Combination of Tissue-Plasminogen Activator With Erythropoietin Induces Blood–Brain Barrier Permeability, Extracellular Matrix Disaggregation, and DNA Fragmentation After Focal Cerebral Ischemia in Mice

Anil Zechariah, MSc; Ayman ElAli, MSc; Dirk M. Hermann, MD

Background and Purpose—After 1 clinical study in which recombinant erythropoietin (EPO) protected against ischemic stroke and improved clinical outcome, the German multicenter EPO trial recently reported increased mortality in stroke patients receiving EPO after tissue-plasminogen activator (t-PA)-induced thrombolysis. The reasons for the adverse effects of EPO in t-PA–treated patients are unknown.

Methods—Mice were submitted to 90 minutes of middle cerebral artery occlusion. Immediately after reperfusion, animals were treated with normal saline or t-PA (10 mg/kg). Animals subsequently received injections of normal saline or EPO that were administered after reperfusion and 12 hours later (2500 IU/kg each). Ischemic injury and brain edema were analyzed at 24 hours after reperfusion by cresyl violet staining and terminal transferase biotinylated-dUTP nick end labeling. Blood–brain barrier integrity was assessed by histochemistry for extravasated serum IgG. Matrix metalloproteinase activity was evaluated by gelatinase zymography.

Results—EPO did not influence ischemic infarct size but reduced brain swelling. This effect was abolished by t-PA, which exacerbated serum IgG extravasation in ischemic tissue. Gelatinase zymographies revealed that EPO promoted matrix metalloproteinase-9 activity that was markedly elevated by t-PA. Add-on treatment with t-PA increased the density of DNA-fragmented cells in ischemic tissue of EPO-treated, but not vehicle-treated, mice.

Conclusions—Our data demonstrate a hitherto unknown interaction of t-PA with EPO at the blood–brain interface, ie, promotion of vascular permeability and extracellular matrix breakdown, which may account for the unfavorable actions of EPO in t-PA–treated patients. After t-PA–induced thrombolysis, EPO may not be suitable as stroke treatment. (Stroke. 2010;41:1008-1012.)

Key Words: blood–brain barrier permeability ■ hematopoietic growth factor ■ neuroprotection ■ stroke ■ thrombolytic

The hematopoietic growth factor erythropoietin (EPO) has strong neuroprotective activity. Promising animal studies using experimental stroke models1–3 rapidly led to a clinical proof-of-concept trial in which EPO was administered to patients with acute ischemic stroke.4 In this study, EPO significantly enhanced neurological outcome and reduced ischemic injury. In view of these findings, the German multicenter EPO trial with >500 patients was conducted between 2003 and 2008.5 More than 60% of patients in this study received thrombolytic treatment. Unexpectedly, in these patients EPO did not improve clinical outcome but rather increased the risk of serious complications, namely death, bleeding, edema, and thromboembolic events.5 Explorative analysis in nonthrombolysed patients reproduced at least some of the promising findings of the first EPO study, indicating that EPO on its own may be attractive for stroke therapy.

The reason why EPO increases mortality in patients treated with tissue plasminogen activator (t-PA) is unknown. For other neuroprotectants and antiinflammatory drugs, namely glutamate antagonists,6 caspase-8 inhibitors,7 free radical scavengers,8 mast cell stabilizers,9 and statins,10,11 synergistic actions had previously been described that supported combination of thrombolytic and survival-promoting treatments. To elucidate the observations in the German multicenter trial, we now submitted mice to intraluminal middle cerebral artery (MCA) occlusion, analyzing effects of t-PA and EPO on ischemic injury, blood–brain barrier integrity, and extracellular matrix proteases. Our data point toward a hitherto unknown interaction of t-PA and EPO, which may explain the complications of EPO in t-PA–treated patients.

Received November 27, 2009; final revision received January 26, 2010; accepted January 27, 2010.

From the Department of Neurology, University Hospital Essen, Germany.

Correspondence to Dirk M. Hermann, MD, Department of Neurology, University Hospital Essen, Hufelandstrasse 55, D-45122 Essen, Germany.

E-mail dirk.hermann@uk-essen.de

© 2010 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.109.574418
Materials and Methods

Animal Groups
All experiments were performed with government approval according to the National Institutes of Health guidelines for the care and use of laboratory animals. Adult male C57BL/6j mice (20–25 grams; obtained from the animal facility of University Hospital Zurich, Switzerland) submitted to 90 minutes of MCA occlusion were randomly assigned to 4 groups (n = 10 animals each). These animals were treated with 200 μL normal saline or t-PA (10 mg/kg; Actilyse, Boehringer-Ingelheim), which was administered immediately after reperfusion via the common carotid artery and 200 μL normal saline or EPO (Epoetin beta; Roche), which was delivered immediately after normal saline/t-PA delivery via the common carotid artery and again at 12 hours of reperfusion by intraperitoneal injection (2500 IU/kg each).

