Hematopoietic Stem Cells Reduce Postischemic Inflammation and Ameliorate Ischemic Brain Injury

Sönke Schwarting, MD; Sara Litwak, PhD; Wenlin Hao, MD; Mathias Bähr, MD; Jens Weise, MD; Harald Neumann, MD

Background and Purpose—Systemic injection of hematopoietic stem cells after ischemic cardiac or neural lesions is one approach to promote tissue repair. However, mechanisms of possible protective or reparative effects are poorly understood. In this study we analyzed the effect of lineage-negative bone marrow-derived hematopoietic stem and precursor cells (Lin−-HSCs) on ischemic brain injury in mice.

Methods—Lin−-HSCs were injected intravenously at 24 hours after onset of a 45-minute transient cerebral ischemia. Effects of Lin−-HSCs injection on infarct size, apoptotic cell death, postischemic inflammation and cytokine gene transcription were analyzed.

Results—Green fluorescent protein (GFP)-marked Lin−-HSCs were detected at 24 hours after injection in the spleen and later in ischemic brain parenchyma, expressing microglial but no neural marker proteins. Tissue injury assessment showed significantly smaller infarct volumes and less apoptotic neuronal cell death in peri-infarct areas of Lin−-HSC–treated animals. Analysis of immune cell infiltration in ischemic hemispheres revealed a reduction of invading T cells and macrophages in treated mice. Moreover, Lin−-HSC therapy counter-regulated proinflammatory cytokine and chemokine receptor gene transcription within the spleen.

Conclusions—Our data demonstrate that systemically applied Lin−-HSCs reduce cerebral postischemic inflammation, attenuate peripheral immune activation and mediate neuroprotection after ischemic stroke. (Stroke. 2008;39:2867-2875.)

Key Words: cell therapy cerebral ischemia hematopoietic stem cells neuroprotection postischemic inflammation

Within 24 hours after ischemia, resident microglia become activated and migrate to the lesion site.1–3 Furthermore, blood and bone marrow (BM)-derived monocytes are recruited to the lesion and locally adopt a microglial-like phenotype.4 Activated microglial cells and invaded macrophages facilitate removal of cellular debris during the acute phase of ischemia but also release cytotoxic molecules, including inflammatory cytokines, complement factors, as well as free radicals.5 Although cytokines released after ischemia are considered to have “proinflammatory” as well as “anti-inflammatory” effects,6 the overall balance of postischemic inflammation appears to be shifted to a proapoptotic outcome resulting in enhanced cell death, exacerbated neuronal loss, and finally, expansion of the infarct size.3 Immune cell attraction from blood circulation and from the spleen toward the ischemic lesion site is mediated by chemokines released by glial cells, which also play a crucial role in recruitment of BM-derived stem and precursor cells.7 Thus, the use of exogenous BM-derived stem and precursor cells offers an attractive therapeutic approach in experimental cerebral ischemia. Unselected BM contains at least 2 populations of multipotent stem and progenitor cells, namely hematopoietic stem cells (HSCs), which are included in the lineage-negative (Lin−-HSCs) population8,9 and marrow stromal cells, also termed mesenchymal stem cells.10 Chopp et al11–14 conducted several experiments with BM-derived stromal cells in experimental cerebral ischemia. Marrow stromal cell injection resulted in functional improvement and attenuated tissue damage after middle cerebral artery occlusion in rats.11–13 Furthermore, stromal cells were shown to migrate into the ischemic boundary zone15 accompanied by reduced neuronal apoptosis and enhanced neoangiogenesis.16,17 Human CD34+ HSCs systematically applied in experimental ischemia of immuno-compromised mice 48 hours before stroke were shown to induce long-term neovascularization in the ischemic zone,18 and human umbilical cord blood cells also acted neuroprotective in experimental cerebral ischemia.19,20

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From Cerebral Ischemia Research Group (S.S., M.B., J.W.), Department of Neurology, University of Göttingen Medical School, Göttingen, Germany; European Neuroscience Institute Göttingen (W.H., H.N.), Göttingen, Germany; Institute of Reconstructive Neurobiology (S.L., H.N.), University Bonn LIFE & BRAIN Center, University Bonn and Hertie-Foundation, Bonn, Germany; Department of Neurology (J.W.), University of Jena Medical School, Jena, Germany.
J.W. and H.N. contributed equally to this article. S.S. and S.L. contributed equally to this article.
Correspondence to Harald Neumann, E-mail hneuman1@uni-bonn.de; or Jens Weise, E-mail JENS.WEISE@med.uni-jena.de
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However, it is unclear how hematopoietic stem or precursor cells could mediate neuroprotection.

