Myoblast-Mediated Gene Therapy Improves Functional Collateralization in Chronic Cerebral Hypoperfusion

Nils Hecht, MD; Aiki Marushima, MD; Melina Nieminen, MSc; Irina Kremenetskaia, MSc; Georges von Degenfeld, MD; Johannes Woitzik, MD; Peter Vajkoczy, MD

Background and Purpose—Direct extracranial–intracranial bypass surgery for treatment of cerebral hemodynamic compromise remains hindered by complications but alternative simple and safe indirect revascularization procedures, such as an encephalomyosynangiosis (EMS), lack hemodynamic efficiency. Here, the myoblast-mediated transfer of angiogenic genes presents an approach for induction of therapeutic collateralization. In this study, we tested the effect of myoblast-mediated delivery of vascular endothelial growth factor-A (VEGF) to the muscle/brain interface of an EMS in a model of chronic cerebral hypoperfusion.

Methods—Permanent unilateral internal carotid artery-occlusion was performed in adult C57/BL6 mice with or without (no EMS) surgical grafting of an EMS followed by implantation of monoclonal mouse myoblasts expressing either VEGF164 or an empty vector (EV). Cerebral hemodynamic impairment, transpial collateralization, angiogenesis, mural cell investment, microvascular permeability, and cortical infarction after ipsilateral stroke were assessed by real-time laser speckle blood flow imaging, 2- and 3-dimensional immunofluorescence and MRI.

Results—VEGF-expressing myoblasts improved hemodynamic rescue by day 14 (no EMS 37±21%, EV 42±9%, VEGF 48±12%; P<0.05 for VEGF versus no EMS and versus EV), together with the EMS take rate (VEGF 60%, EV 18.2%; P<0.05) and angiogenesis of mature cortical microvessels below the EMS (P<0.05 for VEGF versus EV). Importantly, functional and morphological results were paralleled by a 25% reduction of cortical infarction after experimental stroke on the side of the EMS.

Conclusions—Myoblast-mediated VEGF supplementation at the target site of an EMS could help overcome the clinical dilemma of poor surgical revascularization results and provide protection from ischemic stroke. (Stroke. 2015;46:00-00.)

Key Words: cerebral revascularization • cerebrovascular disease • gene therapy

The results of the recently published Carotid Occlusion Surgery Study put the benefit of direct surgical flow augmentation for treatment of atherosclerotic hemodynamic compromise into question and show that extra- to intracranial bypass grafting remains technically challenging and carries a perioperative stroke risk of up to 15%.1,2 Despite this development, however, there still remain clear indications for treatment of cerebral hemodynamic compromise through surgical revascularization, such as multiple vessel disease or Moyamoya disease, in particular.

To reduce the risk of perioperative morbidity, a technically simple and safe solution for extracranial–intracraniial flow augmentation is needed. For this purpose, a variety of indirect revascularization techniques have been developed. One of the most commonly applied procedures for indirect revascularization is termed encephalomyosynangiosis (EMS), which describes the placement of a vascularized temporal muscle graft onto the hypoperfused cortical surface and results in spontaneous transpial collateralization of distal intramuscular branches of the external carotid artery (ECA) with the cortical vasculature of the brain.3–5 Importantly, an EMS has the advantage of being less complex and safer than direct bypass surgery with proven benefit in patients with Moyamoya disease.6–8 Although indirect revascularization seems attractive, it unfortunately lacks hemodynamic effectiveness compared with direct procedures.9,10 For this reason, we hypothesized that a continuous and local boosting of proangiogenic activity at the muscle/brain interface of an EMS may present a novel approach to facilitate indirect revascularization as a technically simple and safe procedure.

The key factor for compensation of hemodynamic compromise is endogenous flow augmentation through outgrowth of...
pre-existing collaterals. This requires an active proliferation of endothelial and perivascular cells, which is naturally limited in the brain. Effective collateralization through an EMS requires the outgrowth and remodeling of pre-existing vessels (arteriogenesis) to form patent collaterals between the extracranial and intracranial vascular beds. In this regard, recent evidence convincingly demonstrated that vascular endothelial growth factor-A (VEGF) acts as a key factor in formation, postnatal maturation, and establishment of adult collateral density in the brain. However, VEGF delivery for the purpose of therapeutic neovascularization is often hampered by the formation of aberrant blood vessels and hemangiomas. In this regard, experimental studies in nonischemic and ischemic tissue have evidenced that an appropriate microenvironmental VEGF concentration is paramount for induction and maintenance of functional neovascularization at a desired target site.

