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

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Background—Colony-stimulating factors (CSFs) have been shown to effectively induce arteriogenesis in the hindlimb. Moreover, clinical trials demonstrated positive effects of CSFs on arteriogenesis in patients with coronary artery disease. However, patients with cerebrovascular disease have not yet profited from treatments aimed at the growth of brain vessels. Thus far, angiogenesis studies have failed to demonstrate improvement of stroke outcome. Arteriogenesis differs from angiogenesis in that it substitutes arterial collaterals for the occluded artery.

Methods and Results—We tested in a novel brain arteriogenesis rat model (occlusion of vertebral plus left carotid artery [3-VO]) the application of CSFs or saline over 7 or 21 days. On 3-VO postmortem, latex perfusion demonstrated a time- and treatment-dependent arteriogenesis of the posterior cerebral artery (PCA). In saline-treated animals, the PCA diameter increased by 39%; in granulocyte-macrophage (GM)-CSF-treated animals, this increase was significantly faster (72% after 1 week). Functionally, saline-treated animals exhibited a decline of CO2 reactivity (mm Hg) from 1.48% to 0.1% compared with GM-CSF–treated animals (1.43% arterial pCO2 change after 1 week). This difference remained significant after 3 weeks. This functional improvement correlated with increased numbers of CD68-positive macrophages in histological sections of the PCA in GM-CSF–treated animals and only a few macrophages in saline-treated animals.

Conclusions—To the best of our knowledge, this is the first report of stimulation of arteriogenesis in the brain. The subcutaneous application of GM-CSF led to functional improvement of brain hemodynamic parameters. (Circulation. 2003;108:610-615.)

Key Words: arteries ■ stroke ■ cerebrovascular circulation ■ drugs

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, Borechen, 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·h⁻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. 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 0.006±0.035%, 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% CO_2. This test evaluates the autoregulatory capacity of the cerebrovascular system under conditions of reduced blood supply. CO_2R 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 caliper on 3-VO; 2. GM-CSF was found to improve functional brain hemodynamic parameters on 3-VO, such as CO_2R; 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, CO_2R 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% CO_2. Comparison of control rats with untreated and GM-CSF-treated animals at 1 week after 3-VO. Note suppression of CO_2-induced increase in blood flow in ipsilateral hemisphere of untreated rat and complete restitution of CO_2R after GM-CSF treatment.

Figure 4. CO_2R 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% CO_2 by laser-Doppler flowmetry (LDF) in parietal cortex of ipsilateral and contralateral hemispheres. After detection of baseline flow, CO_2 was added to ventilation gases; we measured change of LDF flow and calculated percentage of change per 1 mm Hg of arterial pCO_2 (apCO_2). CO_2R is expressed as percent change of LDF per mm Hg increase of arterial pCO_2. Note restoration of suppressed reactivity after GM-CSF treatment. *P<0.05; **P<0.01; † † not significant to control.
dissociated from the ischemic territory. This is in accordance with hindlimb hypoperfusion studies in which arteriogenesis was observed in the proximal oxygen-rich tissue, whereas ischemia was present in the peripheral regions. Angiogenesis, in contrast, is observed mainly within the region of reduced blood flow. In a cohort of 10 patients with survival times of 5 to 92 days after their stroke, capillary sprouting took place mainly in the region of the penumbra, but the physiological relevance of this phenomenon remained unclear. Obviously, an occluded cerebral artery cannot be replaced by capillaries, regardless of the numbers. This is different from arteriogenesis, in which the proliferation of arterial collaterals may compensate for the reduced conductance of a large artery.

The present study therefore focused on the arterial level of the collateral circulation rather than on the capillary network. At the level of the circle of Willis, GM-CSF treatment led to a significant increase in vessel diameter of the PCA. Immunohistochemistry revealed high numbers of periadventitial macrophages compared with untreated 3-VO and control animals, respectively. Similar observations have been made in peripheral arteriogenesis studies. After ligation of the arteria femoralis in the rabbit, shear forces in preexisting collateral arteries increase, which leads to upregulation of intercellular adhesion molecule-1. Additionally, fluid shear stress increases the production of GM-CSF in endothelial cells via mRNA stabilization. These factors combine to effectively recruit circulating cells, particularly monocytes and their pluripotent precursors, to the sites of collateral artery growth. The monocytes mature into macrophages that produce large amounts of growth factors (monocyte chemotactic protein-1 and basic fibroblast growth factor) and degrading enzymes (metalloproteinases). These factors, in turn, create an inflammatory environment that is necessary for arteriogenic remodeling. Increased activation and attraction of circulating mononuclear cells via intra-arterial infusion of proarteriogenic factors into the collateral circulation can significantly influence this process of adaptive muscular collateral artery growth.

From a clinical perspective, the present study points to a novel therapeutic concept for the treatment of patients with stenosis of extracranial and intracranial arteries, who in fact exhibit a high incidence of transitory ischemic attacks or infarction. Despite recent advances in acute management, such as endarterectomy and anticoagulation, as well as primary and secondary prevention of stroke risk factors, noninvasive pharmacological approaches to enhance collateral blood flow of the brain in the presence of arterial stenosis are lacking. GM-CSF is a powerful survival factor for monocytes/macrophages that may induce arteriogenesis in such patients to enhance their collateral capacity and reduce the risk of brain infarction. This is supported by recent findings that demonstrate that GM-CSF treatment reduces the severity of metabolic injury when 3-VO is aggravated by induced hypotension (E. Schnellocch, DVM, et al, unpublished data). Studies are under way to further explore the functional benefits of GM-CSF–induced arteriogenesis on impeding brain ischemia.

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