Effects of Granulocyte-Colony Stimulating Factor After Stroke in Aged Rats

Aurel Popa-Wagner, PhD; Kai Stöcker; Adrian Tudor Balseanu, MD; Andreas Rogalewski, MD; Kai Diederich, PhD; Jens Minnerup, MD; Claudiu Margaritescu, MD; Wolf-Rüdiger Schäbitz, MD

Background and Purpose—In aged humans, stroke is a major cause of disability for which no neuroprotective measures are available. Granulocyte-colony stimulating factor (G-CSF), a member of the cytokine family of growth factors, promotes brain neurogenesis and improves functional outcome after stroke in young animals. We tested the hypothesis that G-CSF provides a restorative therapeutic benefit in aged animals.

Methods—Focal cerebral ischemia was produced by reversible occlusion of the right middle cerebral artery in 19- to 20-month-old male Sprague-Dawley rats. One hour after reperfusion, the aged rats were treated daily with 15 μg/kg G-CSF and for 15 days total. Rats were behaviorally tested and the brains removed for analysis at 28 days poststroke.

Results—G-CSF treatment after stroke exerted a robust and sustained beneficial effect on survival rate and running function. Transient improvement after G-CSF treatment could be observed for coordinative motor function on the inclined plane test and for working memory in the radial-arm maze test. At the cellular level, G-CSF treatment increased the number of proliferating cells in the subventricular zone and dentate gyrus and also increased the number of newborn neurons in the subventricular zone ipsilateral to the lesion.

Conclusions—These results suggest that G-CSF treatment in aged rats has a survival-enhancing capacity and a beneficial effect on functional outcome, most likely through supportive cellular processes such as neurogenesis. (Stroke. 2010; 41:1027-1031.)

Key Words: brain ischemia ■ neuroregeneration ■ stroke in aged rodents ■ stroke recovery

The hematopoietic factor granulocyte-colony stimulating factor (G-CSF) effectively reduces infarct size and improves functional outcome after various types of experimental stroke.1,2 The broad preclinical body of evidence of G-CSF’s efficacy in stroke studies is reflected by a close alignment along the Stroke Therapy Academic Industry Roundtable (STAIR) recommendations. In fact, G-CSF is currently viewed as 1 of the best preclinically studied candidate stroke drugs in recent years that has been translated into clinical development.3 One potential weakness of the preclinical data set is, however, the lack of proof in aged subjects. It is in fact a general drawback of preclinical evaluations of candidate stroke drugs that, due to cost-effectiveness and practicability, most studies have been conducted in young animals. A paucity of data from aged subjects in preclinical studies may at least partly explain the failure of candidate neuroprotective drugs in clinical trials. Relative to the young brain, the aged brain has an enhanced susceptibility to stroke and displays more limited recovery from ischemic injury.4-7 In the present study, we therefore assessed the treatment effects of G-CSF on mortality, behavioral function, infarct volume, and neurogenesis in aged animals.

Materials and Methods

Animals
Nineteen- to 20-month-old male Sprague-Dawley rats, bred in-house, were used for the study (body weight 520 to 600 g, 14 animals for placebo treatment, 15 animals for G-CSF treatment; provided by Department of Neurology, University of Greifswald, Germany). The numbers reported in the results refer to the number of animals that survived the surgery. The experiments reported in this study were conducted in accordance with the statement regarding the care and use of animals and were approved by an animal care committee of the Greifswald University.

Surgery
Cerebral infarction was induced by transcranial interruption of blood flow by transiently lifting the middle cerebral artery with a tungsten hook as previously described.4 Throughout surgery, anesthesia was maintained by spontaneous inhalation of 1% to 1.5% sevoflurane in a mixture of 75% nitrous oxide and 25% oxygen. Body temperature was maintained at 37°C by a Homeothermic Blanket System (Harvard Apparatus) and the tail artery was catheterized to enable the continuous measurement of blood pressure and the withdrawal of

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From the Department of Neurology (A.P.-W., A.B., C.M.), University of Greifswald, Greifswald, Germany; the Department of Molecular Medicine (A.B., C.M.), University of Medicine and Pharmacy, Craiova, Romania; and the Department of Neurology (K.S., A.R., K.D., J.M., W.-R.S.), EVK Bielefeld and University of Münster, Münster, Germany.
Correspondence to Wolf-Rüdiger Schäbitz, Department of Neurology, EVK Bielefeld, Burgsteig 13, Neurologische Klinik EVK Bielefeld, Lehrkrankenhaus der Universität Münster, Bielefeld, Germany 33617. E-mail Wolf.Schaebitz@evkb.de
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blood samples for determination of pH and blood gases (Blutgasystem II, 1620; Instrumentation Laboratory, Munich, Germany) as well as arterial glucose levels (Omnican 7 Balance; B. Braun, Melsungen, Germany) (Supplemental Table I, available online at http://stroke.ahajournals.org). The local changes in blood flow were monitored using a laser Doppler device (Perimed, Stockholm, Sweden), and blood gases were measured at several time points during ischemia. A decrease in the laser Doppler signal to <20% of control values was considered to indicate successful middle cerebral artery occlusion. After 90 minutes, the middle cerebral artery and the common carotid arteries were reopened. Subsequent to survival times of 28 days, the brain was removed, cryoprotected in 15% glycerol, and stored at −70°C until sectioning.