Materials and Methods

Isolation of Lin−HSCs

BM was isolated from green fluorescent protein (GFP) expressing transgenic male mice (C57BL/6-Tg ACTB-EGFP, 10sh/J; stock number 003291; JAX Laboratory, Bar Harbor, Maine) or C57BL/6 mice (6 to 8 weeks of age; Charles River, Sulzfeld, Germany; JAX Laboratory, Bar Harbor, Maine). Erythrocytes were lysed and Lin−HSCs were isolated by removing blood lineage marker-positive cells with immunomagnetic beads (Dynabeads M-450; Dynal; coupled with a sheep antirat antibody) and a cocktail of rat monoclonal antibodies against lineage markers (anti-CD45R/B220, anti-Mac-1, anti-Gr-1, anti-CD4, anti-CD8a, and anti-Ter119, all from BD Biosciences). For flow cytometry analysis (FACScalibur; BD Biosciences), total BM cells or Lin−HSCs were stained with biotin-conjugated anti-CD45, anti-Kit (BD Biosciences), or rat anti-CD11b, anti-TER119, anti-Gr-1, anti-B220, anti-CD8, anti-CD4 monoclonal antibodies, or isotype control antibodies (BD Biosciences), followed by FITC-conjugated streptavidin or FITC-conjugated goat antirat IgG secondary antibody (Dianova). Live gating was performed with propidium iodide (Sigma).

Induction of Focal Cerebral Ischemia and Intravenous Lin−HSC Injection

All experimental procedures were performed according to the EU guidelines for the care and use of laboratory animals and approved by local authorities. Adult male C57BL/6 mice (Charles River; Sulzfeld, Germany) were anesthetized, body temperature was maintained at 37°C using a feedback-controlled heating system, and cerebral blood flow was assessed by recording laser Doppler flow to ensure appropriate ischemia (<30% of initial cerebral blood flow) and reperfusion. Focal cerebral ischemia was induced by transient middle cerebral artery occlusion (45 minutes) of the middle cerebral artery using the intraluminal filament technique as described earlier. Previously, we demonstrated in the same ischemia paradigm that arterial blood pressure, heart rate, arterial blood gases, and pH stay within their physiological range throughout the whole experimental procedure. At 24 hours after onset of ischemia, mice were randomly selected to receive either Lin−HSCs (5 × 10^5) Lin−HSCs in 200 μl phosphate-buffered saline (PBS), pH 7.4) or PBS (200 μl, pH 7.4) intravenously.

Infarct Volume Analysis

Infarct volume analysis was performed in Lin−HSC–injected and PBS-injected animals at 72 hours after treatment in a blinded fashion. Cryostat sections (10 coronal levels, from 2.1 mm anterior to 3.4 mm posterior of Bregma) were stained with cresyl violet and used for planimetric determination of infarct sizes using an image analysis system (NIH Image 3.12). The area of infarction was measured by subtracting the nonlesioned area of the left (infarcted) hemisphere from the area of the right (noninfarcted) hemisphere in all sections. The infarct volume resulted from integration of sequential areas based on distances between analyzed sections according to stereotactic brain coordinates.

TUNEL Staining and Quantification of TUNEL-Positive Cells

The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) method was used to assess postischemic apoptotic cell death. Cryostat sections were fixed, incubated with terminal deoxynucleotidyltransferase enzyme and nucleotide mix, followed by TUNEL blocking reagents according to the manufacturer’s instruction (Roche Diagnostics–Applied Science). After streptavidin-labeling with Alexa Fluor 594 (1:1000; 1% bovine serum albumin, 0.3% Triton-PBS), sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). TUNEL− cells were counted in 9 0.1-mm² areas within the peri-infarct zone at 3 different coronal levels for each animal. Analysis was performed in a blinded fashion. Numbers of TUNEL− cells/mm² are given as mean±SEM for Lin−HSC–treated and PBS-treated animals.

