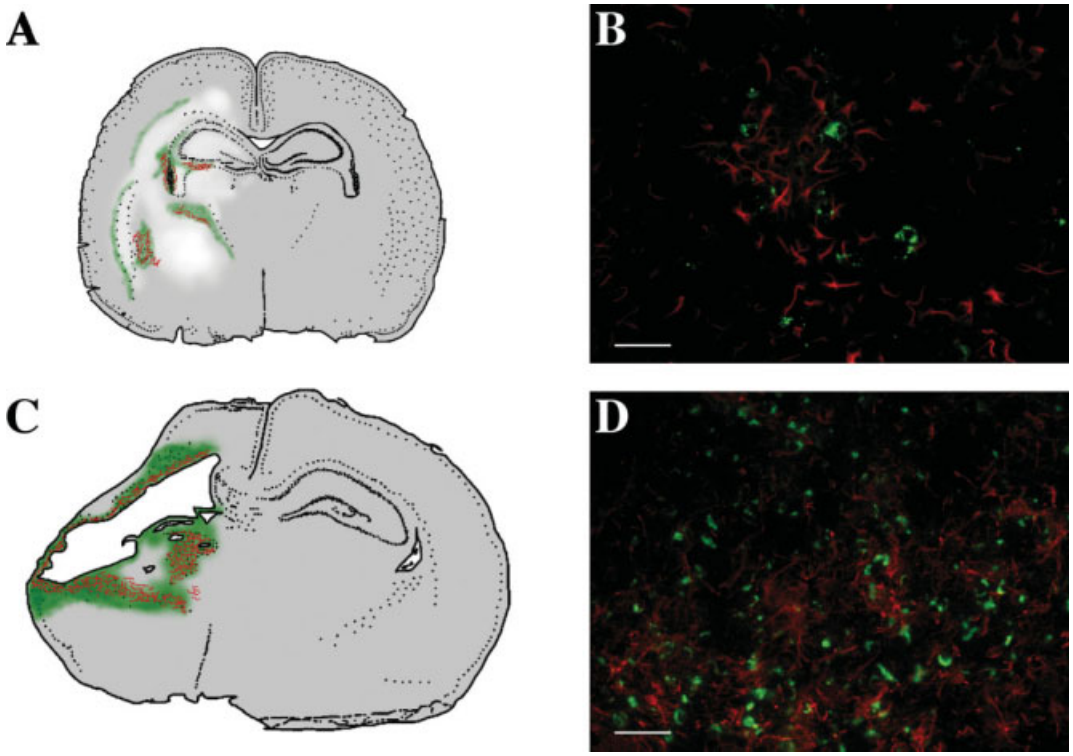

Fig. 2. Location of SDF-1 immunoreactivity with respect to the location of CD68 positive cells. Schematic representation of the distribution of SDF-1 immunoreactivity (red) and CD68 immunopositive cells (green) in hypoxic-ischemic rat brains at P9 (**A**) and P21 (**C**). Photographic documentation of co-immunolabeling for SDF-1 (red) and CD68 (green) in cryosections of P9 (**B**) and P21 (**D**) hypoxic-ischemic rat brains, revealing both proteins located in the same area. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

