Combining Growth Factor and Bone Marrow Cell Therapy Induces Bleeding and Alters Immune Response After Stroke in Mice

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Background and Purpose—Bone marrow cell (BMC)–based therapies, either the transplantation of exogenous cells or stimulation of endogenous cells by growth factors like the granulocyte colony–stimulating factor (G-CSF), are considered a promising means of treating stroke. In contrast to large preclinical evidence, however, a recent clinical stroke trial on G-CSF was neutral. We, therefore, aimed to investigate possible synergistic effects of co-administration of G-CSF and BMCs after experimental stroke in mice to enhance the efficacy compared with single treatments.

Methods—We used an animal model for experimental stroke as paradigm to study possible synergistic effects of co-administration of G-CSF and BMCs on the functional outcome and the pathophysiological mechanism.

Results—G-CSF treatment alone led to an improved functional outcome, a reduced infarct volume, increased blood vessel stabilization, and decreased overall inflammation. Surprisingly, the combination of G-CSF and BMCs abrogated G-CSFs’ beneficial effects and resulted in increased hemorrhagic infarct transformation, altered blood–brain barrier, excessive astrogliosis, and altered immune cell polarization. These increased rates of infarct bleeding were mainly mediated by elevated matrix metalloproteinase-9–mediated blood–brain barrier breakdown in G-CSF- and BMCs-treated animals combined with an increased number of dilated and thus likely more fragile vessels in the subacute phase after cerebral ischemia.

Conclusions—Our results provide new insights into both BMC-based therapies and immune cell biology and help to understand potential adverse and unexpected side effects. (Stroke. 2016;47:852–862. DOI: 10.1161/STROKEAHA.115.011230.)

Key Words: bone marrow cell transplantation ▪ cerebral ischemia ▪ granulocyte colony-stimulating factor ▪ hemorrhagic transformation ▪ macrophage polarization

Stroke is the third leading cause of death and the most frequent cause of adulthood disability worldwide. Cell-based therapies, either transplantation of exogenous cells or stimulation of endogenous cells, are considered a promising means of treating stroke. Approaches using bone marrow–derived cells (BMCs) are particularly attractive because BMCs can be easily obtained and permit autologous transplantation. Besides transplantation of exogenous cells, endogenous BMCs can be mobilized by the granulocyte colony–stimulating factor (G-CSF). A number of studies showed that both BMC and G-CSF therapies improve neurobehavioral and histological outcomes in animal models of cerebral ischemia. BMCs are assumed to exert their actions by the production of cytokines rather than by cell replacement. Cytokines secreted by BMCs feature acute neuroprotective properties and stimulate endogenous repair mechanisms, including neurogenesis and angiogenesis, and support the survival of host neurons. In addition to BMC-mediated mechanisms, G-CSF also exerts direct effects on neurons via neuronal G-CSF receptors. However, in contrast to the large preclinical evidence on G-CSF’s beneficial effects after stroke, a recent clinical stroke trial on G-CSF was neutral. A potential reason might be that the G-CSF-mediated BMC mobilization occurs too late, even when G-CSF stimulation is initiated early after stroke onset. We, therefore, investigated the hypothesis that the combination of G-CSF and BMCs is more effective than either single treatment because transplantation of exogenous BMCs can bridge the gap until G-CSF mobilizes endogenous BMCs into the peripheral circulation (Figure 1A). Vice versa G-CSF might enhance the efficacy of the exogenous BMC therapy according to one of its genuine functions, that is, the increase of survival of BMCs.
Materials and Methods

Animal Care
All animal experiments were conducted in concordance with the local authorities (State Agency for Nature, Environment and Consumer Protection (LANUV), North Rhine-Westphalia, Germany) according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Male 16- to 18-week-old C57BL/6J (Charles River, Germany; n=158) and GFP-transgenic mice (a kind gift by Prof Masaru Okabe, C57BL/6J–GFP, backcrossed with C57BL/6J mice at least 10 times; n=48) were used. Mice were maintained in a pathogen-free laboratory with access to unlimited food and water. Mice were randomly assigned to 4 groups: (1) placebo; (2) G-CSF (10 μg/kg); (3) BMC (5×10⁶ cells); and (4) G-CSF+BMC (n=19 each group/time-point). Surgery, deficit evaluation, and further analyses were done blinded to treatment. We used human G-CSF, which was demonstrated to exhibit a high specificity to the rodent G-CSF receptor.

Transient Focal Cerebral Ischemia
Focal ischemia was induced in 152 C57BL/6J-mice (25–30 g) by occlusion of the middle cerebral artery (MCA) as previously described. Briefly, animals were anesthetized with isoflurane (1.5% in 30% O₂/70% N₂O), and the left common carotid artery was separated through a midline neck incision. A silicon-coated monofilament (0.2±0.1 mm; Doccol) was inserted into the left common carotid artery and advanced distal to the carotid bifurcation for occlusion of the MCA. After 30 minutes, the suture was withdrawn for reperfusion and the wound closed. Before, during, and after middle cerebral artery occlusion (MCAO), cerebral blood flow was monitored with a laser Doppler probe (Periflux 5001; Perimed, Sweden). Sham-operated animals underwent the same procedure without occlusion of the MCA.