Quantitative, Spatial, and Morphological Analysis of GFP+ Cells

Lin−HSC–treated ischemic and nonischemic control mice were perfused with paraformaldehyde (4% in PBS) at 24, 48, or 72 hours after Lin−HSC infusion. Brains and spleens were removed, fixed, and cryosections were prepared. After staining with DAPI, quantitative, spatial, and morphological analysis of GFP+ cells within the brains was performed. Numbers of GFP+ cells per hemisphere were calculated by multiplying cells per analyzed section by the intersectional distance determined using stereotactic coordinates. Spatial analysis of cell distribution within the ischemic hemisphere was performed by categorizing the GFP+ cells as described previously.

Lineage Marker Analysis of Central Nervous System and Spleen Invaded by Lin−HSCs

Immunohistochemistry was performed at 72 hours after cell injection using antibodies against CD11b (1:50; rat monoclonal; Serotec), double cortin (1:100; goat polyclonal; Santa Cruz), and glial fibrillary acid protein, followed by Alexa Fluor 594-conjugated goat antirat (1:500; Molecular Probes) or Cy3-conjugated donkey antigoat (1:200; Jackson ImmunoResearch) as secondary antibody, respectively. For Isolectin B4 (IB4) staining, sections were incubated with biotinylated IB4 (1:25; Vector Laboratories), followed by staining with Alexa Fluor 594-conjugated streptavidin (1:500; Molecular Probes). Spleen sections were refixed and immunostained with rat anti-CD45 monoclonal antibody (1:200; BD Pharmingen), followed by a Cy3-conjugated goat antirat secondary antibody (1:200; Dianova). All slices were counterstained with DAPI.

Evaluation of nDNA Content

A quantitative computer-assisted analysis (Software Axioplan View) of DAPI fluorescence was performed to determine the ploidy of GFP+ cells coexpressing IB4 or CD11b. A DNA index was calculated by measuring the DAPI fluorescence intensity of double-positive cells as previously described. DNA-indices of 60 double-positive cells and 10 surrounding endogenous cells were determined (mean DNA−index±SEM).

Characterization of Postischemic Apoptosis

Sections were incubated with a polyclonal rabbit antiactivated Caspase-3 antibody (1:2500; BD Biosciences) and a monoclonal mouse anti-NeuN antibody (1:200; Chemicon), followed by Cy3-conjugated goat antirabbit secondary antibody (1:400; Jackson ImmunoResearch) and an Alexa Fluor 488 goat antitubulin secondary antibody (1:400; Molecular Probes), and counterstained with DAPI. The number of cells displaying double labeling for activated Caspase-3 and NeuN was quantified in relation to activated Caspase-3–positive cells (in %) from randomly selected fields.

Quantitative Analysis of Postischemic Inflammation

Lin−HSC–treated or PBS-treated mice were perfused with paraformaldehyde (4% in PBS) at 24, 48, or 72 hours after cell or PBS infusion. Brains were sectioned, embedded in paraffin, and IB4 staining was performed using biotinylated IB4 (1:25; Vector Laboratories), followed by incubation with Alexa Fluor 594-conjugated streptavidin (1:500; Molecular Probes). For CD3 staining, sections...
were incubated with a polyclonal rabbit anti-CD3 antibody (1:100; DakoCytomation), followed by goat antirabbit Cy3-conjugated secondary antibody (1:400; Dianova). All sections were counterstained with DAPI. Number of IB4-positive and CD3-positive cells was determined in ischemic basal ganglia of Lin^-HSC-treated and PBS-treated mice in a blinded fashion by an independent observer. To standardize cell counts, areas of visual fields at corresponding coronal levels were predefined by stereotactic coordinates. Data are given as number of IB4-positive or CD3-positive cells/mm^2 (mean±SEM).
Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis of Cytokines and Chemokine Receptor Transcripts in Spleens

Spleens derived from Lin⁻HSC–treated or PBS-treated, ischemic, or nonischemic animals were collected 24 hours after cell injection. RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen). Relative quantification by real-time polymerase chain reaction was performed (ABI Prism 5700 Sequence Detection System; Perkin Elmer). Values were normalized to GAPDH gene transcript levels.