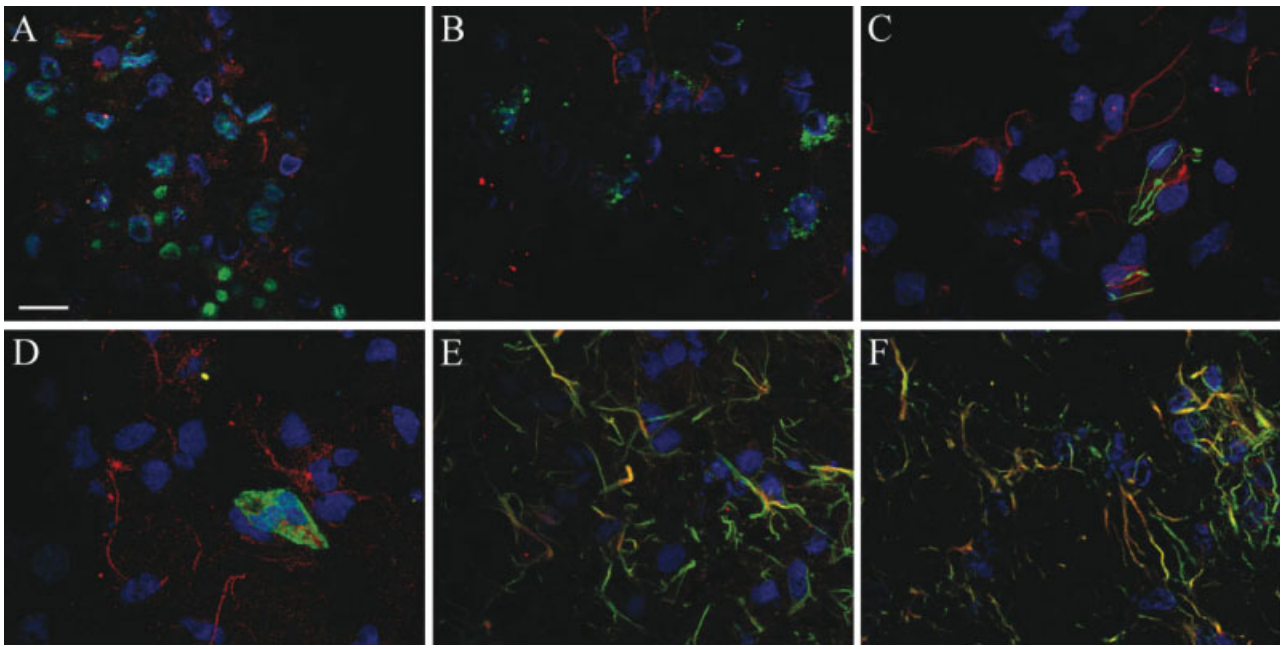

Fig. 3. SDF-1 expression is associated with reactive astrocytes. To determine the cellular source of SDF-1 (red), double staining was performed with marker proteins (all in green) of neurons (NeuN; **A**), microglia (CD68; **B**), vascular endothelial cells (ZO-1; **C**), pericytes (pAPN; **D**), and astrocytes (GFAP; P9: **E**; P21: **F**). Co-localization results in yellow color as evident for GFAP in E and F. Hoechst staining is labeling all nuclei in blue. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