BMC Transplantation and G-CSF Therapy
GFP mice were sacrificed, and femur veins were flushed with phosphate-buffered saline. After erythrolysis, BMCs were washed 3x in phosphate-buffered saline and resuspended at 5×10⁶ cells/mL. BMC-treated mice received 100 μL of the cell suspension (5×10⁶ cells) 90 minutes after occlusion of the MCA by injection into the tail vein of the mouse. Mice treated with G-CSF/G-CSF+BMCs received an immediate additional injection of G-CSF (90 minutes after ischemia induction, 10 μg/kg IP; Figure 1A).

Functional Tests
The rotarod test was used to assess skilled motor performance. Mice were placed on a Rotarod cylinder (4–50 rpm acceleration in 210 s; TSE-Systems, Germany), and the time the animals remained on this cylinder was measured. Mice were given 3 training sessions before MCAO. Mean duration was recorded 1 day prior and daily after MCAO for 7 days. Individual performance was calculated as percent to pre-MCAO value.

Cylinder Test
The cylinder test was used to assess forelimb activity. Mice were placed in a transparent cylinder (10 cm diameter) and videotaped for 2 min before MCAO and on day 1 and 7 post MCAO. Forelimb use was counted, and the first 20 contacts were divided into use of (1) both, (2) right, or (3) left forelimb.

Tissue Preparation and Infarct Volume
One or 7 days after MCAO, animals were perfused through the left ventricle with 6% hydroxyethyl starch (Fresenius, Germany) for 1 minute and 4% buffered paraformaldehyde (pH 7.4) for 10 minutes under xylazine/ketamine anesthesia. Brains were removed, fixed (4% paraformaldehyde, 3 hours), immersed in 10% sucrose, frozen, and stored at −80°C.

Figure 1. Functional outcome and infarct volume assessment revealed beneficial effects of granulocyte colony–stimulating factor (G-CSF) therapy. A, Schematic depicting the experiments rationale and experimental design. B, The rotarod test showed an improved outcome in G-CSF-treated mice compared with the G-CSF+BMC-treated group on day 2 (P<0.05), day 3 (P<0.05), and day 7 (P<0.01; Shapiro–Wilk test for normal distribution, followed by repeated measures analysis of variance [ANOVA]; P<0.0001 and Bonferroni post hoc test, n=15–19; *P<0.05, **P<0.01 (G-CSF vs G-CSF+BMC). C, G-CSF and BMC treatment alone showed a trend toward a more frequent use of the affected limb compared with placebo and G-CSF+BMC therapy in the cylinder test (ANOVA p=0.06). D, G-CSF–treatment resulted in smaller infarct volumes compared with the placebo group on day 1 (P=0.019) and on day 7 post MCAO (P=0.0008, 1-way ANOVA with Bonferroni post hoc test, n=8–10). *P<0.05, **P<0.01. BMC indicates bone marrow cell; and MCAO, middle cerebral artery occlusion.
Mice used for mRNA and protein analyses were perfused with 0.9% NaCl, brains rapidly removed, frozen on dry ice, and stored at −80°C. Coronal sections were cut with a cryostat (Leica CM 3050, Germany), mounted on glass slides, and stored at −20°C. To assess ischemic damage, sections were collected in 300 µm intervals, stained with toluidine blue, and digitized. Infarction was measured with ImageJ (http://imagej.nih.gov/ij/), and infarct volumes were calculated as described.19

Macroscopic Evaluation of Intracerebral Hemorrhage

We evaluated the intracerebral hemorrhage on a macroscopic level using a modified scale according to Henninger et al.20 Intracerebral hemorrhage was defined as (1) no hemorrhage, (2) hemorrhagic infarction (punctate or confluent petechiae within the infarcted tissue), and (3) parenchymal hematoma (large homogenous mass of blood within the infarct).

Gene Expression

RNA purification and reverse transcription were done following the manufacturer’s protocol (RNAsesy, Quantitect RT-Kit, Qiagen, Germany). Primers were purchased from Qiagen: glyceraldehyde-3-phosphate-dehydrogenase; claudin-5; and occludin. Real-time polymerase chain reactions were performed with ABI PRISM 7700 RT-PCR-System (Applied Biosystems). Standard conditions were used and measurements done in duplicates.