Results

Central Nervous System Entry of Lin⁻HSCs After Cerebral Ischemia

Transient focal cerebral ischemia was induced in adult C57BL/6 mice using the middle cerebral artery occlusion model. At 24 hours after reperfusion, animals received an intravenous injection of either $5 \times 10^6$ lineage-negative hematopoietic precursor and stem cells (Lin⁻HSCs) obtained from BM of adult GFP⁺ transgenic mice. Isolated Lin⁻HSCs were CD45⁺ and c-kit antigen-positive but lacked expression of blood lineage markers such as CD11b, TER119, Gr-1, B220, CD8, and CD4 (Figure 1A). GFP⁺ cells were detected at 48 and 72 hours after intravenous injection in the meninges, the perivascular areas, and the parenchyma of the ischemia-affected hemisphere (Figure 1B,C), as well as in peripheral organs such as spleen, lung, liver, and lymph nodes (data not shown). The number of GFP⁺ cells detected within the ischemic hemisphere increased significantly from $146 \pm 43$ cells (mean±SEM; calculated as cells per hemisphere) at 48 hours to $798 \pm 335$ cells at 72 hours after intravenous injection (Figure 1B). In total, $\approx 0.02\%$ of injected cells were estimated to migrate into the ischemic hemisphere at 72 hours after injection.

Systemically Applied Lin⁻HSCs Adopt a Microglia-like Phenotype Within the Central Nervous System

Morphological analysis of central nervous system-migrated Lin⁻HSCs was performed.$^{25}$ At 72 hours after Lin⁻HSC injection, Lin⁻HSCs had differentiated into microglia-like cells, as evidenced by colocalization of IB4 and CD11b with GFP (green), suggesting that Lin⁻HSCs adopt a microglia-like phenotype once in the ischemic tissue. Nuclei were counterstained with DAPI (blue). Scale bar: 10 μm. C, Quantification of markers found to be coexpressed in GFP⁺ cells that had migrated to ischemic hemispheres. Data are presented as mean±SEM; n=6 mice. Number of independent experiments, n=3. D, Representative images of cresyl violet-stained brain sections from Lin⁻HSC–treated (right series; n=6 mice) and PBS-treated (left series; n=7 mice) animals at 72 hours after intravenous injection of Lin⁻HSCs. E, Infarct volume analysis at 96 hours after a 45-minute transient cerebral ischemia and 72 hours after intravenous Lin⁻HSC injection or PBS injection. Infarct volumes are reduced in Lin⁻HSC-injected (26.8±4.6%; mean±SD; n=6 mice) compared to PBS-injected mice (49.3±4.9%; n=7 mice). Infarct volumes are presented as percentage of the contralateral, nonischemic hemisphere. $^*P<0.05$; Student t test.
injection, 34% of GFP/H11001 cells expressed a “ramified” microglia-like phenotype (Figure 1D). Furthermore, GFP/H11001 cells were found positive for microglia/macrophage markers (Figure 2A, B). In detail, 67 ± 16% (mean ± SEM) of GFP/H11001 cells were stained with IB4, and 55 ± 12% were positive for CD11b. GFP/H11001 cells did not colocalize with double cortin or glial fibrillary acidic protein, suggesting a lack of early neuronal or astrocytic differentiation (Figure 2C). Additionally, GFP/IB4 or GFP/CD11b cells were found to be mononucleated, and their mean DNA index was 0.96 ± 0.11 when compared to surrounding endogenous cells, demonstrating that GFP/H11001 cells within the brain did not derive from fusion or phagocytic events.