Hence, the DNA transfer to myogenic precursor cells seems to be an ideal drug delivery strategy to ensure a consistent, long-term and regionally circumscribed overexpression of VEGF for effective induction of neovascularization at the muscle/brain interface of an EMS. Recently, we demonstrated the general feasibility of this approach by successful implantation and fusion of primary monoclonal mouse myoblasts in the nonischemic temporal muscle of an experimental EMS. We now tested whether a myoblast-mediated local delivery of murine VEGF to the muscle/brain interface of an EMS may improve morphological revascularization and functional outcome in a model of cerebral hypoperfusion.

Methods

Ethics Statement

Experiments were permitted by the local ethics committee on animal research (LaGeSo No. G 0262/07, Berlin, Germany) and in conformity with the German Law for Animal Protection and the National Institute of Health Guidelines for Care and Use of Laboratory Animals.

Mouse Myoblast Purification, Retroviral Infection, and Selection

The isolation, culture, and retroviral infection of primary myoblasts is described elsewhere in detail. Briefly, primary mouse myoblasts were transduced with a constitutive LacZ-encoding retrovirus alone (Empty Vector [EV]) or together with a murine VEGF encoding retrovirus (VEGF). For this purpose, flow cytometry-isolated single cells of the VEGF-expressing population were expanded into monoclonal populations and VEGF secretion was monitored periodically by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). On the basis of previous functional and morphological results, a monoclonal VEGF population expressing >70 ng VEGF/10^6 cells/day (defined as the 100% clone in relation to the average VEGF expression level of the polyclonal VEGF parent population) was selected for implantation.

Animals and Experimental Design

Seventy-three male C57/BL6 mice (Charles River WIGA GmbH, Sulzfeld, Germany), aged 12 weeks (28–31 g) were randomized and the following procedures were performed in a blinded fashion:

1. VEGF (n=24): Unilateral (right) internal carotid artery-occlusion (ICA-O) with ipsilateral EMS and implantation of VEGF-expressing myoblasts into the temporal muscle of the EMS (4×10^6 cells).
2. EV (n=25): ICA-O with EMS and implantation of EV myoblasts as biological control (4×10^6 cells).
3. No EMS (n=16): ICA-O and no EMS.
4. Sham (n=8): Sham ICA-O and no EMS.

For all procedures, mice were anesthetized with 70 mg/kg ketamine and 16 mg/kg xylazine and body temperature was maintained at 37°C. Before and after all procedures, mice had free access to food and water. All data about transpial collateralization, cortical perfusion, histology, and stroke volume were obtained and analyzed in a blinded fashion.

EMS and Ipsilateral ICA-O

The ICA-O was performed immediately before the EMS procedure at a time point defined as day 0 and is described elsewhere in detail. Briefly, the animal was positioned supine and the right-sided ICA was permanently ligated with an 8/0 silk suture. Next, the animal was turned to prone position and a right-sided craniectomy was performed along the superior temporal line to the temporal scull base, extending from the bregma to the lambdoid suture using a diamond-tip micro drill (Proxxon GmbH, Warburg/Aist, Austria). The dura was completely excised along the margin of the craniectomy. To secure the temporal muscle above the cortical surface after myoblast implantation (see below), the overlying muscle fascia was sutured to the contralateral aponoeurys and the skin was readapted with 6/0 Nylon.

Myoblast Implantation

For implantation, cultured myoblasts were trypsinized and resuspended in phosphate-buffered saline with 0.5% bovine serum albumine. On the basis of previous results, we implanted either 4×10^6 EV or VEGF myoblasts per animal in two 5-μL cell suspensions (each containing 2×10^5 cells) by injection into the temporal muscle using a Hamilton microsyringe with a 26-gauge needle (Hamilton Co., Reno, NV) immediately before completing the EMS procedure on day 0.

Laser Speckle Imaging and Cerebrovascular Reserve Capacity

Baseline (resting) perfusion and hemodynamic impairment was quantified on days 3, 7, 14, and 21 by assessment of the acetazolamide-specific cerebrovascular reserve capacity (CVRC) with laser speckle imaging as described previously in Sham- (n=8), no EMS- (n=10), EV- (n=15), and VEGF (n=14)-treated animals (please see the online-only Data Supplement).

FITC-Lectin Perfusion and Assessment of Transpial Collateralization

On day 21, transpial collaterals in the muscle/brain border region of the EMS were determined by 3-dimensional confocal microscopy after an in vivo fluorescein isothiocyanate (FITC)-lectin perfusion in a random subset of animals (no EMS n=6; EV n=11; VEGF n=10). To determine the blood delivery via the muscle graft and the transpial collaterals, we injected FITC-lectin via the ECA ipsilateral to the side of the EMS (please see the online-only Data Supplement). For analysis, 60-μm coronal cryosections were obtained from the bregma level ±0.0 to −4.5 mm in 0.5 mm intervals and observed under a laser-scanning microscope (LSM710, Zeiss, Oberkochem, Germany). To better characterize the positive or negative development of transpial collaterals, we defined the EMS take rate parameter: In each section, the total number of sections with positive transpial collateralization was determined positive only after direct observation (≥45% of the vascular space covered by the transpial collaterals to the cortex) and (2) a distinct association of these vessels with the resident vasculature of the cortical region below the EMS. The total number of sections with positive transpial collaterals was counted in each animal and the individual EMS was only rated positive if collaterals were noted in ≥50% (≥25/10) of all
section levels. The EMS take rate was calculated as the percentage of positive EMS and compared between animals with no EMS, EV, or VEGF treatment.