Protein Preparation and Western Blot

Western blot analyses were done with brain lysates of the infarcted hemisphere. Lysates were prepared by ultrasonic homogenization in lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5% sodium-deoxycholate, 1% NP-40, protease inhibitor; Roche, Switzerland). After centrifugation (12000 g, 20 min, 4°C), lysates were collected and protein adjusted to 1 mg/mL (BCA Assay, Pierce Chemical). Lysate (12 µL) was separated on 12.5% SDS-polyacrylamide gels and transferred to 4% to 20% gradient gels (MiniProtean, BioRad, Germany). After blocking (5% wt/vol milk powder, TBS-T, 1 h), membranes were incubated with antibodies rabbit anti-albumin (Serotec, Germany; 1:1000), rabbit anti-claudin-5 (Abcam, UK; 1:100), rabbit anti-occludin (Invitrogen; 1:200), and rabbit anti-CD31 (Abcam; 1:100). Detection of antibodies was done with horse-raddish peroxidase–conjugated anti-rabbit antibody (1:5000, 45 min). Protein bands were visualized with Lumi-Light® (Roche).

Immunofluorescence Microscopy

Brain sections (bregma 0–1 mm) were pretreated in 3% H2O2/methanol (10 min) and Blocking Reagent (Roche Diagnostics, Germany; 15 min). Primary antibodies (Table I in the online-only Data Supplement) were applied overnight (4°C). Neuronal nuclei (NeuN) and glial fibrillary acidic protein (GFAP) were visualized with anti-mouse-AlexaFluor594 (Molecular Probes; 45 min, 1:100) and Laminin with anti-rabbit-AlexaFluor594 (Molecular Probes; 1:100, 4 °C, overnight). Signal amplification of CD16/32, F4/80, 7/4, matrix metalloproteinase-9 (MMP-9), and CD31 was done with horse-radish peroxidase–conjugated streptavidin (DAKO, Denmark; 1:100, 45 min) and biotinyl tyramide (1:100, 10 min). Visualization was done with streptavidin-conjugated dye (Alexa Fluor 594, Molecular Probes, the Netherlands; 1:100, 45 min). 4′-6-diamidino-2-phenylindole was applied for nuclear counterstain (DAPI; Vector). Non-confocal images were taken with Nikon Eclipse 80i microscope (Nikon GmbH, Germany). Confocal images were taken with Axiovert microscope (Zeiss, Germany).

Quantification of Astrogliosis and Macrophage Polarization

GFAP (as astroglis marker) and CD16/32 (as exemplary M1 marker) intensity were measured with densitometric analysis technique described elsewhere.21 Briefly, whole hemisphere images (8 bit) underwent 2 processing steps using ImageJ: background noise was reduced with a 20 pixel wide rolling-ball subtraction followed by median filter (2 pixel wide). Remaining pixels between gray values 5 to 105 were considered GFAP- or CD16/32-positive and summed for each brain. Signal of placebo-treated mice was set as reference (100%).

Fluorescence-Activated Cell Sorting Analysis

Blood of BMC-transplanted mice was collected from the right atrium before perfusion. Samples were washed in phosphate-buffered saline for 3 times, and GFP− cells were quantified by fluorescence-activated cell sorting analysis.

MMP-9, High-Mobility-Group-Box-1, and Interferon-γ In Vivo and In Vitro

MMP-9 concentration was measured using MMP-9 ELISA Kit (BosterBio), high-mobility-group-box-1 (HMGB1) using HMGB1 ELISA kit (Biosol, Germany), and interferon-γ (IFN-γ) with IFN-γ ELISA kit (R&D Systems).

MMP-9, HMGB1, and IFN-γ In Vivo

Brain tissue protein concentration was evaluated by applying 5 µg total protein (n=4 per group) to each well.

Evaluation of Blood Vessel Diameter

Ipsilateral vessel diameter was measured within 3 regions (cortex, infarcted core, piriform cortex at bregma 0.25 mm; size of each measured area=65.2×10^3 µm^2) 7 days after MCAO using ImageJ analysis. Vessel diameter means were calculated of 10 vessels per area per animal (n=5 per group).

Statistical Analysis

Statistics were done with GraphPad Prism (5.01, GraphPad Software). Evaluations of intergroup effects were done with analysis of variance after checking data for normal distribution (Shapiro–Wilk). When a significant effect could be detected, a Bonferroni post hoc test for multiple comparisons was applied. For comparison of 2 groups, unpaired 2-tailed student t tests were applied (quantification of immigrated GFP+ cells and fluorescence-activated cell sorting analysis). Frequency of parenchymal hematoma was evaluated by using the chi-square test. Results are presented as means±SEM unless otherwise stated. A P value of <0.05 was considered significant.

Results

Functional Outcome, Mortality, and Infarct Volume Quantification

Rectal temperature, body weight, and cerebral blood flow were not different between groups (see Table II in the online-only Data Supplement). Four placebo, 1 G-CSF−, 5 BMC−, and 6 G-CSF+BMC-treated mice died during the survey and were excluded. For evaluation of functional outcomes, each mouse underwent the Rotarod and cylinder test. Animals treated with G-CSF alone showed increased running time compared with the G-CSF+BMC-treated mice (analysis of variance
Both G-CSF and BMC treatment alone showed a trend toward a more frequent use of the affected limb compared with placebo and G-CSF+BMC therapy in the cylinder test (analysis of variance, \( P=0.06 \); Figure 1C). Furthermore, infarct volumes were reduced in G-CSF-treated animals on day 1 and day 7 compared with placebo-treated mice (Figure 1D).

