The adaptive proliferation of preexisting collateral pathways (arteriogenesis) is an effective biological rescue system against detrimental effects of arterial stenosis. We have recently demonstrated that occlusion of the left carotid artery (CA) and both vertebral arteries induced a significant redistribution of blood flow via the left posterior cerebral artery (PCA), which increased its diameter 2-fold. Importantly, the induced hemispheric hypoperfusion was not severe enough to produce expression of the angiogenic factor VEGF (vascular endothelial growth factor) or histological injury. On the functional level, we demonstrated that this adaptive arteriogenesis led to a significant improvement of the hemodynamic capacity of the hypoperfused brain. Thus, we proposed a novel concept of brain protection by arteriogenesis: collateral artery growth as a means to prevent cerebral ischemia during progressing cerebrovascular disease.1

Several experimental studies have shown that the speed of arteriogenesis is not limited to its natural time course. The infusion of CC chemokines (monocyte chemotactic protein-1),2 fibroblast growth factors,3 or granulocyte-macrophage colony-stimulating factor (GM-CSF) into the peripheral or coronary collateral circulation led to a significant increase in collateral conductance compared with untreated animals. The proarteriogenic effect of the latter was explained by the prolongation of the life cycle of monocytes/macrophages, which invade proliferating collateral pathways.4 A recent clinical trial demonstrated a positive effect of GM-CSF on therapeutically enhanced arteriogenesis in a small cohort of patients with coronary artery disease.5

Patients with cerebrovascular disease have not yet profited from treatments aimed at the growth of brain vessels. Although several reports documented the therapeutic stimula-
tion of angiogenesis in the brain, these studies failed to demonstrate improvement of stroke outcome. This is not surprising, because angiogenesis is too slow to compensate for the sudden decline of flow after acute vascular occlusion.

In the present study, we therefore focused on arteriogenesis, which differs from angiogenesis in that it substitutes arterial collaterals for the occluded artery. In contrast to capillaries, these arterial pathways are potentially able to act as conduits that supply enough blood from outside the risk region to compensate for the increased resistance of the obstructed or occluded artery.

Here, we tested the hypothesis that arteriogenesis, beyond its natural time course, can be therapeutically enhanced in the brain. To the best of our knowledge, this is the first experimental study to investigate the effects of therapeutic arteriogenesis in the brain.

Methods
Experiments were performed according to the German Law for Protection of Animals and the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

Arteriogenesis Brain Model
A nonlethal brain hypoperfusion model (3-vessel occlusion [3-VO]) was used to investigate cerebral arteriogenesis. Sprague-Dawley rats (weight, 290 to 330 g; Harlan-Winklemann, Borchem, Germany) were submitted to occlusion of the left CA and both vertebral arteries and enrolled into the study as follows: control group, nonoperated (n=8); untreated (saline) group (n=8); and treated group (GM-CSF; 40 μg · kg−1 · d−1; n=13), which were compared at 1 and 3 weeks after 3-VO. Solutes were applied intraoperatively by a single intra-arterial dose into the nonoccluded CA and subsequently by subcutaneous injection every second day. At the end of experiments, animals were perfused with either colored latex for visualization of their cerebrovascular anatomy or 4% paraformaldehyde for histological evaluation.

CO2 Reactivity of Cerebral Blood Flow
We tested cerebrovascular reserve by ventilating animals with 6% CO2 and recording the associated changes in cerebral blood flow. CO2 reactivity (CO2R) was expressed as percent increase of blood flow in parietal cortex per mm Hg arterial PCO2 change. Flow changes were recorded from both hemispheres by laser-Doppler flowmetry. Measurements of CO2R were performed 3 times per animal; between each measurement, the arterial PCO2 level was allowed to return to the physiological range.

Visualization of Cerebral Angioarchitecture
At the end of experiments, cerebrovascular anatomy was studied by a modification of the postmortem latex perfusion method of Maeda et al.10 The external diameter of the PCA was measured with a stereozoom microscope with a calibrated eyepiece micrometer. The diameter of Heubner’s anastomoses connecting the peripheral branches of the anterior and middle cerebral arteries was assessed on the dorsal surface of the brain.