**Reduced Infarct Volumes and Attenuated Neuronal Apoptosis in Lin^−^-HSC–Treated Animals**

Mice treated with Lin^−^-HSCs showed smaller infarct volumes than did PBS-injected animals as determined at 72 hours after treatment (Figure 2D). In detail, infarct volumes were 49.3 ± 4.9% (mean ± SEM) of noninfarcted hemispheres in PBS–control mice, whereas infarct volumes were reduced to 26.8 ± 4.6% of noninfarcted hemispheres in Lin^−^-HSC–treated mice (Figure 2E). The number of TUNEL^+^ cells within the peri-infarct area was significantly reduced from 1450 ± 204 TUNEL^+^ cells/mm² (mean ± SEM) in PBS-treated to 955 ± 27 TUNEL^+^ cells/mm² in Lin^−^-HSC–treated animals (Figure 3A–C). Double-labeling against the neuronal marker NeuN and activated Caspase-3 demonstrated that 97 ± 3% (mean ± SD) of apoptotic cells were of neuronal origin (Figure 3C). Thus, stem cell therapy by Lin^−^-HSCs prevented neuronal apoptosis in the peri-infarct zone.

**Lin^−^-HSC Treatment Reduces Postischemic Inflammation**

Histochemistry for the macrophage/microglial marker IB4 and the T-cell marker CD3 revealed a significant reduction in IB4^+^ and CD3^+^ cells in peri-infarct tissues of Lin^−^-HSC–treated mice compared to PBS controls at 72 hours after treatment (Figure 4A-C). Lin^−^-HSC treatment also resulted in reduced numbers of IB4^+^ cells (762 ± 57 vs 972 ± 84 cells/mm² in PBS controls) at 48 hours and CD3^+^ cells (377 ± 45 vs 467 ± 68 cells/mm² in PBS controls) at 24 hours after treatment; however, these differences were not statistically significant (Figure 4A–C).

**Lin^−^-HSCs Counter-Regulate Ischemia-Induced Inflammation in the Spleen**

Reduced numbers of inflammatory IB4^+^ and CD3^+^ cells in ischemic hemispheres of Lin^−^-HSC–treated mice were observed before detection of intravenously injected Lin^−^-HSCs in the brain. To detect possible homing of Lin^−^-HSCs to immune organs, analysis of spleens was performed. The
GFP<sup>+</sup> cells were already detected within the spleen at 24 hours after Lin<sup>−</sup>-HSC injection at a density of 86±23 per cm<sup>2</sup> (mean±SEM) for ischemic and 67±28 per cm<sup>2</sup> for normal mice (Figure 5A, B). Analysis of ischemia-mediated effects on postischemic proinflammatory and chemokine receptor gene transcription profiles in the spleen revealed that relative gene transcript levels of tumor necrosis factor-α and IL-1β increased 30.3±16.9-times (mean±SEM) and 15.0±6.2-times (mean±SEM) at 48 hours after lesion onset, respectively (Figure 5C). Furthermore, chemokine receptor gene transcripts chemokine receptor 2 and CX3CR1 were upregulated at 48 hours after cerebral ischemia (Figure 5D). These ischemia-induced effects on proinflammatory cytokine and chemokine receptor levels within the spleen were counterregulated by Lin<sup>−</sup>-HSCs at 24 hours after intravenous injection. In detail, spleens obtained from Lin<sup>−</sup>-HSC–treated mice...
Discussion

In our study, central nervous system entry of GFP+ cells derived from BM occurred at 48 to 72 hours after systemic injection. Migration of BM-derived cells into the central nervous system after transplantation has been observed before.27,28 In contrast to our data, infiltration of BM-derived cells into the central nervous system after transplantation has been observed because of irradiation-induced alterations of the nervous tissue allowing this earlier attraction and central nervous system entry of BM-derived cells.

We only observed homing of GFP+ cells to the spleen within the first 24 hours after injection, which was in accordance with reports showing early and primary homing of hematopoietic progenitor and stem cells to BM and spleen after transplantation.29,30 Several BM transplantation approaches in injury models provided evidence of stem cell differentiation and fusion. Whereas transplantation of hematopoietic stem and precursor cells led to engraftment of microglia-like cell types within the healthy central nervous system,27,28 direct injection of selected (Sca-1+ Thy-1+ c-kit+) HSCs in a model of spinal cord injury resulted in cell expression of markers typical of astrocytes, oligodendrocytes, and neural precursors.31 In our model of experimental cerebral ischemia, Lin-HSCs showed a macrophage or microglia-like phenotype after central nervous system entry at 72 hours after transplantation, and DNA-indices of GFP+ cells indicated absence of cell fusion events. However, transdifferentiation into neuronal or astrocytic cell types cannot be excluded to occur at later time points.