G-CSF Treatment and Bromodeoxyuridine Labeling
G-CSF treatment (n=15) was started 1 hour after restoring cerebral blood flow at a dose of 15 μg/kg G-CSF subcutaneously (Amgen, Munich, Germany) and continued daily for 15 days. The control group (n=14) was treated with physiological saline. In this study, we chose a dosage scheme that appears clinically safe and feasible for a treatment period of weeks, 15 days of 15 μg/kg. This application scheme is similar to the 1 used in the previous experiments using young rats.\(^3\) To label newly generated cells, rats were additionally treated with once-daily injections of bromodeoxyuridine (BrdU; 50 mg/kg body weight, intraperitoneally; Sigma-Aldrich, Munich, Germany) beginning in the first week poststroke and continued at alternating days for a total period of 14 days.

Behavioral Tests
The testing procedure involved 2 experimenters, 1 who performed the surgery and was in charge of handling the animals according to group assignment and another 1 who carried out the behavioral tests and who was not aware of the animals’ group assignments. To evaluate changes in neurological function associated with ischemia, the rats were subjected to a variety of somatosensory, motor, learning, and memory tests before and after surgery.\(^5\) All testing was performed from 9 to 11 AM. For the rotating pole task, the rats were trained daily for 2 weeks before stroke to master the task. Functional recovery was expressed as percent recovery relative to the presurgery baseline. For the inclined plane and radial-arm maze tests, no training before surgery was undertaken.

Rotating Pole
The rotating pole task assesses coordination and sensorimotor function in the middle cerebral artery occlusion model. Each rat was tested for its ability to cross a rotating (6 rpm) horizontal rod. The score assessment was done as previously described.\(^5\)

Inclined Plane
We tested the ability of each animal to maintain its position at a given angle on an inclined plane.\(^5\) The relative angle at which the rat could no longer maintain its position was taken as a measure of functional impairment. This test was conducted once before surgery and daily thereafter.

Spatial Working Memory: Radial-Arm Maze
Rats were tested on an 8-arm radial maze that was elevated 60 cm above the floor and had an octagonal central area. One of the 8 arms served permanently as a starting compartment, whereas the other 7 arms served as goal arms.\(^5\)

Determination of Infarct Volume
To assess the size of the infarct induced by permanent focal ischemia, every 20th section was stained for neuronal nuclei using NeuN antibodies (Millipore, Schwalbach, Germany). Digital images of the stained sections were taken to cover the entire infarcted area, which was then calculated as the sum of partial areas using the Scion image analysis software. Integration of the resulting partial volumes (partial areas × no. of sections × section thickness × section intervals) yielded the total volume of the ipsilateral hemisphere along with the total volume of the infarct. In previous studies, we have found that the disappearance of NeuN immunoreactivity is a reliable indicator that neurons have been lost.\(^4,5\)

BrdU Immunohistostaining
For detection of incorporated BrdU, we used cryostat, free-floating sections as previously described in detail.\(^6\) For double immunofluorescence, sections were incubated with NeuN antibody at 4°C overnight. The next day, sections were incubated with the secondary antibody for 4 hours. Then sections were brought onto Superfrost Plus slides and mounted with Vectashield Mounting medium (Vector Laboratories). The following antibodies were used: rat monoclonal to BrdU (1:250; Abcam), Alexa Fluor 594 goat antirat IgG (1:250; Invitrogen, Karlsruhe, Germany), mouse anti-NeuN (1:500; Millipore, Schwalbach, Germany), and Alexa Fluor 488 goat antimouse IgG (1:500; Invitrogen).