**Analysis of Transplanted BMCs**

Fluorescence-activated cell sorting quantification of circulating transplanted BMCs (GFP\(^+\)) showed no significant changes in cell counts between the investigated groups (\( P=0.18 \); Figure 2A). Intracerebral evaluation of GFP\(^+\) cells showed an increased number of grafted cells within the brains of G-CSF+BMC-treated animals (\( P=0.039 \); Figure 2A). GFP\(^+\) cells were mainly located within the infarcted striatum and cortex and were increased in G-CSF+BMCs-treated mice compared with BMC monotherapy. Transplanted cells were frequently seen in groups of 2 to 3 cells (Figure 2B). GFP\(^+\) cells occasionally expressed the microglia/macrophage marker F4/80 (Figure 2C) and numerous showed ramified shape. Some F4/80\(^+\)/GFP\(^+\) cells were intermingled with NeuN\(^+\) cells, suggesting phagocytosis of defect neurons (Figure 2D). GFP\(^+\) cells were rarely seen within vessels of the contralateral hemisphere. Random evaluation of spleen, liver, and lung showed numerous GFP\(^+\) cells within the respective tissue (not shown).

**G-CSF+BMC Combination Induces Hemorrhagic Transformation and BBB Damage**

During the preparation of the coronal brain slices, it came to our attention that occasionally brains showed an unusual pronounced petechial or even parenchymal intracerebral bleeding. To our surprise, parenchymal bleeding was only seen in mice which received BMCs or G-CSF+BMCs (G-CSF+BMC post 7 days; \( P=0.031 \); Figure 3A). To investigate mechanisms of hemorrhagic transformation, we performed BBB leakage evaluation via quantification of intracerebral albumin. We measured increased albumin in G-CSF+BMCs-treated mice on day 1 compared with placebo and G-CSF-treated mice (Figure 3B; see Figure 1 in the online-only Data Supplement for whole blots). To further evaluate BBB integrity, we quantified mRNA and protein of claudin-5 and occludin. No differences of occludin expression could be found on day 1. On day 7, either placebo or G-CSF+BMC-treated animals showed a marked downregulation of occludin compared with the G-CSF- and BMC-treated group. Protein analyses revealed an increase of occludin signal in all investigated groups on day 1. Seven days after MCAO, G-CSF+BMC-treated animals showed reduced occludin protein compared with the placebo group, indicating a critical influence of G-CSF+BMC on post ischemic BBB opening and restructuring (Figure 4A). Claudin-5 expression revealed no significant changes between the groups, whereas protein analysis showed elevated claudin-5 protein in BMC- and G-CSF+BMC-treated mice compared with the placebo and G-CSF-treated group (Figure 4B). We next investigated mechanisms of BBB damage. MCAO resulted in pronounced MMP-9 secretion within the infarct. Combination of G-CSF+BMCs caused increased MMP-9 secretion on day 1 (Figure 4C). The vast majority of MMP-9\(^+\) cells were identified as neurons, endothelial cells, and GFP\(^+\) BMCs. In contrast, in vitro studies of cultured BMCs showed markedly decreased MMP-9 in cells treated with G-CSF and G-CSF+albumin (Figure 4D). MMP-9 was normalized for cell counts. Proliferation rates compared
with placebo-treated cells were 138.46% (G-CSF), 111.54% (albumin), and 208.65% (G-CSF + albumin).

Astrogliosis and Immune Cell Response
Evaluation of GFAP signal intensity indicated increased astrogliosis in mice treated with G-CSF+BMCs compared with placebo- and G-CSF-treated animals 7 days after MCAO (Figure 5A). However, as intracerebral bleeding is known to cause astrocyte activation, we investigated a potential association between bleeding intensity and GFAP level. Such association could not be observed (Figure II in the online-only Data Supplement). Because G-CSF+BMC treatment led to an increased astrocyte activation, equating a more intense inflammatory response, we investigated a potential altered polarization of involved immune cells. Hence, we decided to evaluate CD16/32 as representative M1-macrophage marker. Indeed, CD16/32 signal intensity showed a marked upregulation in BMC- and G-CSF+BMC-treated mice 7 days after MCAO (Figure 5B). One day after MCAO, CD16/32 signal could be mainly observed surrounding the infarcted core, whereas after 7 days, CD16/32+ cells could be seen particularly within the infarct core and, in case of BMC- and G-CSF+BMC-treated mice, also within the adjacent cortex (Figure 6C). Quantification of CD16/32+-cells showed no difference between treatments on day 1. Seven days after MCAO, however, CD16/32+ cell counts were significantly increased in BMC- and G-CSF+BMC-treated animals (Figure 6D).