MCA Occlusion
Animals were anesthetized with 1.5% isoflurane (30% O2, remainder N2O). Rectal temperature was maintained between 36°C and 37°C using a feedback-controlled heating system. Focal ischemia was induced using an intraluminal technique. Briefly, a midline neck incision was made and the left common and external carotid arteries were isolated and ligated. A microvascular clip (Aesculap; Tuttilingen, Germany) was temporarily placed on the internal carotid artery and an 8-0 silicon-coated nylon monofilament was directed through the internal carotid artery until the origin of MCA. The monofilament was left in place for 90 minutes and then withdrawn. During the experiment, laser Doppler flow (LDF) was monitored using a flexible fiberoptic probe (Perimed) attached to the skull overlying the core of the MCA territory. LDF was measured before and during ischemia, up to 30 minutes after reperfusion. At that time, anesthesia was discontinued and animals were allowed to recover. Twenty-four hours later, animals were reanesthetized and transcardially perfused with normal saline. Brains were removed, frozen on dry ice, and cut into 20-μm coronal cryostat sections.

Analysis of Infarct Size and Brain Swelling
Sections 1.5-mm apart were stained with cresyl violet. The border between infarcted and healthy tissue was outlined with an image analysis software (Image J; National Institutes of Health) and the area of infarction was quantified by subtracting the area of nonlesioned ipsilateral hemisphere from that of the contralateral side. All measurements were performed by 2 examiners, out of which mean values were formed. Infarct areas from various rostrocaudal brain levels were integrated for infarct volume analysis. Brain swelling was calculated as absolute difference between the ischemic and nonischemic hemisphere at mid striatal levels (ie, at the level of the bregma).

Evaluation of Serum IgG Extravasation
With gentle stirring, brain sections obtained from the mid striatum were rinsed for 10 minutes at room temperature in 0.1 mol/L phosphate-buffered saline, to remove intravascular IgG, and fixed in 4% paraformaldehyde. After blocking of endogenous peroxidase and immersion in 0.1 mol/L phosphate-buffered saline containing 5% bovine serum albumin and normal swine serum (1:1000), sections were incubated for 1 hour in biotinylated goat anti-IgG (1:200; Santa Cruz) and stained with avidin peroxidase kit (Vectastain Elite; Vector Labs) and diaminobenzidine. Sections were scanned, converted into gray values, and densitometrically analyzed by subtracting optical densities in the contralateral from the ischemic hemisphere, thus evaluating IgG extravasation in the core of the MCA territory.

Matrix Protease Activity
Tissue samples were obtained from the ischemic and contralateral nonischemic MCA territory (striatum and parietal cortex). Samples pooled from animals belonging to the same group were homogenized, lysated, supplemented by 5% protease inhibitor cocktail, and sonicated. Protein concentrations were measured using the Bradford assay kit. For assessment of matrix metalloproteinase (MMP)-9 (gelatinase-B) and MMP-2 (gelatinase-A) activity, 25 μg protein were mixed with 5× nonreducing loading buffer and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis using 9% acrylamide-bis gel containing 0.1% gelatin. Gels were removed and washed and incubated for 1 hour at room temperature with slight shaking in modified enzymatic activation buffer (50 mmol/L Tris-HCl, 6 mmol/L CaCl2, 1.5 μmol/L ZnCl2; pH 7.4) containing 2.5% Triton X-100 to remove sodium dodecylsulfate and restore gelatinase activity. Gels were incubated at 37°C for 24 hours in modified enzymatic activation buffer. Gels were stained in Coomassie brilliant blue R-250 (Bio-Rad) and immersed in destaining solution and 2% acetic acid. Gels were dried and digitized. Three different gels were performed, with each gel being loaded with 3 samples from each group, so that 9 samples were densitometrically analyzed for each experimental condition.

Analysis of DNA Fragmentation
Adjacent sections were fixed with 4% paraformaldehyde and used for terminal transferase biotinylated-dUTP nick end-labeling using the fluorescein in situ cell death detection kit (11684795910; Roche). After labeling, sections were counterstained with 4′,6-diamidino-2-phenylindole and cover-slipped. Sections were evaluated blinded for the experimental condition by counting DNA fragmented cells in 6 regions of interest of the striatum and 3 regions of interest of the cortex (each measuring 250,000 μm²), as described. Mean values were determined for both regions.