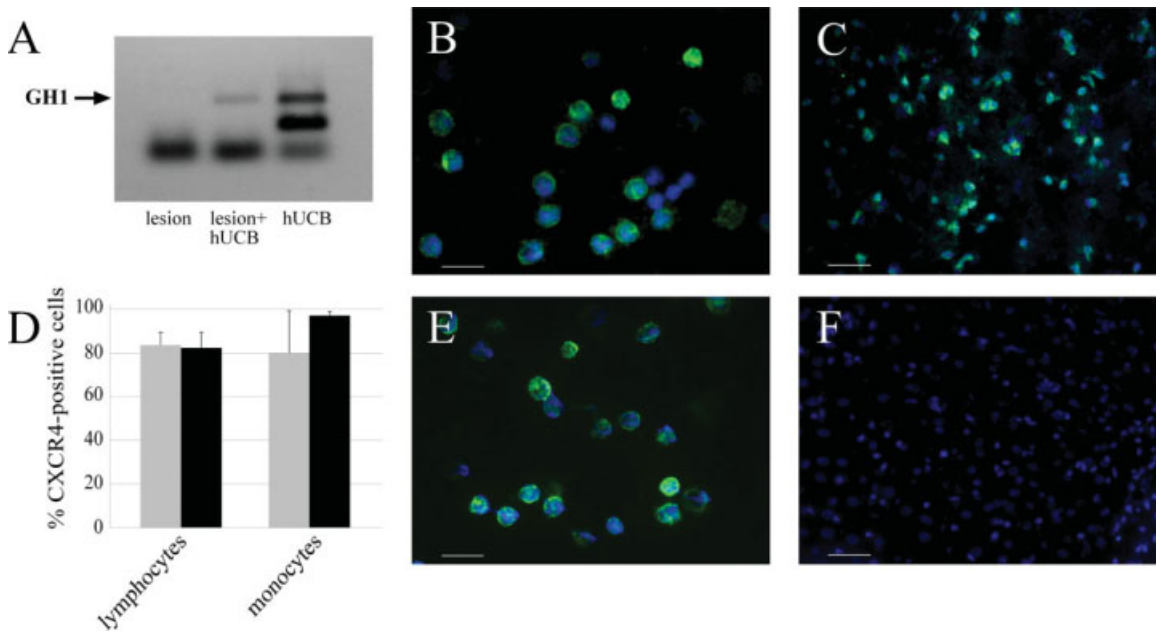

Fig. 4. Identification of hUCB cells in vitro and in vivo. For detection of hUCB cells PCR analysis was performed on P21 rat brains, using primers specific for human growth hormone 1 (GH1) (**A**). The expression of CXCR4 was investigated on human umbilical cord blood and mononuclear cells derived from cord blood. (**B**) Immunocytochemical detection of CXCR4 (green) in cytospins of hUCB-derived mononuclear cells. (**C**) Immunostaining of CXCR4 (green) in a P9 rat brain with hypoxic-ischemic lesion followed by transplantation of hUCB cells. CD45 immunolabeling of cytospins prepared of hUCB cells. (**D**) Fluorescence-activated cell analysis of CXCR4 in the lymphocyte and monocyte fraction of full blood (grey columns) and of isolated mononuclear cells (black columns). (**E**), and of a lesioned rat brain (P9) with transplanted hUCB cells (**F**). Nuclei (blue) are labeled with Hoechst staining. Scale bar in B,E = 20 μm; C,F = 50μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Transplanted hUCB Cells are Located at SDF-1 Expressing Brain Areas

Migration of hUCB cells was investigated performing immunohistochemical analysis with human leukocyte antigen (HLA)-DR antibodies and PCR analysis with primers specific for human growth hormone 1 (GH1) at P9 and P21. Induction of a hypoxic-ischemic brain lesion resulted in specific 'homing' of hUCB-derived mononuclear cells to the lesioned brain within one day, i.e. at P9 (Fig. 1H), and transplanted cells were still detectable at the lesion site fourteen days after the insult (Fig. 4A). These cells were exclusively located at lesioned areas and could not be detected in non-lesioned hemispheres (data not shown). Double-immunostaining with SDF-1 and HLA-DR antibodies revealed hUCB cells residing mainly in regions expressing SDF-1 protein (P9: Fig. 1H; P21: data not shown). However, we also detected regions at the lesion site containing SDF-1, which were devoid of hUCB cells.

Remarkably, the expression profile of SDF-1 protein in lesioned brain hemispheres with transplantation of hUCB cells showed a further increase of SDF-1 compared to lesioned hemispheres without transplantation. This increase was significant at P21 ($P < 0.05$), but not at P9 (Fig. 1C,E,G).

CXCR4, the first receptor identified for SDF-1, was detected in isolated mononuclear cells by RT-PCR (data not shown) and immunolabeling of cytospins (Fig. 4B). In addition, FACS analysis on full blood and isolated mononuclear cells revealed expression of CXCR4 on hUCB cells, with a ratio of 82.24% of lymphocytes and 96.99% of monocytes expressing CXCR4 on their surface (Fig. 4D). One day after transplantation of hUCB cells we were able to detect CXCR4 positive cells in lesioned brain hemispheres in vivo (Fig. 4C).

An additional marker protein expressed by hUCB cells in vitro was CD45 (Fig. 4E). As determined with immunocytochemical analysis (this study) and fluorescence-activated cell analysis (Neuhoff et al., 2007), about 90% of hUCB-derived mononuclear cells were positive for CD45. But in contrast to expression of CXCR4 we were not able to detect CD45 immunopositive hUCB cells at the lesion site after transplantation (Fig. 4F).