HMGB1 and IFN-γ
Because we observed an increased bleeding susceptibility of BMC- and G-CSF+BMC-treated mice in combination with increased astrogliosis, we investigated whether these mechanisms could be mediated by the damage-associated molecular pattern named HMGB1. HMGB1 is known to be
released by reactive astrocytes and to promote neurovascular remodeling during stroke recovery. 23 No differences could be seen between treatment groups on day 1. However, 7 days after MCAO, G-CSF+BMC-treated animals showed a trend toward a suppressed HMGB1 release ($P=0.07$) compared with the placebo group (Figure 6A). Concomitantly, in vitro analysis also showed a reduced HMGB1 release by BMC after G-CSF+albumin treatment, which could further indicate an altered inflammatory milieu triggered by the G-CSF stimulated cell graft. We next checked whether increased astrogliosis, CD16/32+-cells, and altered HMGB1 release led to an altered overall inflammation. We chose IFN-γ as representative marker because microglia and immigrating macrophages can be stimulated by IFN-γ to the pro-inflammatory M1-phenotype. 24 G-CSF and, to our surprise, G-CSF+BMC treatment resulted in a decreased IFN-γ concentration within the infarcted hemisphere 7 days after MCAO (Figure 6B). In vitro analysis confirmed the reduced IFN-γ release in cultured cells after G-CSF and albumin treatment.

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**Figure 4.** A, Occludin expression/protein concentration 1 and 7 days after MCAO (mRNA: analysis of variance [ANOVA] $P<0.0001$; $n=5$ each. Protein: ANOVA $P=0.030$; $n=4$ each). B, Claudin-5 expression/protein concentration 1 and 7 days after MCAO (mRNA: ANOVA $P>0.05$; $n=5$ each. Protein: ANOVA $P<0.001$; $n=4$ each). C, Matrix metalloproteinase-9 (MMP-9) levels within the infarct obtained by ELISA (ANOVA $P=0.0211$; $n=4$ each [dotted line: sham values±SEM]). IHC, Autofluorescent erythrocytes within the infarcted striatum in close vicinity to MMP-9-stained cells. The majority of MMP-9+-cells were identified as neurons, administered GFP+, and endothelial cells. D, MMP-9 protein concentration in supernatants of cultured bone marrow cells (BMCs). MMP-9 concentration was normalized for cell numbers in each well (ANOVA $P=0.010$; $n=6$ each, data shown as ng/10^3 cells). Protein and mRNA levels are shown as relative change compared with sham group. IHC indicates immunohistochemistry; and MCAO, middle cerebral artery occlusion. *$P<0.05$, **$P<0.01$, ***$P<0.001$; Bonferroni post hoc test.
Combination Therapy Increases Vessel Diameters Within the Ischemic Hemisphere

For further investigation of mechanisms underlying hemorrhagic transformation, we evaluated poststroke vessel integrity. Mice treated with G-CSF+BMCs showed increased mean blood vessel diameter compared with placebo and G-CSF-treated animals (Figure 6C). Analysis of CD31, which is mainly expressed by endothelial cells, showed a diminished reduction of CD31 protein in G-CSF-treated mice compared with placebo, BMCs, and G-CSF+BMCs groups on day 1 (Figure 6D).

Discussion

The present study confirms that G-CSF treatment results in smaller infarct volumes and improved functional outcome. According to our hypothesis, G-CSF co-treatment increased the number of transplanted BMCs within the ischemic brain compared with BMC therapy alone. Because G-CSF is noticeably upregulated as early as 2 hours after ischemia and earlier treatment is usually associated with an improved outcome, we decided to first examine an early treatment initiation with G-CSF. As the combination therapy, in contrast to our hypothesis, showed detrimental effects, we refrained from investigating further times of treatment initiation but evaluated mechanisms underlying the observed deteriorated outcomes. The neurological deterioration after combination therapy was frequently accompanied by hemorrhagic infarct transformation. We identified MMP-9-mediated BBB loss as reason for the increased susceptibility to intracerebral bleeding. We also found excessive astrogliosis and altered immune cell polarization after co-treatment of G-CSF and BMCs.