Data Analysis
LDF recordings were evaluated by repeated-measurement analysis of variance (ANOVA) with values determined at 15-minute intervals during MCA occlusion and at 5-minute intervals after reperfusion. Because significant group×time interaction effects were observed in an analysis covering the reperfusion phase (repeated measures analysis from 5–30 minutes after reperfusion), 1-way ANOVA followed by least significant differences tests were also performed with data obtained at individual time points. Significant results from this analysis are indicated in the graphs. Infarct size, brain swelling, IgG extravasation, MMP-9 activity, and DNA fragmentation were analyzed by 1-way ANOVA, followed by least significant differences tests. Significant group differences were again shown in the graphs. In addition, 2-way ANOVA was computed for brain swelling, IgG extravasation, MMP-9 activity, and DNA fragmentation, of which significant treatment interaction effects are reported in the text. All data are presented as mean±SD. P<0.05 was considered significant.

Results
LDF
To evaluate cerebral blood flow changes induced by t-PA and EPO, LDF recordings were analyzed. On induction of ischemia, LDF reproducibly decreased to 10% to 20% in all groups, remaining stable throughout the 90 minutes of MCA occlusion (Figure A). Reperfusion was associated with a restoration of blood flow to ≈95% of baseline values in vehicle-treated control animals (Figure A). Compared with control mice, LDF values were significantly reduced in animals receiving t-PA combined with EPO (Figure A). Repeated-measurement ANOVA for the reperfusion phase revealed significant group×time interaction effects (F[3,36]=4.248; P<0.05).

Infarct Size, Brain Swelling, and Serum IgG Extravasation
To analyze effects of t-PA and EPO on ischemic injury and blood–brain barrier (BBB) permeability, brain infarcts and edema were outlined and IgG extravasation was measured. When delivered alone, t-PA did not influence infarct volume (Figure B), infarct area at the rostrocaudal level of the mid
t-PA reverses the attenuation of brain edema induced by EPO in mice submitted to 90 minutes of MCA occlusion, exacerbates serum IgG extravasation, and induces extracellular matrix disaggregation. LDF (A) showing postischemic hypoperfusion in animals treated with t-PA and EPO. Infarct volume (B) and infarct area at the level of the mid striatum (C) are not influenced by EPO, but brain swelling (D) is reduced. This vascular preservation is abolished by concomitant t-PA delivery (D) that exacerbates serum IgG extravasation in ischemic brain tissue (E) and promotes MMP-9 activity (F). Although DNA fragmentation is not significantly influenced by EPO, add-on treatment with t-PA exacerbates cell injury (G, H). Data are means±SD (n=10 animals per group, A–E, G, H; n=9 samples per group, F). *P<0.05, **P<0.01 compared with vehicle; †P<0.05, ††P<0.01 compared with EPO.
Matrix Proteinase Activity

To rule out whether and how t-PA and EPO influence extracellular matrix dissociation, MMP-9 and MMP-2 zymographies were evaluated. Alone, t-PA did not influence the activity of MMP-9 and MMP-2 at 24 hours after reperfusion (Figure F). EPO increased MMP-9 (Figure F) but not MMP-2 (not shown) activity. Combined treatment with t-PA and EPO further increased the activity of MMP-9, so that activity levels were significantly higher than in animals receiving EPO only (Figure F). Two-way ANOVA exhibited a significant interaction effect for both therapies (F[1,8] = 5.440; P < 0.05), indicating that the combined treatment with t-PA and EPO induces different responses than t-PA and EPO when individually applied.

DNA Fragmentation

To assess how the altered MMP-9 activation affects DNA fragmentation, which is a hallmark of apoptotic injury, terminal transferase biotinylated-dUTP nick end-labeling stainings were analyzed. When administered alone, t-PA did not have any influence on cell injury (Figure G, H). EPO alone delivered after the stroke slightly, but not significantly, reduced the density of DNA-fragmented cells. Our data are in contrast to previous observations in rats in which EPO1–2,14 and its neuroprotective, but not hematopoietic, variants14–16 reduced ischemic and hemorrhage, in t-PA–treated patients.5 In our study, EPO passes the BBB.4 Whether this is also true in mice and whether systemic delivery allows achievement of therapeutic tissue levels remains unknown.

Although it is well-established that EPO decreases postischemic brain edema and BBB leakage,3,19,20 our data for the first time show reversal of the antiedematous effects of EPO by t-PA. When administered alone after 90 minutes of intraluminal MCA occlusion, t-PA does not influence brain swelling.5,10,21 Dose-dependent increases in BBB permeability by t-PA have been reported in models of permanent or prolonged transient (180 minutes) MCA occlusion by others in mice22 and rats.23 In this model of less severe ischemia, we could not find any effect of t-PA on BBB integrity. That t-PA promoted BBB breakdown when administered together with EPO raises the question about responsible mediators.