**Immunohistochemistry**

In the remaining subset of animals (no EMS n=4, EV n=4, VEGF n=4), vessel density and pericyte coverage in the muscle/brain border region of the EMS on day 21 were determined by immunohistochemistry. Coronal cryosections (6 μm) of nonperfused, snap-frozen, whole-head specimens were obtained from the anterior, middle, and posterior region of the EMS (Bregma −0.5, −1.5, and −3.0 mm, respectively) and a combined CD31/Desmin stain was performed. Vessel density within the cortical area below the EMS and the associated temporal muscle was calculated as CD31-positive vessels per mm². Pericyte coverage was expressed as the percentage of CD31-positive vessels with Desmin colocalization. In 3 VEGF-treated animals that underwent FITC-lectin perfusion, cell proliferation at the muscle brain interface and in the cortical region below the EMS was visualized with a combined CD31/Ki67 staining. In a second series of animals (EV n=4, VEGF n=4), half of which underwent middle cerebral artery filament-occlusion (MCA-O) on day 21, the effect of VEGF on vascular permeability in the ischemic/nonischemic cortex below the muscle/brain interface was assessed after in vivo Evans Blue perfusion and CD31 staining. Sections were mounted and observed under a fluorescence-enhanced microscope (Axio Imager 2; Zeiss; please see the online-only Data Supplement).

**Middle Cerebral Artery Occlusion and Cortical Stroke Volume Assessment**

In a third series of animals (no EMS n=6; EV n=6; VEGF n=6), cortical stroke volume was assessed by MRI after 60-minute MCA-O and 23-hour reperfusion on day 21 (please see the online-only Data Supplement). Laser Speckle Imaging was used to visualize the effect of VEGF on the cortical perfusion pattern over the affected hemisphere during the 60-minute occlusion in the second series of mice used for assessment of vascular permeability (EV n=2; VEGF n=2; see above). The timeline of the experiments is depicted in Figure 1.

**Statistical Analysis**

Data are presented as mean±SD or percentage. Statistics were performed with GraphPad Prism for Mac (Version 5.0f, GraphPad Software, San Diego, CA). Statistical significance was set at *P*<0.05. For a comprehensive description, please see the online-only Data Supplement.

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**Results**

**Transpial Collateralization at the Muscle/Brain Interface**

On day 21 after ICA-O, animals with no EMS did not show signs of spontaneous extracranial–intracranial collateralization across the interface of the temporal muscle and the brain after FITC-lectin perfusion through the ECA on the side of ICA-O (Figure 2A). In contrast, grafting of an EMS with implantation of EV myoblasts yielded patent FITC-positive vessel bridges crossing from the temporal muscle into the brain as a sign of spontaneous EMS collateralization with an 18% EMS take rate among the EV-treated animals. This 18% EMS take rate improved significantly to 60% in animals that received an EMS with implantation of VEGF<sub>164</sub> expressing myoblasts (*P*<0.05 versus EV) with distinct signs of proangiogenic outgrowth, such as a more tortuous morphology of

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**Figure 2.** Vascular endothelial growth factor-A (VEGF)<sub>164</sub>-expressing myoblasts improve encephalomyosynangiosis (EMS) take rate. A. Fluorescein isothiocyanate (FITC)-lectin injection in an animal without EMS and only internal carotid artery-occlusion (ICA-O; no EMS) fails to show FITC-lectin positive fluorescence within the cortical vasculature. B. Vasculature at the muscle/brain interface and within the cortical parenchyma below the EMS shows positive FITC-lectin fluorescence and tortuous transpial vessel sprouts transgressing from the temporal muscle into the cortex after implantation of both empty vector (EV) myoblasts (upper panels) and VEGF<sub>164</sub> expressing myoblasts (VEGF; lower panels). The dashed rectangle indicates the area of detail enlargement on the right. C. Bar graph illustrating the significantly higher EMS take rate after treatment with VEGF myoblasts compared with EV myoblasts (*P*<0.05). b indicates brain; m, muscle; asterisk, bone; dashed line, muscle/brain interface; bar=1000 μm.
the transpial vessel bridges connecting the vasculature of the EMS interface to the underlying cortical vessels (Figure 2B and 2C).