Several previous studies have shown that grafting of either mononuclear or menenchymal bone marrow cells promotes neuroprotection and angiogenesis. Furthermore, it has been shown that immigrating bone marrow cells are capable to respond to local epigenetic stimuli and to differentiate into neural and glial phenotypes. In this study, application

Figure 5. Evaluation of astroglia and CD16/CD32− cell activation/distribution. A, GFAP immunohistochemistry showed increased astrogliosis in mice treated with G-CSF+BMC (analysis of variance [ANOVA] P=0.0005; n=8–10). B, CD16/CD32− staining revealed an increased signal intensity in BMC or G-CSF+BMC-treated mice 7 days after MCAO (black and white inserts: densitometric surface plot [ANOVA P<0.0001; n=8–9]; dotted line: placebo value=100%±SEM). C, Heat map depicting the distribution pattern of CD16/CD32− cells and revealed an increased area of CD16/CD32 expression in BMC- and BMC+G-CSF-treated mice 7 days after MCAO (ANOVA P=0.0052; n=8–10). D, BMC and G-CSF+BMCs treatment resulted in increased CD16/CD32− cell counts within the infarcted hemisphere (ANOVA P<0.0001; n=8–9); *P<0.05, **P<0.01, ***P<0.001; *vs placebo, †vs G-CSF, ‡vs BMC, Bonferroni post hoc test. BMC indicates bone marrow cell; G-CSF, granulocyte colony-stimulating factor; GFAP, glial fibrillary acidic protein; and MCAO, middle cerebral artery occlusion.
of BMCs resulted in minor improved functional outcome but failed to reduce infarct volume. Injected BMCs immigrated into the ischemic tissue and could be found beyond vessel structures. However, none of the engrafted cells coexpressed neuronal markers, likely because of the early investigation time point. Numerous cells coexpressed the macrophage marker F4/80 and showed ramified morphology. Additional G-CSF stimulation resulted in an increase of intracerebral GFP cells, indicating either G-CSF-mediated enhanced proliferation or survival of grafted cells.

Figure 6. A, High-mobility-group-box-1 (HMGB1) protein analysis within the infarcted hemisphere showed no differences between the investigated groups on day 1 post MCAO and a trend toward decreased HMGB1 in BMC+G-CSF-treated mice compared with placebo. HMGB1 protein concentration in supernatants of cultured BMCs revealed a decreased HMGB1 secretion by G-CSF+albumin-treated cells (analysis of variance [ANOVA] \( P=0.0225; n=6 \) each). B, G-CSF and G-CSF+BMC treatment led to a reduced interferon-\( \gamma \) (IFN-\( \gamma \)) secretion in mice subjected to MCAO (post 7 days, ANOVA \( P=0.0002; n=4 \) each). Sole G-CSF or albumin treatment resulted in a reduced interferon-\( \gamma \) (IFN-\( \gamma \)) release by cultured BMC (ANOVA \( P=0.0058; n=6 \) each). HMGB1 and IFN-\( \gamma \) protein concentration were normalized for cell numbers in each well (n=6 wells each, data shown as ng/10^3 cells). C, G-CSF+BMC-treated mice showed an increased number of dilated blood vessels (ANOVA \( P=0.001; n=5 \) vessels per animal measured, n=5 per group). D, CD31 levels obtained by Western blot analysis (ANOVA \( P=0.033; n=4 \) each; dotted line: sham value=100\%±SEM). *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \); *vs placebo, #vs G-CSF, $vs BMC; Bonferroni post hoc test.

BMC indicates bone marrow cell; G-CSF, granulocyte colony–stimulating factor; and MCAO, middle cerebral artery occlusion.
Previous studies revealed a crucial role of bone marrow cells for the development of hemorrhagic infarct transformation.\(^{29,30}\) Subsets of monocytes/macrophages were demonstrated to be essential for integrity maintenance of the neurovascular unit after cerebral ischemia.\(^{30}\) Depletion of circulating monocytes/macrophages or prevention of CCR2-dependent recruitment of monocytes caused hemorrhagic transformation in murine stroke models. The finding of an increased hemorrhagic transformation after combination of G-CSF therapy and BMCs is of great importance from a translational perspective because bleeding in the infarct is associated with worse neurological outcomes in stroke patients. We, therefore, investigated potential underlying mechanisms of this phenomenon.