Histology and Immunohistochemistry
The brains of 3-VO rats were examined histologically to exclude neuronal damage. Cerebral vasculature was perfused with 4% paraformaldehyde at 3 days after 3-VO, embedded in paraffin, and sectioned for immunohistochemistry. Antibody against Ki-67 antigen (Dako) was used to identify cell proliferation after heat-induced epitope retrieval. Antibody recognizing ED-1 antigen of rat monocytes and macrophages (Acris) was used to identify adventitial mononuclear cells.

Data Analysis
All data are given as mean±SD. Group differences for hemodynamic, morphological, and general physiological measurements were analyzed for statistical significance by Student’s t test. Statistical significance was assumed for P<0.05.

Results
Cerebral Angioarchitecture of Dorsal Brain Vessels After 3-VO
Given that GM-CSF enhances arteriogenesis in the rabbit hindlimb model after subcutaneous injection, we investigated whether the growth of preexisting collateral pathways was inducible in the brain via the same route of GM-CSF administration. GM-CSF treatment did not influence the arterial angioarchitecture on the dorsal brain surface (Figure 1). After systemic injection of colored latex, there was no detectable difference of Heubner’s anastomoses connecting the dorsal branches of the anterior and middle cerebral arteries. In the nonischemic control group, we measured an average diameter of 43.0±3 μm. At 1 week after 3-VO, the untreated group showed a mean diameter of 47.7±9 μm compared with 47.3±7 μm after GM-CSF treatment.

PCA as Main Collateral Pathway
At the level of the circle of Willis, untreated 3-VO led to a significant increase in the diameter of the left PCA, measured after maximal vasodilation by latex infusion (Figure 1). Compared with the nonischemic control animals (187±27 μm), there was a marked increase after 1 week (260±37 μm) and even more so after 3 weeks (322±50 μm). The increase in diameter during natural arteriogenesis could be therapeutically enhanced by application of GM-CSF. In rats treated every second day with GM-CSF, the diameter of the PCA was 62 μm larger (322±33 μm) after 1 week and 59 μm larger (380±107 μm) after 3 weeks than in the untreated 3-VO group (Figure 2).

GM-CSF Restores CO2R of Cerebral Blood Flow
To define the functional importance of changes in diameter, we measured blood flow in both hemispheres by laser-Doppler flowmetry under normal air ventilation and during ventilation with additional 6% CO2 (Figure 3). The increase in arterial PCO2 under CO2 ventilation reached comparable levels in all 3 groups. In nonischemic control animals, laser-Doppler flowmetry increased during 6% CO2 ventilation by 1.48±0.3% and 1.1±0.2% per mm Hg arterial PCO2 in the left and right hemisphere, respectively. One week after 3-VO, untreated animals showed a severe reduction of CO2R. In the left hemisphere (ie, ipsilateral to the occluded CA), CO2R declined to 0.00±0.35%, and in the right hemisphere, it declined to 0.18±0.6% per mm Hg arterial PCO2. These values represent 4% and 16% of the control response, respectively. At 3 weeks after 3-VO, CO2R of untreated animals improved slightly on the left side to 0.48±0.08% and on the right side to 0.30±0.39% per mm Hg arterial PCO2, representing 32% and 27% of the control response, respectively.

After GM-CSF treatment, the hemodynamic reserve of the brain almost completely recovered. In the left hemisphere, CO2R...
returned to $1.43 \pm 0.68\%$ per mm Hg arterial $pCO_2$ after 1 week and to $1.16 \pm 0.44\%$ per mm Hg after 3 weeks, ie, 97% and 78% of the control response, respectively. In the right hemisphere, the corresponding values were $1.12 \pm 1.1\%$ and $0.7 \pm 0.16\%$ per mm Hg arterial $pCO_2$ after 1 and 3 weeks. These values represent 101% and 64% of control and reflect the substantial therapeutic improvement by GM-CSF (Figure 4).

**ED-1–Positive Macrophages Invade Proliferating Collateral Pathways**

Immunohistochemistry with antibody recognizing ED-1 antigen from rat revealed significant differences in the numbers of macrophages accumulating in the adventitia of proliferating collateral pathways. In the nonischemic control group, mononuclear cells were rarely detectable. 3-VO without treatment resulted in the appearance of a small number of macrophages in the adventitia of the PCA. With GM-CSF treatment, the number of these mononuclear cells increased markedly (Figure 5). Cell proliferation (Ki-67 staining of PCA) was also more prominent in GM-CSF–treated animals than in untreated rats.