Hemodynamic Effect of Increased Transpial Collateralization After Treatment With VEGF<sub>164</sub> Myoblasts

Laser Speckle Images of typical cortical blood flow responses to acetazolamide on day 21 are illustrated in Figure 3A. To get an idea of the physiological cortical perfusion and blood flow response after acetazolamide, we first performed a series of laser speckle measurements in sham-operated animals that did not undergo ICA-O or an EMS procedure. The total mean baseline perfusion in these animals was determined at 565±89 Flux with a mean 49±11% increase in blood flow after acetazolamide stimulation, which is illustrated as the dashed gray line in Figure 3B.

Next, we compared baseline perfusion among treatment groups: In line with previous results, ICA-O alone (no EMS) did not relevantly influence baseline perfusion compared with sham-treated animals at each individual observation time point (data not shown). Furthermore, baseline perfusion did not differ between animals with no EMS, EV, or VEGF (Figure 3B, upper graph).

We then focused on the cortical perfusion response after acetazolamide: Over the untreated hemisphere, acetazolamide led to a marked perfusion increase in all groups (Figure 3A). Over the treated (right) hemisphere, CVRC did at first not differ among groups but remained 24% to 39% below the physiological blood flow response of sham-treated animals. By day 7, we observed a first sign of hemodynamic recovery in EV-treated animals with a significantly higher CVRC compared with animals with no EMS (no EMS 26±14%, EV 41±9%; *P<0.05). Later, however, animals with no EMS also showed signs of spontaneous CVRC recovery, whereas EV-treated animals showed no further improvement of CVRC (Figure 3B, lower graph).

In contrast, animals that received an EMS with VEGF myoblasts showed a significant 21% to 25% better CVRC recovery beginning at day 14 (no EMS 37±21%, EV 42±9%, VEGF 48±12%; *P<0.05 for VEGF versus no EMS and #P<0.05 for VEGF versus EV) until day 21 (no EMS 36±22%, EV 38±11%, VEGF 48±9%; *P<0.05 for VEGF versus no EMS and #P<0.05 for VEGF versus EV). Importantly, hemodynamic improvement after VEGF did not result in an overshooting CVRC response or cortical hyperperfusion but reached the physiological parameters of sham-treated animals (Figure 3A and 3B).

Vessel Density and Pericyte Coverage

Next, we searched for signs of proangiogenic activity and vessel maturation at the muscle/brain interface. Because, we wanted to distinguish between capillaries—mainly responsible for nutritive perfusion—and the larger pre- and postcapillary vessels, CD31-positive vessels were grouped according to their diameter. As a sign of positive proangiogenic activity in the cortex below the EMS, treatment with VEGF resulted in a significant 21% increase in the parenchymal microvascular density compared with animals that only received EV myoblasts (no EMS 194±32 1/mm<sup>2</sup>, EV 174±14 1/mm<sup>2</sup>, VEGF 220±50 1/mm<sup>2</sup>; *P<0.05 for VEGF versus EV; Figure 4A and 4B). This proangiogenic activity in the cortex and around FITC-lectin perfused transpial collaterals was confirmed by Ki67/CD31 colocalization at the muscle/brain interface of the EMS, indicating a marked increase in endothelial cell proliferation in the VEGF myoblast group (Figure 4D).

Figure 3. Encephalomyosynangiosis (EMS) and vascular endothelial growth factor-A (VEGF<sub>164</sub>) expressing myoblasts improve cerebral hemodynamic rescue. A, Real-time laser speckle images of cortical perfusion before (left) and after (right) acetazolamide challenge on day 21 after EMS and implantation of empty vector (EV) myoblasts or VEGF<sub>164</sub>-expressing myoblasts (VEGF). Relative perfusion in the arbitrary perfusion unit CBF-Flux (Flux) is mapped as a color-coded image of cortical cerebral blood flow. The higher relative perfusion increase after acetazolamide after EMS and VEGF can be noted on the right. The dashed rectangle shows the area of perfusion assessment. R indicates right. B, Line graphs illustrating the mean resting (baseline) perfusion (CBF-Flux; upper panel) and mean cerebrovascular reserve capacity (CVRC; percent change in CBF-Flux; lower panel) in animals with internal carotid artery-occlusion (no EMS) and no EMS and in animals treated with EMS and EV or VEGF myoblasts over the 21-day monitoring period (day 7: *P<0.05 EV vs no EMS; day 14 and 21: *P<0.05 VEGF vs no EMS and #P<0.05 VEGF vs EV). The dashed horizontal lines show the mean resting perfusion and CVRC of sham animals without ICA-O or EMS.
determines the vascular maturity and stability. In addition, pericytes are involved in cerebrovascular flow regulation. Thus, it is noteworthy that cortical angiogenesis was accompanied by a significant 8% increase in the pericyte coverage of nutrient vessels in the cortical parenchyma after EMS and VEGF, and vascular pericyte coverage (%) in vessels <9 mm (left) and >9 mm (right) in diameter in animals with no EMS and in animals treated with EMS and EV or VEGF myoblasts (*P<0.05). C. Positive CD31/Desmin colocalization at the muscle/brain interface of the EMS after VEGF-myoblast implantation confirms localized pericyte coverage as a sign of vessel maturity (left). The image on the right shows a 3-dimensional confocal microscope reconstruction of a 60 μm section at high magnification to confirm the intimate cell–cell interaction between green-stained pericytes wrapping around red-stained endothelial tubes. D. Angiogenic remodeling of the cortical vasculature at the muscle/brain interface of an EMS after VEGF-myoblast treatment is confirmed by Ki67 (red)/CD31 (blue) colocalization in an animal that underwent fluorescein isothiocyanate (FITC)-lectin perfusion (green) to visualize the patent vasculature at the muscle/brain interface of the EMS. Bar in A, C (left)=100 μm; bar in C (right)=50 μm.