Therapy by Lin-HSCs acted neuroprotectively and reduced the infarct size. To understand whether Lin-HSCs mediated changes in the cerebral growth factor, micromilieu might be responsible for the observed neuroprotection we performed in vitro in gene transcript analysis of neurotrophic factors (brain-derived neurotrophic factor, basic fibroblast growth factor, glial-derived neurotrophic factor, nerve growth factor, neurotrophin-3, vascular endothelial growth factor, and transforming growth factor-β) and cytokines (tumor necrosis factor-α, interferon-γ, IL-1β) of Lin-HSCs before injection. However, we were not able to detect substantial gene transcript levels of trophic factors in Lin-HSCs in vitro (data not shown). Furthermore, gene transcript analysis of these neurotrophic factors and cytokines within GFP+ cell-containing ischemic hemispheres did not reveal any significant changes at 72 hours after Lin-HSC injection (data not

showed significantly reduced relative gene transcript level of TNF-α from 30.3 ± 16.9 to 4.6 ± 1.2 (mean ± SEM), of IL-1β from 15.0 ± 6.2 (mean ± SEM) to 3.3 ± 1.1, of chemokine receptor 2 from 11.9 ± 4.7 to 2.8 ± 1.4, and of CX3CR1 from 10.3 ± 3.8 to 3.7 ± 1.9, respectively (Figure 5C, D).

Figure 5. Antiinflammatory effect of Lin-HSCs in spleen. A, GFP+ cells were detected by confocal microscopy in spleen at 24 hours after injection in an ischemic mouse. GFP+ cells were counterstained by a CD45-specific antibody (red). Scale bar: 10 μm. B, Quantification of GFP+ cells in spleens 24 and 72 hours after HSC injection in normal (HSC; n=5 mice per time point) and ischemic mice (iHSC; n=3 mice per time point). Ischemic mice showed increased numbers of GFP+ HSCs at 72 hours after injection. Data are presented as mean ± SEM. Number of independent experiments, n=3 for 24 and 72 hours. *P<0.05, Student t test. C, Real-time reverse-transcription polymerase chain reaction analysis of proinflammatory cytokines in spleens 24 hours after Lin-HSC or PBS injection of normal (PBS or HSC) or ischemic mice (iPBS or iHSC). Treatment with Lin-HSCs counter-regulated cerebral ischemia-induced increase in gene transcripts of TNF-α and IL-1β. Data are presented as mean ± SEM. Number of independent experiments, n=6. *P<0.05, ANOVA followed by Bonferroni multiple comparison test. D, Real-time reverse-transcription polymerase chain reaction analysis of chemokine receptors in spleens 24 hours after Lin-HSC or PBS injection of normal (PBS or HSC) and ischemic mice (iPBS or iHSC). Cerebral ischemia induced upregulation of gene transcripts for chemokine receptors chemokine receptor 2 and CX3CR1. Treatment with Lin-HSCs counter-regulated cerebral ischemia-induced increase in gene transcripts for chemokine receptor 2 and CX3CR1. Data are presented as mean ± SEM. Number of independent experiments, n=6. *P<0.05, ANOVA followed by Bonferroni multiple comparison test.
shown). Therefore, a local stem cell-mediated trophic factor support could not be detected in our experiments.

Several reports described a contribution of inflammation to cerebral injury in animal models of cerebral ischemia. Activated microglia and macrophages were increased at 24 hours, T cells were increased at 72 hours, and neutrophils were increased up to 96 hours after ischemia. We observed that Lin- HSCs attenuate the peripheral postischemic immune response, reduce immune cell infiltration into ischemic hemispheres, and mediate neuroprotection in the subacute phase after ischemic stroke.

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Disclosures
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