Erythropoietin in Combination of Tissue Plasminogen Activator Exacerbates Brain Hemorrhage When Treatment Is Initiated 6 Hours After Stroke

Longfei Jia, MD; Michael Chopp, PhD; Li Zhang, MD; Mei Lu, PhD; Zhenggang Zhang, MD, PhD

Background and Purpose—Erythropoietin (EPO), a hematopoietic cytokine, exerts neuroprotective effects in experimental stroke. In the present study, we investigated the effect of recombinant human EPO (rhEPO) in combination with tissue plasminogen activator (tPA) on embolic stroke.

Methods—Rats subjected to embolic middle cerebral artery occlusion (MCAO) were treated with rhEPO (5000 U/kg) in combination with tPA (10 mg/kg) at 2 or 6 hours after MCAO. Control groups consisted of ischemic rats treated with rhEPO (5000 U/kg) alone, tPA (10 mg/kg) alone, or saline at 2 or 6 hours after MCAO.

Results—The combination therapy of rhEPO and tPA initiated 6 hours after MCAO did not reduce the ischemic lesion volume and significantly (\(P<0.05\)) increased the incidence of brain hemorrhage measured by frequency of gross hemorrhage and a quantitative spectrophotometric hemoglobin assay compared with rats treated with rhEPO alone and tPA alone. However, when the combination therapy was initiated 2 hours after MCAO, the treatment significantly (\(P<0.05\)) reduced the lesion volume and did not substantially increase the incidence of hemorrhagic transformation compared with saline-treated rats. Immunostaining analysis revealed that the combination therapy of rhEPO and tPA at 6 hours significantly (\(P<0.05\)) increased matrix metalloproteinase-9, NF-κB, and interleukin-1 receptor-associated kinase-1 immunoreactive cerebral vessels compared with rats treated with rhEPO alone and saline.

Conclusions—EPO exacerbates tPA-induced brain hemorrhage without reduction of ischemic brain damage when administered 6 hours after stroke in a rat model of embolic MCAO and that matrix metalloproteinase-9, NF-κB, and interleukin-1 receptor-associated kinase-1 upregulated by the delayed combination therapy may contribute to augmentation of brain hemorrhage. (Stroke. 2010;41:00-00.)

Key Words: focal ischemia ■ thrombolysis ■ hemorrhage

Recombinant human tissue-type plasminogen activator (tPA) is an effective treatment for acute ischemic stroke only when given within 4.5 hours of stroke onset.\(^1\) However, tPA treatment increases the incidence of hemorrhagic transformation.\(^2,3\) Experimental studies show that neuroprotective or antithrombotic agents used in conjunction with tPA increase safety and efficacy of thrombolytic therapy in experimental stroke.\(^4-6\)

Erythropoietin (EPO) is a naturally occurring cytokine and has been used for treatment of anemia for \(>2\) decades.\(^7\) Administration of exogenous recombinant human EPO (rhEPO) after focal or global cerebral ischemia augments the cytoprotective and restorative EPO response pathway leading to a substantial improvement in neurobehavioral outcome.\(^8-10\) However, a recent report on the Phase III clinical trial for the treatment of acute ischemic stroke with EPO demonstrated increased mortality and hemorrhage in EPO-treated patients compared with control subject.\(^11\) The reasons for the apparent adverse effects of the EPO treatment stand in contrast to the robust preclinical data indicating a therapeutic benefit of EPO treatment.\(^12,13\) Careful review of the trial shows that 63% of patients enrolled in this trial also received tPA and that many of these patients were treated at or beyond the therapeutic window for tPA.\(^11\) Data from the clinical trial also indicate that it was these patients who drove the adverse response to EPO.\(^11\) Consistent with the imperative to test preclinically the interaction between tPA and EPO and as part of our ongoing studies on the neuroprotective effects of EPO treatment, we investigated the effects of combination treatment with EPO and tPA in a model of embolic stroke in the rat. We demonstrate that the combination treatment performed early after stroke (ie, 2 hours) is neuroprotective; however, outside the therapeutic window (ie, 6 hours), combination tPA and EPO significantly exacerbates hemorrhagic transformation and negates any benefit of EPO therapy.
Methods
All experimental procedures were approved by the Henry Ford Hospital Committee for the Care of Experimental Animals.