Cortical T2 Signal Hyperintensity and Regional Perfusion After MCA-O on Day 21
Having shown that local VEGF expression improved transpial collateralization, hemodynamic recovery and cortical angiogenesis, we next sought to determine whether these findings also translated into protection from ischemic stroke after temporary vessel occlusion. Thus, we applied a protocol with 60-minute occlusion of the ipsilateral MCA on day 21 followed by a 23-hour reperfusion period before determining the cortical stroke volume by MRI. All animals subject to MCA-O survived the 23-hour reperfusion period and underwent MRI. In all cases, apparent signs of intracerebral hemorrhage were not observed. The mean volume of the total right-hemispheric cortex did not differ among groups (no EMS 77±3 mm³, EV 74±8 mm³, VEGF 80±2%; *P<0.05 for VEGF versus EV; Figure 4A and 4B). Moreover, animals treated with EMS and VEGF showed a dense mural Desmin coverage of the vasculature bordering the muscle/brain interface, which we confirmed by a confocal microscope-generated reconstruction of the cortical vasculature at the muscle/brain interface adjacent to the myoblast implantation site (Figure 4C).
74.4±9%, VEGF 56.2±16%; *P<0.001; Figure 5). Moreover, VEGF-treated animals also showed areas of sustained cortical perfusion during the MCA-O period compared with animals that only received EV myoblasts (Figure 6A).

Microvascular Permeability Under Regional VEGF Supplementation
Finally, we assessed the effect of regional VEGF supplementation on microvascular permeability in ischemic and nonischemic cortex at the muscle/brain interface. Most importantly, VEGF supplementation for 21 days in nonischemic animals without MCA-O did not result in perivascular Evans Blue extravasation, supporting our finding of functional but also nonleaky microvessels. In ischemic animals with MCA-O, however, VEGF treatment was associated with notable Evans Blue extravasation around the cortical microvasculature compared with EV-treated mice (Figure 6B).

Discussion
In this proof-of-concept study, we explored the potential of VEGF164-expressing myoblasts as a novel proangiogenic gene therapy for treatment of chronic cerebral hypoperfusion in combination with indirect vasoreconstructive surgery. Implantation of VEGF164-expressing myoblasts into the temporal muscle of an EMS significantly improved extracranial–intracranial collateralization at the muscle/brain interface, as well as parenchymal angiogenesis in the cortical region below the EMS. Morphological findings were in line with functional results showing better hemodynamic rescue and an attenuated cortical stroke volume in living mice. Together, this demonstrates that the myoblast-mediated delivery of recombinant angiogenic growth factor to the target site of an EMS may be harvested as a novel translational approach to facilitate indirect revascularization in patients at risk of ischemic stroke because of chronic cerebral hypoperfusion.

Myoblast-Mediated Gene Delivery
Myoblast-mediated gene delivery offers several advantages over other gene delivery approaches: On intramuscular injection, myoblasts fuse stably with pre-existing muscle fibers, providing long-term, possibly lifelong, highly localized gene product expression without the need for immune suppression or the propensity to proliferate in an uncontrolled manner.18–20,22,25 Furthermore, myoblasts can be characterized in vitro before implantation, allowing, for example, the quantification of gene expression. In view of the potentially high complication rate after direct bypass grafting (ie, in patients with atherosclerotic cerebrovascular disease), myoblast-mediated gene delivery in combination with an EMS represents a novel approach to improve functional and morphological collateralization after indirect revascularization, which remains to lack hemodynamic effectiveness compared with direct bypass procedures.