Counting Procedures
BrdU-positive cells were analyzed in 3 brain regions: the peri-infarct area, subventricular zone (SVZ), and dentate gyrus of both the contralateral and ipsilateral hemispheres. For the peri-infarct area, 4 squares (300 μm × 300 μm) adjacent to the infarct area were stereologically analyzed (Bregma 1 mm to −0.4 mm; counting frames of 70 μm × 70 μm) using a semiautomatic stereology system (StereoInvestigator; MicroBrightField, Colchester, Vt). Counting of BrdU cells in the SVZ was performed stereologically (counting frames of 70 μm × 70 μm) using the same 4 sections per animal as used to assess the peri-infarct area. BrdU-positive cells were counted in the whole area of the dentate gyrus using 3 sections per animal (Bregma 2.6 to −3.4). To estimate the percentage of neurons among the newly generated cells, 20 randomly selected BrdU-positive cells per region and animal were analyzed for BrdU/NeuN coimmunopositivity. Multiplying the total number of BrdU-positive cells with the percentage of NeuN/BrdU double-positive cells yielded the number of newborn neurons.\(^8,9\)

Statistical Analysis
The main effects of treatment and time as well as interactions of the 2 factors on recovery were evaluated using a mixed-model analysis of variance for each measure with treatment as between-subjects variables and time as a within-subject variable. The nonparametric data analysis was conducted using the Kruskal-Wallis test that is designed for multiple independent measures followed by a Bonferroni correction for alpha errors (SPSS Inc, Chicago, Ill). For rank analysis of mortality, we used the Mantel-Cox test. Neurogenesis results were analyzed by 1-way analysis of variance. A probability value of 0.05 was considered statistically significant.

Results
Mortality Rate and Infarct Volume
One of the remarkable effects of G-CSF treatment was a significant decrease in the mortality rate. In the control group, 3 rats died, 1 each on Days 7, 10, and 12, but none died in the treatment group (Figure 1A). There was no significant effect of G-CSF on reducing infarct volumes (G-CSF treated group 264±62 mm³ versus the control group at 215±36 mm³).

Behavioral Tests
After infarction, all rats had diminished performance on the first 3 days postsurgery, which was at least partially attributable to the surgical procedure itself. The bilateral sensorimotor coordination was assessed in 2 tasks: rotating beam-walking test (rotating pole) and inclined plane. On the inclined plane, a significant beneficial effect of the G-CSF treatment was evident between Days 6 and 9 poststroke (Figure 1B, open circles). In this test, control rats started recovery on Day 9 poststroke as...
compared with G-CSF-treated animals, which started to recover by Day 3, reaching the recovery levels of the G-CSF-treated rats on Day 12 (Figure 1B, filled circles). Thereafter, both groups recovered to a similar extent (96% on Day 28 poststroke).

After an abrupt decline in performance on the rotating pole on Day 3 poststroke, the G-CSF-treated rats began to improve, recovering to 90% of the optimum on Day 28 (Figure 1C, open circles). Control rats began to recover after a delay of 9 days as compared with G-CSF-treated animals (which started to recover by Day 3) and reached 78% of the baseline performance on Day 28. An overall significant, beneficial effect of the G-CSF treatment on the recovery of bilateral sensorimotor coordination was noted between Days 6 and 28 poststroke due primarily to the earlier recovery of the G-CSF-treated group (Figure 1C, open circles).

Recovery of spatial learning and memory based on positive reinforcement was assessed in the radial-arm maze. Performance of control animals in the radial maze deteriorated progressively during the first 9 days poststroke. Although control rats temporarily recovered to some extent between Days 9 and 15, the number of failures in this test remained high (Figure 1D, filled circles). Treatment with G-CSF significantly reduced the number of errors during the first 9 days of treatment (Figure 1D, open circles). At later time points, however, both groups performed similarly with a substantial rate of failures.

Neurogenesis Assessment
To assess whether G-CSF treatment led to an increased number of newborn cells in the ischemic peri-infarct area, we first counted the number of BrdU-positive cells. G-CSF treatment exerted no significant effect on the total number of BrdU-labeled cells in the peri-infarcted area compared with vehicle treatment (Figure 2B). However, stereological analysis of the number of BrdU-labeled cells in the SVZ showed a significant increase in the number of BrdU-positive cells in G-CSF-treated animals as compared with vehicle-treated animals (Figure 2A).

The analysis of neurogenesis in the dentate gyrus revealed a significant increase in the number of BrdU-positive cells in the infarcted hemisphere of G-CSF-treated animals as compared with vehicle-treated animals in the SVZ of the infarcted hemisphere was significantly increased by the G-CSF treatment (Figure 2E–F).

Discussion
G-CSF treatment in aged rats led to a reduced mortality, an improved functional recovery of motor function, and an increased number of proliferating cells and newborn neurons in the SVZ of the infarcted hemisphere.