Animal Model
Male Wistar rats weighing 350 to 450 g (n = 194, Charles River Breeding Co, Wilmington, Mass) were subjected to embolic middle cerebral artery occlusion (MCAO) by placement of an embolus at the origin of the middle cerebral artery as described previously.14

Experimental Protocols
Recombinant human EPO (epoietin α; AMGEN) was given (intraperitoneally) at a dose of 5000 U/kg 2 or 6 hours after embolic MCAO and was followed by a second and third dose of 5000 U/kg 24 and 48 hours after the first dose. Recombinant human tPA (Genentech) was infused intravenously at a dose of 10 mg/kg (10% bolus 2 or 6 hours after MCAO and the remainder at a continuous infusion over a 30-minute interval using a syringe infusion pump; Harvard Apparatus). After MCAO, animals were randomly assigned to monotherapy with rhEPO at 2 hours (n = 27) or 6 hours (n = 24), tPA at 2 hours (n = 26) or 6 hours (n = 26), combination therapy with rhEPO and tPA at 2 hours (n = 39), 6 hours (n = 36), or saline (n = 26). Rats were killed at 1 or 7 days after MCAO.

Measurements of Infarct Volume and Hemorrhage
Seven days after MCAO, infarct volume and gross hemorrhage were measured as previously described14 (see Supplemental Methods for more details; available at http://stroke.ahajournals.org).

Spectrophotometric Measurement of Intracerebral Hemorrhage
The hemorrhage volume was quantified with a spectrophotometric hemoglobin assay 24 hours after stroke (see Supplemental Methods).15

Immunohistochemistry and Western Blots
Immunohistochemistry and Western blots were performed on brain tissue from rats euthanized 24 hours after ischemia. The following antibodies were used in the present study: a mouse anti-matrix metalloproteinase-9 (MMP-9; 1:100; Chemicon), a mouse anti-fibrinogen antibody (1:1000; Accurate Chemical & Scientific), a mouse antiendothelial barrier antigen (1:1000, SMI 71; Sternberger Monoclonals Inc), a mouse anti-NF-κB (P65, 1:150; Chemicon), which is specific for the detection of activated NF-κB,16,17 and a mouse anti-interleukin-1 receptor-associated kinase-1 (IRAK-1; 1:50; Santa Cruz; see Supplemental Methods).

Statistics
Data were evaluated for normality. Ranked data were used in the analysis when they were not normally distributed. The 2 × 2 factorial design and 2-way analysis of variance were used for each rhEPO and tPA combination at each administration time. The analysis started testing for overall group effect/treatment interactions followed by the pairwise group comparisons if the overall group effect/treatment interaction was detected at the 0.05 level; otherwise, the pairwise group comparisons would be considered as exploratory analysis. P < 0.05 was considered a significant difference. All values are presented as mean ± SE.

Results

Mortality
The mortality rates were 15%, 20%, 17%, 30%, 33%, and 38% for rats treated with saline, EPO at 2 hours, EPO at 6 hours, tPA at 6 hours, and the combination of EPO and tPA at 2 and 6 hours, respectively. No significant differences were detected among the groups. The majority of animals died within 24 hours of onset of MCAO. Rats that died were excluded from further evaluation.

The Effect of rhEPO in Combination With tPA on Ischemic Lesion Volume
To examine whether a combination therapy of rhEPO and tPA has a neuroprotective effect, ischemic rats were treated 2 or 6 hours after MCAO and killed 7 days after MCAO. When the treatment initiated at 2 hours of onset of stroke, monotherapy of rhEPO or tPA significantly (P < 0.05) reduced ischemic lesion volume (Figure 1), which is consistent with published studies.13,18 The combination therapy with rhEPO and tPA also significantly (P < 0.05) reduced the lesion volume compared with rats treated with saline (Figure 1), but no treatment interaction (synergistic effect) or additive effect on the lesion volume was detected compared with the monotherapy. However, when the combination of rhEPO with tPA was administered 6 hours after stroke, the combination did not reduce the lesion volume compared with the saline group. As expected in this model,13 monotherapy of rhEPO but not tPA given, 6 hours after stroke substantially reduced the lesion volume (Figure 1). These data indicate that the efficacy of 6-hours treatment of stroke with rhEPO is negated by tPA.