Spontaneous Extracranial–Intracranial Collateralization After Indirect Revascularization
First, we investigated whether an EMS results in spontaneous transpial collateralization in a mouse model of chronic cerebral hypoperfusion. We sought to assess transpial vessel sprouts at the muscle/brain interface because a functional EMS is characterized by development of patent extracranial–intracranial anastomoses in form of transpial collaterals crossing from the temporal muscle into the brain.1,4,7 To assess the patency of these collaterals and ensure an exclusive staining of the vasculature connected to the EMS, we established an in vivo FITC-lectin perfusion through the ipsilateral ECA feeding the temporal muscle. As a first interesting finding, patent extracranial–intracranial collaterals were also detected after EMS without VEGF supplementation, which is in line with a previous report4 and confirms the hypothesis that cortical hypoperfusion alone may act as an inducer of extracranial–intracranial collateralization. The fact that the FITC-lectin positive vasculature in the brain was only noted in circumscribed cortical areas associated with the EMS and not in the brain of animals with no EMS excluded an incidental filling of intracranial vessel segments through more proximal preformed collaterals of the ECA and ICA.

Stimulated Neovascularization Through VEGF
Collateral outgrowth is initially governed by physical forces (ie, fluid shear-stress) that activate the endothelium of pre-existing arterioles. One recently suggested pathway lies in the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which leads to stabilization of hypoxia-inducible factor-1α expression with a
VEGF being a key factor, however, functional collateralization relies on a complex interaction of several growth factors (ie, fibroblast growth factor and colony-stimulating factors), cytokines (ie, monocyte chemoattractant protein-1), cell types (ie, monocytes and endothelial progenitor cells), as well as a multitude of proteolytic enzymes (ie, matrix-metalloproteinases).

Although previous reports suggest that implantation of the VEGF-expressing myoblasts mainly results in proangiogenic activity at the target site of implantation, at this point, we can only speculate on a more distant effect of our treatment, for example, on the native collateral vasculature. Possibly, the localized VEGF supplementation could stimulate outward remodeling of the preformed leptomeningeal or pial collaterals but it remains unclear, whether our localized VEGF delivery might also have influenced the more proximal collateral vasculature, such as the Circle of Willis, particularly in the setting of chronic cerebral hypoperfusion.

**Hemodynamic Improvement After Myoblast-Mediated VEGF Therapy**

Previous experimental VEGF gene-therapy studies for cerebral ischemia have mainly focused on the treatment of acute focal stroke and VEGF-induced angiogenesis has been shown to result in structural neuroprotection and functional recovery. However, whether the beneficial effects of VEGF were a consequence of improved hemodynamics because of neovascularization remains unknown since proangiogenic strategies aimed at increasing the global vascular density may worsen rather than improve cerebral hemodynamics. Therefore, we next addressed whether VEGF-mediated neovascularization at the muscle-brain interface also translated into improved cerebral hemodynamics. Typically, nonischemic cerebral hypoperfusion is characterized by a normal or at best mild reduction of baseline (resting) cerebral blood flow. In this study, semiquantitative baseline perfusion (CBF-Flux) between animals with no EMS, EV, or VEGF treatment did not differ and remained within the physiological range of sham-treated animals. Most importantly, the improved morphological and functional results that we observed after VEGF delivery were not accompanied by an overshooting increase of regional cortical blood flow compared with the physiological blood flow response of sham-treated animals, which is essential for our translational approach to avoid the dilemma of postoperative hyperperfusion syndrome in hemodynamically compromised patients undergoing surgical revascularization.

In clinical practice, the degree of cerebrovascular reactivity remains one of the most important parameters to assess the patients’ risk of hemodynamic ischemic stroke, as well as to determine hemodynamic improvement after a surgical intervention. In adult C57BL6 mice, we previously demonstrated that assessment of the acetazolamide-specific CVRC is practical for quantification of hemodynamic impairment after unilateral ICA-O. In this study, the acetazolamide-associated blood flow increase within the macro- and microcirculation of the untreated hemisphere most likely represents an intact CVRC due to the dilation of precapillary resistance vessels in response to acetazolamide with a resulting blood flow increase in the draining veins and sagittal sinus. Over
the affected hemisphere, however, CVRC in animals with ICA-O and no EMS was markedly reduced and within the same range as reported previously,24 tending toward spontaneous hemodynamic recovery but still remaining 27% below the physiological CVRC response of sham-treated animals. Although this CVRC recovery was somewhat better in animals that received an EMS and EV myoblasts, only VEGF supplementation resulted in complete functional recovery. Importantly, the time frame of this better hemodynamic rescue also seemed to be in line with the previously reported time required for stable host integration of the myoblasts, as well as the first reported observation time point of positive vascular remodeling between days 7 and 14 after VEGF<sub>164</sub>-myoblast implantation.18,19