**Discussion**

The potential of the circle of Willis to provide alternate flow routes in case of diminished arterial supply to the brain has been known since Sir Thomas Willis first described the collateral function of the arterial anastomoses in 1664. It has also long been known that the luminal width of anastomoses is a major determinant of the quality of blood perfusion in the territory of the occluded artery and of the amount of tissue that can be protected from infarction by collateral circulation.11

In addition to these passive hemodynamic properties of collateral anastomoses, previous reports documented an active adaptation by arteriogenesis that was not limited to its natural time course but could be enhanced therapeutically.12 In a rabbit hindlimb hypoperfusion model (femoral artery ligation), the continuous intra-arterial application of CSFs significantly enhanced collateral artery growth measured via the fluorescent microsphere technique (5-fold increase compared with control).4

These data prompted us to investigate the role of arteriogenesis in the brain. As previously demonstrated, 3-VO produces a nonlethal type of brain hypoperfusion...
that, with ongoing ischemia time, leads to a gradual improvement of collateral blood supply. However, in many cases, the spontaneous proliferation of collateral pathways does not prevent or only partially prevents the detrimental effects of vascular occlusion, because the speed of arteriogenesis is too slow to compensate for a sudden blood flow deficit.

The functional end point in the present study was the hemodynamic reserve of the brain, as determined by measuring the change of blood flow during ventilation with 6% CO₂. This test evaluates the autoregulatory capacity of the cerebrovascular system under conditions of reduced blood supply. CO₂R fails as soon as brain vessels are fully dilated, indicating that the brain vasculature is no longer able to compensate for any further reduction of blood supply. Although the present study does not provide the “gold standard” technique of collateral conductance measured via microspheres, we present 4 novel findings: (1) GM-CSF was found to induce an enlargement of PCA caliber on 3-VO; (2) GM-CSF was found to improve functional brain hemodynamic parameters on 3-VO, such as CO₂R; (3) the morphological and functional changes observed could be induced via subcutaneous application of GM-CSF; and (4) GM-CSF was found to enhance the invasion of mononuclear cells (macrophages) at the site of vascular collateral proliferation. We propose that the effects seen most likely reflect arteriogenesis.

In the present 3-VO model, CO₂R was completely suppressed shortly after vascular occlusion, but under GM-CSF treatment, we observed a return to normal within 1 week. To the best of our knowledge, this is the first study that demonstrates an improvement of brain hemodynamic parameters after such treatment. Our angiographic studies demonstrate that this improvement was mainly due to enlargement of the PCA, a vessel that is far from the area of reduced blood flow. Arteriogenesis is therefore spatially

Figure 3. Representative recordings of laser-Doppler flow in parietal cortex during ventilation with 6% CO₂. Comparison of control rats with untreated and GM-CSF–treated animals at 1 week after 3-VO. Note suppression of CO₂-induced increase in blood flow in ipsilateral hemisphere of untreated rat and complete restitution of CO₂R after GM-CSF treatment.

Figure 4. CO₂R of blood flow in normal rat (control) and at 1 and 3 weeks after 3-VO without and with GM-CSF treatment. Blood flow was measured during ventilation with 6% CO₂ by laser-Doppler flowmetry (LDF) in parietal cortex of ipsilateral and contralateral hemispheres. After detection of baseline flow, CO₂ was added to ventilation gases; we measured change of LDF flow and calculated percentage of change per 1 mm Hg of arterial pCO₂ (apCO₂). CO₂R is expressed as percent change of LDF per mm Hg increase of arterial pCO₂. Note restoration of suppressed reactivity after GM-CSF treatment. *P<0.05; **P<0.01; ***not significant to control.
enzyme activity, as well as reduced expression of proarteriogenic factors. These findings support the concept that GM-CSF treatment could enhance collateral blood flow in the presence of arterial stenosis.

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References


Therapeutic Induction of Arteriogenesis in Hypoperfused Rat Brain Via Granulocyte-Macrophage Colony-Stimulating Factor

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