The Effect of rhEPO in Combination With tPA on Hemorrhage
A major concern of thrombolysis is the incidence of hemorrhagic transformation.3 To examine whether the combination of rhEPO with tPA affects the incidence of brain hemorrhage, we measured frequency of the gross hemorrhage and quantified brain hemorrhagic volume with a spectrophotometric hemoglobin assay 24 hours after stroke.19 Neither the combination therapy of rhEPO with tPA (35%) nor monotherapy of rhEPO (25%) or tPA (28%) initiated 2 hours after stroke significantly increased the incidence of hemorrhagic transformation compared with saline-treated rats (14%). In contrast, the combination of rhEPO and tPA at 6 hours significantly increased (P < 0.05) the incidence of gross hemorrhage from 14% in the saline group to 59% in the combination-treated group. The EPO monotherapy at 6 hours did not significantly
increase the incidence of gross hemorrhage (25%) compared with saline-treated rats. The monotherapy of tPA at 6 hours resulted in a trend toward increase in the incidence of gross hemorrhage (43%), which did not reach statistical significance. However, quantitative data analysis of the brain hemorrhage revealed that the tPA monotherapy at 6 hours significantly \((P<0.05)\) increased the hemorrhagic volume \((4.4 \, \mu L; \text{Figure 2})\) compared with the volume in the saline group \((1.4 \, \mu L; \text{Figure 2})\). Moreover, the combination of rhEPO and tPA further augmented \((P<0.05)\) the hemorrhage volume \((9 \, \mu L; \text{Figure 2})\) compared with the tPA-alone group (Figure 2). The monotherapy of rhEPO did not significantly increase the hemorrhage volume compared with the volume in the saline group (Figure 2).

Brain parenchymal deposition of the large plasma protein fibrin/fibrinogen is an indicator of blood–brain barrier leakage. To further examine vascular leakage, we measured fibrin/fibrinogen deposition in ischemic brain. The combination of rhEPO and tPA at 6 hours substantially increased the number of cerebral vessels with extravascular fibrin deposition compared with ischemic rats treated with saline or the combination therapy at 2 hours (Figure 3), suggesting that the delayed combination therapy exacerbates blood–brain barrier disruption.

### The Effect of rhEPO in Combination With tPA on Expression of MMP-9, NF-\(\kappa\)B, and IRAK1

Upregulation of MMP-9 contributes to hemorrhagic transformation after stroke. To examine whether the delayed (6-hour) combination therapy of rhEPO and tPA affects MMP-9 expression, immunohistochemical staining with antibodies against MMP-9 was performed on coronal brain sections obtained from rats euthanized 24 hours after stroke. Combination treatment with rhEPO and tPA at 6 hours significantly increased the number of NF-\(\kappa\)B-immunoreactive vessels exhibited parenchymal fibrin/fibrinogen deposition (Figure 3). The anti-NF-\(\kappa\)B antibody used in the present study is specific for the detection of activated NF-\(\kappa\)B. Thus, our data suggest that the delayed combination treatment upregulates IRAK-1 and activates NF-\(\kappa\)B, which may trigger MMP-9 expression, leading to brain hemorrhage.

Activation of endothelial nitric oxide synthase enhances thrombosis and reduces brain hemorrhage in the ischemic brain. EPO elevates levels of phosphorylated endothelial nitric oxide synthase protein in cerebral vessels. To examine whether the combination of rhEPO and tPA affects endothelial nitric oxide synthase levels, Western blot analysis was performed: The combination treatment with EPO and tPA at 2 or 6 hours had no effects on endothelial nitric oxide synthase protein levels (Figure 3).

### Discussion

The present study demonstrated that the combination of rhEPO and tPA initiated 6 hours after embolic MCAO failed to reduce ischemic lesion volume and exacerbated hemorrhagic transformation, whereas the combination therapy started 2 hours after stroke significantly reduced ischemic damage without increasing the hemorrhagic transformation. These data suggest that rhEPO exacerbates tPA-induced brain hemorrhage when the combination treatment starts 6 hours after stroke in a rat model of embolic MCAO.