**Parenchymal Angiogenesis After Myoblast-Mediated VEGF Therapy**

Compared with animals that only underwent ICA-O, an EMS with EV myoblast implantation did not result in a higher proangiogenic activity. Implantation of VEGF<sub>164</sub>-expressing myoblasts, however, resulted in cortical angiogenesis of the nutritive vasculature, which supports the idea of a direct and regionally localized VEGF effect at the muscle/brain interface of the EMS and may be beneficial for protection from ischemic stroke. Next to the mere de novo formation of a vascular network, however, a functional vasculature also requires pericyte recruitment for regulation of vessel diameters and blood flow. In this regard, implantation of VEGF<sub>164</sub>-myoblasts resulted in a significant increase in the pericyte coverage of the resident and newly formed microvasculature. This indicates that high-dose local delivery of VEGF did not simply result in immature and nonfunctional blood vessels but may in fact regulate the formation of mature vessels in the brain.31 However, a previous report demonstrated that VEGF alone may also act as a negative regulator of pericyte function and vessel maturation,35 which underlines that blood vessel stability depends on the co-ordinated and balanced interaction of multiple signaling pathways in the endothelial and perivascular cells. A key factor that mediates pericyte recruitment and vessel maturation is platelet-derived growth factor-BB.36 Therefore, future studies could address whether a balanced codelivery of VEGF and platelet-derived growth factor-BB might further improve the positive effects that we observed.

**Cortical Stroke and Vascular Permeability After VEGF<sub>164</sub>-Treatment in Combination With EMS**

The main goal of an EMS is the reduction of subsequent ischemic events through improvement of collateral flow at the level of the leptomeningeal vasculature because the degree of leptomeningeal collateralization is a major determinant in the severity of stroke.17 Although the endogenous leptomeningeal collaterals were not directly addressed in this study, the positive VEGF effect on transpial collateralization underlines that VEGF not only increases parenchymal angiogenesis30 but also act as an important mediator of pial collateral formation and maintenance in the adult mouse brain.12,13 Interestingly, VEGF seems not to significantly change the time line in which new vessels are formed.10 Therefore, VEGF probably has negligible effect on brain hemodynamics when applied at the time point of an acute ischemic event but may rather protect the brain against subsequent ischemic episodes. Consequently, we tested whether our morphological findings also translated in regional ischemic protection. The significant 25% reduction of cortical infarct volume next to the circumscribed area of higher cortical perfusion following MCA-O on day 21 after treatment with EMS and VEGF<sub>164</sub>-expressing myoblasts are in line with this hypothesis. Although VEGF increased the microvascular leakiness after MCA-O, no leakiness was noted in animals without focal stroke and the overall effect on blood flow and tissue survival was beneficial, which is in line with previous results.18,19 Possibly, the increased permeability after MCA-O could be explained by the fact that the supplementation of only VEGF may render the vasculature more fragile and susceptible to hypoxic injury or additional endogenous VEGF, which is known to accumulate in response to focal stroke. Again, this highlights that future studies should address the important question whether a balanced and controlled myoblast-mediated codelivery of multiple exogenous growth factors, such as VEGF and platelet-derived growth factor-BB, may further improve collateralization and hemodynamic rescue, microvascular remodeling and ischemic protection.

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**Disclosures**

None.

**References**

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Nils Hecht, Aiki Marushima, Melina Nieminen, Irina Kremenetskaia, Georges von Degenfeld, Johannes Woitzik and Peter Vajkoczy

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SUPPLEMENTAL MATERIAL

Myoblast-mediated gene therapy improves functional collateralization in chronic cerebral hypoperfusion

1Nils Hecht, MD; 1Aiki Marushima, MD; 1Melina Nieminen, MSc; 1Irina Kremenetskaia, MSc; 2Georges von Degenfeld, MD; 1Johannes Woitzik, MD; 1Peter Vajkoczy, MD

1Department of Neurosurgery and Center for Stroke research Berlin (CSB), Charité - Universitätsmedizin Berlin, Berlin, Germany.

2Baxter Laboratory for Stem Cell Biology, Stanford University, Stanford, CA, USA. Current address: Common Mechanism Research, Bayer Healthcare AG, Wuppertal, Germany and Institute for Operative Medicine, University of Witten/Herdecke, Cologne, Germany.
SUPPLEMENTAL METHODS

Laser Speckle Imaging and cerebrovascular reserve capacity
After positioning of the laser speckle device (MoorFLPI, Moor Instruments, Devon, England), a five-minute baseline measurement of cortical resting perfusion (CBF-Flux measured in arbitrary perfusion units) was recorded within a 6x4 mm region of interest (ROI) over the right middle cerebral artery (MCA) territory, which permitted a combined arterial, venous and parenchymal perfusion and blood flow assessment. A 120-second CBF-Flux plateau was calculated as baseline perfusion. Next, 50mg/kg acetazolamide (Diamox, Goldshield Pharmaceuticals Ltd., Surrey, England) was injected intraperitoneally and the acetazolamide-specific cerebrovascular reserve capacity (CVRC) was calculated as the percent perfusion change between the baseline plateau and a 120-second CBF-Flux plateau after a maximum rise in CBF-Flux.