Analysis of intracerebral albumin as indicator for BBB leakage revealed a markedly increased albumin influx in BMC- and G-CSF+BMC-treated mice already 24 hours after MCAO. Furthermore, altered claudin-5 and occludin levels in G-CSF+BMC-treated mice point toward unwanted side effects, respectively increased BBB damage triggered by BMC application. Regarding the formation of ischemia-induced BBB damage, MMPs are well-known to contribute to BBB breakdown by degrading endothelial basal lamina.\(^{4,31,32}\) We, therefore, investigated the secretion of MMP-9 and found a noticeable increase of intracerebral MMP-9 in all groups that was markedly elevated in mice treated with G-CSF+BMC. Immunohistological analysis revealed particularly neurons and endothelial cells as MMP-9 source, confirming the results of an earlier study.\(^{33}\) In addition, grafted BMCs were also identified as MMP-9 source in vivo. However, our in vitro analyses of cultured BMCs showed that G-CSF and G-CSF+albumin treatment led to decreased MMP-9 production. We thus conclude that the adjacent cells trigger either infiltrating BMCs to an alternate state or that BMCs alter the cytokine milieu within the injured tissue, resulting in an increased neuronal and endothelial MMP-9 release. The idea of a BMC-mediated cytokine release modification is supported by the finding of a noticeably increased reactive astrogliosis and altered immune cell polarization toward the pro-inflammatory M1 phenotype in G-CSF+BMC-treated animals. Increased astrogliosis has been recently linked to enhanced angiogenesis via the HMGB1, a protein secreted by BMC application.\(^{34}\) Additionally, HMGB1 induces toll-like-receptor-4-mediated MMP-9 release and augments inflammation by increasing the release of tumor necrosis factor-\(\alpha\), interleukin-1\(\beta\), and other cytokines.\(^{33}\) To our surprise though, G-CSF+BMC treatment resulted in a decreased HMGB1 and IFN-\(\gamma\) release. Furthermore, G-CSF+BMC treated mice showed increased numbers of dilated blood vessels. As MMP-9 secretion is known to further support neovascularization\(^{35}\) in combination with reduced angiogenesis-promoting HMGB1, this could point toward insufficient formation of neovessels or impaired vessel repair and further increased susceptibility to hemorrhagic transformation after G-CSF+BMC treatment. The idea of a potential adverse role of the cell graft on BBB integrity is supported by recent findings of dela Peña et al.,\(^{36}\) who showed that sole application of G-CSF exerts crucial effects on vascular stability, leading to attenuated delayed tissue plasminogen activator--induced hemorrhagic transformation and enhanced vascular- and angiogenesis.

Evaluation of microglia and macrophages showed no differences in cell numbers between the investigated groups but an enlarged area of activated microglia within the infarct after G-CSF treatment, despite smaller infarct volumes (see Figure III in the online-only Data Supplement). This could indicate a beneficial role of early microglial activation by G-CSF, as suggested in a current study on spinal cord injury.\(^{37}\) Today it is suggested that microglia and infiltrating macrophages show a dynamic response to ischemia by either polarizing toward an anti-inflammatory and angiogenesis-promoting (M2) or to a detrimental pro-inflammatory M1 phenotype.\(^{38}\) The reduced CD16/32\(^+\) cells in G-CSF-treated mice compared with the C-CSF+BMC group could indicate a microglial shift towards less detrimental or even beneficial action during the acute phase. Clearly, further studies need to address whether G-CSF and BMCs exercise influence on glial and leukocyte polarization.

Our data showing increased claudin-5 and occludin protein in connection with increased BBB leakage in G-CSF+BMCs-treated mice is counterintuitive at a first glance. However, in a previous study, we could show that extended occlusion time of 45 minutes\(^{39}\) resulted in minor changes of claudin-5 and occludin protein concentration. These findings were supported by the results of Stamatovic et al.,\(^{40}\) which suggest that claudin-5 and occludin are, in consequence of oxygen shortage, not decomposed by proteinases, but stored away within endothelial endosomes. This would make both proteins available for later reestablishment of the BBB. Therefore, our finding of increased BBB protein could be as a result of demasking of the unbound proteins within endosomes, making them more susceptible to antibody staining.

Our finding that transplantation of BMCs abrogates G-CSFs’ beneficial effects confirm results of our ancestor study.\(^{41}\) In this previous study, we found that G-CSF+BMC treatment results in accumulation of endogenous granulocytes and graft cells within the spleen, which in turn led to splenic overload, granulocyte clearance failure, and increase of circulating and intracerebral granulocytes. In the present study, we also observed increased granulocyte numbers within the ischemic lesion of G-CSF+BMC-treated animals, but not in mice treated with G-CSF alone. The finding that BMC administration abrogates the beneficial effects of G-CSF after murine stroke might provide a potential explanation why G-CSF monotherapy has been beneficial in a number of animal studies, but failed to improve neurological outcome in a clinical study.\(^{14}\) Mice have lower numbers of circulating granulocytes compared with humans and G-CSF-induced neutrophilia is more pronounced in humans compared with mice.\(^{14,42,43}\) Therefore, growth factor--combined cell therapy might cause excessively increased leukocyte numbers in mice that resemble effects of G-CSF monotherapy on peripheral white blood count in humans. The assumption that such excessive increased peripheral leukocytes are detrimental and abolish potential neuroprotective effects of G-CSF after stroke is also supported by a previous study, which found particularly high doses of G-CSF to be detrimental after cerebral ischemia in mice.\(^{44}\) Therefore, future preclinical investigations applying
bone marrow cell–based therapies should consider differences in blood composition in between rodents and humans. Our study has strengths and limitations. Strengths are large animal numbers and multimodal mechanistic experimental approaches. A potential limitation of this work regarding its comparability to other studies using BMCs in animal stroke studies could be the preparation of the transplanted cells. Many promising preclinical studies describing beneficial effects during stroke recovery used a plethora of different subsets, for example, mesenchymal, hematopoietic, or endothelial progenitor cells, which could be a reason why sole injection of BMC resulted only in minor functional improvement. However, as the preparation needs prior culturing lasting up to several days, our aim was to study the efficacy of primary cells prepared directly from the bone marrow, which are available within hours. In addition, we cannot rule out adverse effects driven by a potential mismatch of the applied human clinical grade G-CSF (Neupogen) and the mice-derived BMC, leading to the observed altered immune response. We also assert that further adjusting of the G-CSF application timing in relation to the cell grafting may reduce the adverse effects of the combination therapy. A further limitation is based on the fact that we cannot account for pretreatment stroke severity, which could potentially differ between groups.