The ultimate goal of stroke treatment is restoration of neurological function. Recent developments indeed focused not only on brain tissue protection, but rather on recovery-enhancing mechanisms of drug candidates for stroke treatment. Well-explored examples are, for instance, the hematopoietic growth factors G-CSF and erythropoietin, both effective compounds that interact multimodally within stroke pathophysiology. G-CSF in particular has been shown to improve neurological function after various types of focal cerebral ischemia. A limitation of this considerable data set, however, is that all studies to date have been executed in young adult animals.
For the first time, we have shown in the present study a robust recovery of running function in aged animals over the entire testing period of 28 days. Significant improvements also were seen for coordinative motor function (inclined plane) and cognitive function (radial-arm maze). However, this effect lasted just for the first 12 days after stroke onset, corresponding approximately to the active G-CSF treatment period. The results suggest that, at least in aged animals, longer and more intensive G-CSF treatment periods could be necessary to maximize the effect on improvement of poststroke recovery of neurological function. In addition to enhanced recovery of poststroke motor and cognitive function, G-CSF treatment clearly reduced mortality in aged rats after focal cerebral ischemia: 3 animals in the control group died on Days 7, 10, and 12, whereas none of the treated animals died. These results corroborate the findings of earlier studies in young mice and rats, in which a single intravenous treatment with G-CSF reduced mortality after middle cerebral artery occlusion.1 In general, this survival-enhancing capacity of G-CSF in older subjects may be interpreted as a strong treatment effect inasmuch as aged rats have an increased stroke susceptibility and a higher mortality rate after stroke.4-7

Meta-analysis of previous studies in young rats suggests that G-CSF treatment can reduce infarct size up to 0.84% and 2.06% per 1 μg/kg body weight2. In the present study, an infarct-reducing effect of G-CSF treatment was not observed, most likely because the low dose of G-CSF (15 μg/kg body weight) may not be sufficient for neuroprotection. In addition, necropsies performed after death have revealed that the control rats that died prematurely had large infarcts, which may help to explain why the infarct volumes in surviving animals were similar in the 2 groups. In all studies in which G-CSF exerted a clear neuroprotective and infarct-reducing effect, doses of 50 to 60 μg/kg were used. These findings are supported by the recent meta-analysis that shows a clear dose-related effect of G-CSF’s infarct-reducing capacities. Furthermore, the functional relevance of infarct size for behavioral recovery has been questioned in a recent MRI study that compared lesion size and neurological function at longer poststroke intervals, showing a lack of correlation between lesion size and functional deficit.13

Recent data indicate that neurological function is more impaired by stroke in aged rats than in young rats, limiting the recovery of neurological function in older animals.5 Further-
more, aged rats show an accelerated infarct development in the first week poststroke as compared with their young counterparts. All of these factors together complicate therapeutic strategies in the elderly. Indeed, 1 recent study has shown that coadministration of a plasminogen activator inhibitor type 1-derived peptide, EEIIMD, with tissue plasminogen activator, a drug currently used for thrombolysis in stroke units, produced no improvement in total infarct volume, edema formation, or functional outcome in aged rats.

Improvement of function under treatment may suggest a direct effect of G-CSF on recovery of motor function and may be similar to our recent findings showing that G-CSF is an essential factor for the development of normal running function in wild-type and G-CSF−/− mice.9 Potential mechanisms include a relative shift in favor of activation (upregulation of NMDA receptors, downregulation of GABA receptors) and improved behavioral performance associated with G-CSF stimulation. G-CSF administration may improve neuronal survival also indirectly by inhibition of apoptosis,15,16 increased supportive angiogenesis17 and/or by its ability to inhibit excitotoxicity after ischemic stroke.18 Likewise, administration of G-CSF alone or in combination with stem cell factor during chronic stroke improves functional outcomes, possibly by facilitating the proliferation of endogenous neural stem cells and enhancing cytokine-induced generation of neuronal cells from bone marrow-derived cells.19,20

G-CSF treatment was recently shown to induce substantially more neural progenitor cells and immature neurons in subcortical regions adjacent to the infarcted area. G-CSF also increased neurogenesis in the dentate gyrus of the hippocampus.13 This cytoregenerative effect in young adult animals was confirmed in the present study in aged rats in the SVZ ipsilateral to the lesion. Although G-CSF treatment in aged rats increased the number of proliferating cells in the dentate gyrus and in the SVZ, we found more newborn neurons only in the SVZ of the damaged hemisphere as compared with the ipsilateral hemisphere of vehicle-treated rats.

In conclusion, the results of our study show, for the first time, that G-CSF treatment in aged rats after stroke enhances survival, functional neurological recovery, and induces neurogenesis. These findings are important for the further clinical development of the drug in elderly patients with stroke. Future studies should focus on the optimization of the G-CSF treatment schedule to achieve optimal poststroke recovery enhancement in aged subjects.

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Disclosures
W.R.S. is an inventor on a patent regarding the neuroprotective effects of G-CSF.

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