Functional Inhibition of Cell Migration by Application of SDF-1 Antibodies In Vivo

For the assignment of relative hUCB cell numbers to functional inhibition of SDF-1, HLA-DR positive cells were determined in eleven selected regions of brain sections (Fig. 5A). Intraperitoneal application of SDF-1 neutralizing antibodies, simultaneous with transplantation of hUCB cells, significantly reduced the number of HLA-DR positive cells in those regions of the lesioned

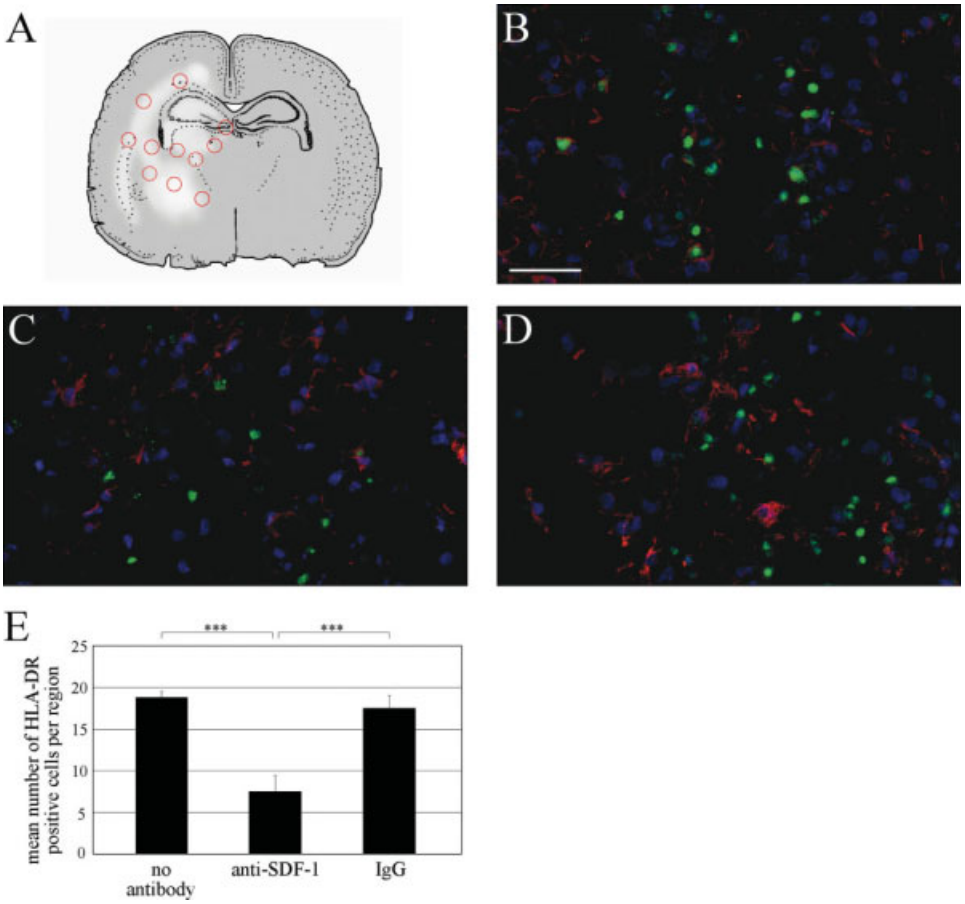

Fig. 5. Functional inhibition of hUCB cell migration by application of neutralizing antibodies to SDF-1 in vivo. For quantification of transplanted hUCB cells, immunohistochemistry for the HLA-DR (green) was carried out in eleven selected regions (**A**). For identification of the lesion site double-immunostaining with GFAP (red) was performed on P9 brains of animals that had sustained hypoxic-ischemic lesion and transplantation of hUCB cells (**B**), plus additional application of neutralizing SDF-1-antibodies (**C**), or immunoglobulins G (IgG; **D**). Hoechst staining (blue) labeled all nuclei. Scale bar = 50 μm. **E**) Quantification of the mean number of HLA-DR positive cells counted in eleven regions per section. Significant differences ($P < 0.001$) are indicated by ***. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

brain ($P < 0.001$; Fig. 5C,E). In contrast, control immunoglobulins did not affect cell migration significantly (Fig. 5D) as compared to non-treated animals (Fig. 5B).