FITC-lectin perfusion and assessment of transpial collateralization
The external carotid artery was cannulated with a polyethylene catheter (inner diameter 0.28mm; outer diameter 0.61mm) connected to a micro syringe. The ipsilateral common carotid artery was proximally ligated and a solution of 50µl (100µg) FITC-lycopersicon esculentum (tomato) lectin (Vector Laboratories Inc., Burlingame, CA, USA) and 200µl PBS was injected. The mice were decapitated within 2 seconds after the injection and whole-head specimens were snap-frozen at -80°C.

Immunohistochemistry
The cerebral vasculature was detected by a rat anti-mouse CD31 antibody (1:50 dilution in 0.5% Casein; PECAM-1, BD Biosciences, Franklin Lakes, NJ, USA) and a donkey anti-rat IgG conjugated with CyTM3 (1:200 dilution in 0.5% Casein; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) antibody. Pericytes were detected with a rabbit anti-desmin polyclonal antibody (Dilution 1:100 in 0.5% Casein; Abcam, Cambridge, UK) detected by CyTM5-conjugated donkey anti-rabbit IgG antibody (Dilution 1:100 in 0.5% Casein; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Proliferating cells were visualized with a monoclonal rabbit anti-mouse Ki67 antibody (Dilution 1:200 in 0.5% Casein; Thermo Scientific, Waltham, MA, USA) detected by CyTM5-conjugated donkey anti-rabbit IgG (Dilution 1:100 in 0.5% Casein; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA).

Middle cerebral artery occlusion and cortical stroke volume assessment
For temporary middle cerebral artery occlusion (MCA-O), animals were turned to supine position and the midline neck incision was reopened. The carotid sheath was carefully dissected and the right internal carotid artery (ICA) was incised distal to the site of the previous ICA-occlusion. Next, a 7/0 silicone-rubber coated monofilament (Doccol monofilament, length 20mm, diameter with coating 0.21±0.01mm; Doccol cooperation, Sharon, MA, USA) was smoothly inserted up to the level of the ICA/MCA bifurcation. After 60 minutes, the filament was removed and the ICA was permanently ligated proximal to the incision. Twenty-three hours later, the volume of the ischemic cortical tissue and of the total cortex ipsilateral to the EMS was determined in a 7-tesla animal MRI (Bruker Pharmascan 70/16 with a 20mm radio frequency volume resonator, Bruker Biospin, Ettlingen, Germany) and analyzed with purpose-designed biomedical imaging software (Analyze 10.0, Biomedical Imaging Resource, Mayo Clinic, Rochester, MN, USA) according to signal hyperintensity in serial T2-weighted coronal images.
**Evans Blue perfusion**

For assessment of vascular permeability in ischemic and non-ischemic cortical microvessels on day 21, an additional series of mice with ICA-O and EMS were randomized to undergo Empty Vector (EV) or VEGF myoblast implantation (n=4 per group) with or without additional MCA-O on day 21 (n=4 per group). For visualization of vascular permeability, a 2% Evans Blue (Sigma #E2129, Sigma-Aldrich, St. Louis, Missouri, USA) stock solution (diluted in 0.9% saline) was prepared. The animals were anesthetized 4 hours prior to their scheduled sacrifice (in the case of MCA-O: 19 hours after the 60-minute filament occlusion) and a solution of 5µl (500µg) per gram bodyweight Evans Blue was injected into the tongue vein with a micro syringe. Four hours after injection (in the case of MCA-O: 23 hours after the 60 minute filament occlusion) the animals were anesthetized again and a lateral skin incision was performed through the abdominal wall to expose the diaphragm and left cardiac ventricle. Next, a 26-gauge needle affixed to a syringe loaded with Phosphate Buffered Saline (PBS) was inserted into the ventricle and the animals were perfused with 20ml PBS after incision of the vena cava to allow venous outflow. Whole-head specimens were snap-frozen at -80°C and coronal cryosections (20µm) of snap-frozen, whole-head specimens were obtained from the anterior, middle and posterior region of the EMS (Bregma -0.5mm, -1.5mm and -3.0mm, respectively). The cerebral vasculature was detected immunohistochemically by a rat anti-mouse CD31 antibody (1:50 dilution in 0.5% Casein; PECAM-1, BD Biosciences, Franklin Lakes, NJ, USA) and a donkey anti-rat IgG conjugated with Cy3 (1:200 dilution in 0.5% Casein; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) antibody.

**Statistical analysis**

Baseline perfusion and CVRC were compared by a two-way ANOVA for repeated measures with subsequent pair-wise comparison of means by Fisher’s least projected difference test. The EMS take rate was compared by a non-parametric Kruskal-Wallis test with Dunn’s post-test for multiple comparisons. Vessel density, pericyte coverage and cortical stroke volume were compared by a one-way ANOVA with Bonferroni’s multiple comparison tests.