Using matrix protease zymography, we demonstrated that t-PA stimulates MMP-9 activation in EPO-treated animals, indicating that the combined treatment with t-PA and EPO facilitates extracellular matrix breakdown. In focal cerebral ischemia, MMP-9 activation closely accompanies BBB changes.22,23 By disrupting cell matrix interactions, MMP-9 predisposes to brain swelling and intracerebral hemorrhage.22,24 In fact, pharmacological inhibition of MMP-9 reverses the BBB permeability changes induced by t-PA after prolonged focal cerebral ischemia25 and reduces the incidence of brain bleeding.26 It has previously been shown that endothelial cells activated by EPO release the matrix protease MMP-9.27 By demonstrating that combination treatment with t-PA and EPO goes along with more pronounced MMP-9 activation, we provide a mechanism for the BBB changes. That the increased MMP-9 activation in animals receiving t-PA and EPO was also accompanied by exacerbated DNA fragmentation furthermore suggests that MMP-9 activation unfavorably affects neuronal injury.

Conclusion

Although the beneficial effects of t-PA–induced thrombolysis are unquestioned in embolic stroke—via recanalization of occluded vessels t-PA leads to tissue reperfusion, which is a sine qua non requirement for the survival of ischemic brain tissue11,28,29—t-PA has previously been shown to exacerbate brain injury in nonembolic models of focal cerebral ischemia.6–10,21–23 Several mechanisms have been suggested to contribute to the promotion of ischemic injury by t-PA, such as extracellular matrix degradation,22 activation of inflammatory pathways (eg, reflected by upregulation of inducible nitric oxide synthase),8 secondary hemodynamic abnormalities (eg, reflected by the downregulation of endothelial nitric oxide synthase10 leading to secondary hypoperfusion27), as well as a shift of death

striatum (Figure C), brain swelling (Figure D), or IgG extravasation (Figure E) at 24 hours after reperfusion. Similarly, EPO alone, when administered after the stroke, did not change infarct size (Figure B, C) but reduced brain swelling (Figure D), yet without significantly reducing IgG extravasation. Importantly, the edema preventive effect of EPO was abolished by t-PA (Figure D). Add-on treatment with t-PA furthermore increased IgG extravasation in EPO-treated ischemic mice (Figure E).

Discussion

Our study shows a hitherto unknown interaction of t-PA with EPO, ie, promotion of brain edema, BBB permeability, and extracellular matrix breakdown, which may explain the adverse effects of EPO, ie, increased risk of death, brain edema, and hemorrhage, in t-PA–treated patients.5 In our study, EPO alone reduced brain edema, yet without significantly reducing IgG extravasation in ischemic brain tissue. Cotreatment with t-PA exacerbated the BBB permeability above levels in EPO-treated mice, closely accompanied by an increased activation of the matrix protease MMP-9. As a consequence, the density of DNA-fragmented cells increased.

Notably, we did not observe a reduction in infarct size by EPO and only found a minor and nonsignificant reduction in the density of DNA-fragmented cells. Our data are in contrast to previous observations in rats in which EPO1,2,14 and its neuroprotective, but not hematopoietic, variants14–16 reduced ischemic damage. Only few mouse studies assessed effects of EPO on histological injury. After exogenous intraventricular and intraperitoneal delivery, reduced brain infarcts and apoptotic injury were reported when animals were treated 24 hours17,18 or 30 minutes19,20 before their strokes. Similarly, in transgenic mice constitutively overexpressing human EPO, we previously observed decreased infarct volumes and inhibition of caspase-3–dependent disseminated neuronal death.3 To our best knowledge, there are no mouse studies available that examined how EPO influences brain injury when delivered after a stroke. The question arises whether systemically administered EPO accumulates in the brain at sufficiently high concentrations to exert its function. Cerebrospinal fluid studies in humans suggested that EPO passes the BBB.4 Whether this is also true in mice and whether systemic delivery allows achievement of therapeutic tissue levels remains unknown.
Acknowledgments
The authors thank Beate Karow for technical assistance.

Sources of Funding
Supported by the Roche Foundation for Anemia Research (RoFAR).

Disclosures
None.

References
Combination of Tissue-Plasminogen Activator With Erythropoietin Induces Blood–Brain Barrier Permeability, Extracellular Matrix Disaggregation, and DNA Fragmentation After Focal Cerebral Ischemia in Mice
Anil Zechariah, Ayman ElAli and Dirk M. Hermann

Stroke. 2010;41:1008-1012; originally published online April 1, 2010; doi: 10.1161/STROKEAHA.109.574418

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/41/5/1008

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/