In summary, our results confirm the efficacy of both G-CSF treatment and BMC monotherapy in experimental stroke. Contrary to our hypothesis, the combination of G-CSF+BMC application led to poorer functional outcome and surprisingly increased intracerebral bleeding. The latter resulted from G-CSF+BMC co-therapy–mediated upregulation of cerebral MMP-9 with subsequent BBB damage and altered inflammation. Our findings provide new insights into bone marrow–based stroke therapies and reveal potential side effects.

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Disclosures
Dr Schäbitz is an inventor on a patent claiming the use of granulocyte colony-stimulating factor in stroke treatment. The other authors report no conflicts of interest. This work forms part of the doctoral thesis of Joanna Olk.

References


Combining Growth Factor and Bone Marrow Cell Therapy Induces Bleeding and Alters Immune Response After Stroke in Mice
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Combining growth factor and bone marrow cell therapy induces bleeding and alters immune response after stroke in mice

Supplementary figures

**Supplementary Fig. I**: Western blot analysis of CD31, albumin, claudin-5 and occludin protein amounts within the ipsilateral hemisphere of placebo, G-CSF, BMC and G-CSF + BMC-treated animals. Whole protein lysates (12 µg per lane) were applied to each lane. Band intensities were measured using NIH ImageJ. Each bands’ intensity was additionally corrected using the individual actin-band density. All gels were run at the same time under equal conditions except for the third occludin gel (far right gel, post 7, G-CSF, BMC, G-CSF + BMC) which had to be redone.
Supplementary Fig. II: No correlation between astrocyte activation and bleeding severity could be observed. Image showing bleeding severity on the x-axis and GFAP-signal intensity obtained by densitometric analysis on the y-axis. (7 days post-MCAO, each group n=8, only PFA-perfused animals).
**Supplementary Fig III:** Activation and immigration of innate inflammatory cells.

(A) Quantification of F4/80\(^+\)-cells, indicating macrophages and microglia, showed no differences between treatments. However, all groups showed a marked increase of F4/80\(^+\)-cells on day 7 compared to day 1. Analysis of F4/80\(^+\)-cell inhabited areas revealed no differences between the investigated groups (ANOVA \(p=0.054\), \(n=7-10\), Fig IIIa). (B) Quantification of 7/4\(^+\)-neutrophil granulocytes showed no differences in cell density, except for a trend towards elevated cell counts after G-CSF+BMC-treatment on day 1 (\(p=0.09\)). Analysis of 7/4\(^+\)-inhabited areas showed a reduced area in G-CSF-treated mice on day 1 (ANOVA \(p=0.0069\), \(n=7-10\), Fig.IIIb, mean±SEM). ***\(p<0.01\); Bonferroni post-hoc test.
<table>
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<th>Antibody</th>
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<th>Manufacturer</th>
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<td>7/4*</td>
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*denotes applied signal amplification.
Table II: body weight, body temperature and cerebral blood flow during surgery

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<tr>
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<th>Weight [g]</th>
<th>T$_{pre}$ [°C]</th>
<th>T$_{MCAO}$ [°C]</th>
<th>T$_{post}$ [°C]</th>
<th>CBF$_{pre}$ [%]</th>
<th>CBF$_{MCAO}$ [%]</th>
<th>CBF$_{post}$ [%]</th>
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<tr>
<td>Placebo</td>
<td>27.56±0.80</td>
<td>36.49±0.55</td>
<td>36.64±0.49</td>
<td>36.64±0.49</td>
<td>100±12.33</td>
<td>12.29±2.21</td>
<td>83.23±6.00</td>
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<td>G-CSF</td>
<td>27.27±0.79</td>
<td>36.43±0.36</td>
<td>36.53±0.39</td>
<td>36.61±0.35</td>
<td>100±12.39</td>
<td>12.45±2.28</td>
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<td>BMC</td>
<td>26.66±1.76</td>
<td>36.53±0.34</td>
<td>36.60±0.30</td>
<td>36.63±0.27</td>
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<td>G-CSF+BMC</td>
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<td>100±13.24</td>
<td>11.23±2.25</td>
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</table>

Body weight of mice prior to occlusion, rectal temperature (T) and cerebral blood flow (CBF) prior (T$_{pre}$, CBF$_{pre}$), during (T$_{MCAO}$, CBF$_{MCAO}$) and after (T$_{post}$, CBF$_{post}$) occlusion of the MCA.