DISCUSSION

In this study, we demonstrated that the alpha-chemokine SDF-1 (CXCL12) constitutes a functional cue in the 'homing' of transplanted hUCB-derived mononuclear cells to the site of hypoxic-ischemic brain lesion in perinatal rats. Our evidence for a putative role of SDF-1 at lesioned brain sites arose from the observation of a significantly increased SDF-1 protein expression in response to a hypoxic-ischemic brain lesion. Both time and location of SDF-1 expression matched those anticipated for chemotactic proteins. The up-regulation of SDF-1 expression was detected two and fourteen days after the insult, i.e. the earliest and latest time-point investigated in our study, and was confined to areas structurally impaired by the lesion. An increase of SDF-1 has also been detected in other models of ischemic injury (Hill et al., 2004; Miller et al., 2005), the period of increased expression of SDF-1 in those studies ranged from 10 to 30 days. Interestingly, the time of maintaining high SDF-1 expression levels in response to ischemic brain lesion seems to correlate with the age of animals at the time of injury. As extrapolated from those studies, SDF-1 expression caused by ischemic injury apparently lasts longer in adult than in neonatal animals. In addition, constitutive expression of SDF-1, which is not lesion induced, has been described in various studies and was also observed in non-lesioned hemispheres analyzed in our model. Nevertheless, the description of constitutive expression levels varies in these studies from very low (Hill et al., 2004) to high (Tham et al., 2001). In our study, basic levels were low and seemed unchanged at all ages investigated. Lesion induced expression of SDF-1 mRNA seemed to reflect local changes in cortex and hippocampus (Wang et al., 2008) and might therefore not have been detected in our RNA isolation approach using whole hemispheres. Furthermore, the cellular source of SDF-1 is also of debate. Reports include the description of neuronal and oligodendroglial (Gleichmann et al., 2000), endothelial (Stumm et al., 2002), or astroglial (Ohtani et al., 1998) SDF-1 expression. Discrepancies between these observations might be due to identification of different SDF-1 isoforms as well as to detection of mRNA versus protein. As determined by co-localization studies, we detected SDF-1 expression mainly associated with astrocytes nearby the hypoxic-ischemic brain lesion. Thus, our data point to an expression and up-regulation of SDF-1 in reactive astrocytes

following (hypoxic) injury, an effect, which was also observed in response to application of pro-inflammatory agents (Guillemin et al., 2003; Hill et al., 2004; Krumbholz et al., 2006). Induction of SDF-1 in response to brain injury may serve different functions, which might include effects on angiogenesis/vasculogenesis and therefore remodeling of the brain (Hiasa et al., 2004; Mirshahi et al., 2000), and/or chemoattraction of exogenous and endogenous cells (Bleul et al., 1996; Aiuti et al., 1997; Gupta et al., 1998; Hill et al., 2004; Tabatabai et al., 2005). As demonstrated in this model, SDF-1 contributes to the chemoattraction of an unselected population of hUCB-derived mononuclear cells, as these systemically applied hUCB cells specifically migrated to areas of SDF-1 protein expression at hypoxic-ischemic lesion site within one day and were still detectable in the lesioned hemisphere two weeks after transplantation. Interestingly, transplanted cells detected at the lesion site were devoid of CD45, although freshly prepared hUCB cells expressed this marker protein. It is feasible that, possibly due to the CNS impairment a non-hematopoietic hUCB cell population migrated to the brain lesion or that the cells underwent changes in antigen presentation during migration.

The paper gives, for the first time, evidence for hUCB cells being present for a long time in an immunocompetent host organism. Although the long-term survival of hUCB cells is remarkable, it maybe explained by the presence of mesenchymal stem cells (MSCs) in cord blood. It was shown that MSCs have immunosuppressive properties on T cell proliferation and suppress graft versus host disease (GvHD) through paracrine effects (Aggarwal and Pittenger, 2005; Sensebe and Bourin, 2009). Recently, it was published that mesenchymal stem cells from cord blood also exhibit these immunomodulatory characteristics (Yoo et al., 2009). The fact that we observed only the migration of CD45 negative hUCB cells to the brain lesion (see above) might be due to the presence of umbilical MSCs within the migrating population and therefore explain the long duration of hUCB cell viability in the lesioned brain area. Although the presence of cells at the lesion site coincides with the beneficial functional effects observed in our model (Meier et al., 2006), causality between these two events has not been demonstrated.