Previous experimental studies in the treatment of acute ischemic stroke demonstrated that tPA in combination with neuroprotective agents such as neuroserpin, minocycline, and atorvastatin given 4 to 6 hours after embolic stroke enhances the neuroprotective effect by attenuating the side effects of...
thrombolysis.\textsuperscript{28–30} Because the efficacy of EPO in experimental stroke has been well demonstrated, we hypothesized that the combination treatment of rhEPO with tPA extends the therapeutic window for tPA. However, to our surprise, the present study showed that the combination of rhEPO and tPA initiated 6 hours after stroke substantially augmented the incidence of brain hemorrhage compared with monotherapy of rhEPO or tPA given at the same time point. The efficacy of the monotherapy of rhEPO at 2 and 6 hours observed in the present study is consistent with published reports.\textsuperscript{13} In this model of embolic stroke, we have demonstrated that the therapeutic window for tPA is $<4$ hours after stroke onset, and delayed tPA treatment increases the incidence of hemorrhagic transformation.\textsuperscript{28,31} The present study shows that monotherapy of tPA at 6 hours augmented the incidence of brain hemorrhage. However, rhEPO in combination with tPA at 6 hours aggravated the hemorrhage, suggesting that EPO likely exacerbates tPA-induced hemorrhage. These experi-
mental results are consistent with observations made from the recent double-blind, placebo-controlled, randomized German Multicenter EPO Stroke Trial,11 reporting that with a high proportion of tPA-treated patients enrolled (63%), there was a significant increase in death rate when patients with stroke received EPO within 6 hours of the onset of stroke compared with the death rate in the placebo group.11 The elevated tPA protocol violation rate (50%), including treatment beyond the 3-hour time window, may have contributed to the high death rate in the EPO-treated patients.

Mechanisms underlying the exacerbation and the increased incidence of hemorrhage are currently unknown when the combination therapy of rhEPO and tPA was initiated 6 hours after stroke. Thrombolysis with tPA upregulates MMP-9, a protein-digesting enzyme that degrades the major extracellular matrix components and thereby exacerbates blood–brain barrier disruption and hemorrhagic transformation.12–14 The present study shows that tPA in combination with rhEPO at 6 hours but not 2 hours significantly increased the MMP-9 levels, which was concurrent with an increase in the number of leaking vessels, suggesting that upregulation of MMP-9 contributes to augmentation of brain hemorrhage. NF-κB triggers upregulation of MMP-9 expression.35 EPO regulates the NF-κB signaling pathway that mediates EPO-induced neuroprotection.36 Thus, EPO could activate the NF-κB signaling pathway leading to an upregulation of MMP-9 expression when EPO is administered in combination of tPA. Indeed, the delayed (6-hour) combination treatment substantially activated NF-κB signaling pathway in cerebral vessels, although our Western blot analysis did not reveal significant differences of NF-κB levels among experimental groups either at an acute (2 hours after initiated treatments) or delayed (24 hours after initiated treatments) time point. Moreover, the delayed combination treatment upregulated IRAK-1 in cerebral vessels. IRAK-1 plays an important role in signal transduction of Toll-like receptors and interleukin-1 receptors.25 IRAK-1 along with other molecules activate NF-κB.25 Collectively, we speculate that the delayed combination therapy may activate the NF-κB signaling pathway and IRAK-1, leading to upregulation of MMP-9 that mediates augmentation of brain hemorrhage observed in the present study. A possible limitation of the present study is that the dose of tPA used was 10 times higher than the dose used in humans, and this dose may upregulate IRAK-1 and NF-κB and thereby contributes to hemorrhagic transformation. Future studies focusing on investigating the cause-effect of the NF-κB signaling pathway and IRAK-1 upregulated by the delayed combination of rhEPO and tPA on exacerbating brain hemorrhage are warranted.

In summary, the present study indicates that the combination treatment with rhEPO and tPA carries a higher risk of intracerebral hemorrhage without reduction of ischemic brain damage when administered 6 hours after embolic stroke, whereas the combination therapy initiated 2 hours after stroke does not exacerbate brain hemorrhage and reduces ischemic cell damage.

Acknowledgments
We thank Cindi Roberts and Qing-e Lu for technical assistance.

Sources of Funding
This work was supported by National Institute of Neurological Disorders and Stroke grants PO1 NS23393 and RO1 HL64766.

Disclosures
None.

References


Erythropoietin in Combination of Tissue Plasminogen Activator Exacerbates Brain Hemorrhage When Treatment Is Initiated 6 Hours After Stroke
Longfei Jia, Michael Chopp, Li Zhang, Mei Lu and Zhenggang Zhang

Stroke. published online July 29, 2010;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

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/early/2010/07/29/STROKEAHA.110.586198.citation

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2010/07/29/STROKEAHA.110.586198.DC1

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/