There are also reports that improvement of symptoms after cell transplantation occurs without a noteworthy number of cells being present in the lesioned brain area (Borlongan et al., 2004; Yasuhara et al., 2009). Nevertheless, in our model and with our detection systems human transplanted cells were identified in the rat brain. In any case, the observed functional improvement is likely to be due to indirect mechanisms for the instance the release of cytokines (Neuhoff et al., 2007), possibly resulting in neuroprotective effects of hUCB cells as described in a model of hypoxic-ischemic brain injury (Pimentel-Coelho et al., 2009). In addition, the spatial proximity of neuronal cells and hUCB cells was shown to be crucial for the exertion of neuroprotective effects in vitro (Hau et al., 2008; Reich et al., 2008).

As expression of SDF-1 might facilitate the attraction of cells to the lesion site in the first place (see above), the potential responsiveness of hUCB cells to SDF-1 was further investigated. HUCB cell were shown to express the CXCR4 protein, i.e. the signal transduction receptor for all SDF-1 isoforms. In turn, interaction of SDF-1 with this receptor is known to induce multiple intracellular signaling pathways, such as phosphatidylinositol-3 kinase and extracellular signal regulated kinase 1/2 (Ganju et al., 1998; Sotsios et al., 1999; Bajetto et al., 2001), both of which are involved in the control of cell proliferation and differentiation. Consequently, the attraction of CXCR4 carrying hUCB cells by SDF-1 might exert beneficial effects on the lesioned brain.

Remarkably, the transplantation of hUCB cells in lesioned animals yielded in a further increase of SDF-1 expression. Transplanted cells might either stimulate the expression of endogenous SDF-1, or, alternatively, express SDF-1 themselves. Data obtained in vitro support the view that hUCB cells are capable of secreting SDF-1 under certain culture conditions (Neuhoff et al., 2007). As the antibodies employed did not distinguish between SDF-1 of different species, the source of transplantation-related SDF-1 could not be determined.

In this study, however, we focused on elucidating the role of the SDF-1/CXCR4 axis in directed migration of hUCB cells to SDF-1 expressing lesion sites. The physiological relevance was demonstrated by significantly reduced numbers of HLA-DR positive hUCB cells at the lesion site in response to functional inhibition of SDF-1 in vivo. Nevertheless, about 30% of HLA-DR positive cells were still present in the lesioned hemisphere, which might either be caused by technical limitations, for instance the amount of antibody applied and the incubation time, or be due to additional signaling molecules involved in directed migration of transplanted cells. Chemoattraction of immune cells in general can be mediated by a variety of chemokines, including monocyte chemotactic protein (MCP)-1 (Carr et al., 1994; Taub et al., 1995) and macrophage inflammatory protein (MIP)-1 alpha (Schall et al., 1993; Maghazachi et al., 1994). As expression of both of these beta-chemokines is increased following ischemic brain injury (Kim et al., 1995; Che et al., 2001; Cowell et al., 2002), MCP-1 and MIP-1 alpha qualify as additional candidates for the induction of hUCB cell 'homing'. In a recently published study, MCP-1 and MIP-1 alpha were shown to be chemoattractant for hUCB cells in a stroke model in adult rats (Jiang et al., 2008).

In summary, chemoattraction of transplanted hUCB cells in response to hypoxic-ischemic injury is likely to depend, at least in part, on the presence of SDF-1 protein. The potential synergistic action of additional chemokines is feasible, and remains to be demonstrated in our lesion paradigm. Identification of factors that induce the specific migration of umbilical cord blood cells transplanted for a cell therapy is crucial to

improve the therapeutic action of these cells. Potential applications in therapy of perinatal hypoxic-ischemic brain injury may include injection of specific chemokines to boost the number of cells migrating toward a lesion site, and, thus indirectly improve and accelerate functional benefits of these cells.

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