Effects of a Selective CD11b/CD18 Antagonist and Recombinant Human Tissue Plasminogen Activator Treatment Alone and in Combination in a Rat Embolic Model of Stroke

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Background and Purpose—We evaluated the neuroprotective effect of UK-279,276 (also referred to as recombinant neutrophil inhibitory factor), a selective CD11b/CD18 antagonist, in combination with thrombolytic therapy on focal cerebral ischemia.

Methods—Male Wistar rats (n=88) were subjected to embolic middle cerebral artery occlusion. Animals were randomly assigned to the following groups (n=11 in each group): vehicle treatment alone at 2 or 4 hours, UK-279,276 treatment alone at 2 or 4 hours, recombinant human tissue plasminogen activator (rhtPA) treatment alone at 2 or 4 hours, or the combination of UK-279,276 and rhtPA at 2 or 4 hours. Infarct volume, neurological function, hemorrhagic transformation, neutrophil accumulation, and parenchymal fibrin deposition were measured 7 days after middle cerebral artery occlusion.

Results—Treatment with UK-279,276 significantly (P<0.05) improved neurological severity scores, an index of neurological functional deficit, but had no effect on infarct volume compared with vehicle-treated animals. Treatment with rhtPA alone at 2 but not 4 hours significantly (P<0.05) reduced infarct volume and improved neurological function compared with vehicle-treated animals. Combination treatment with UK-279,276 and rhtPA at 2 or 4 hours significantly (P<0.01) reduced infarct volume and enhanced recovery of neurological function compared with control. Neutrophil accumulation and fibrin deposition in the brain parenchyma of combination-treated rats at 2 and 4 hours after stroke were significantly reduced (P<0.05) compared with corresponding vehicle-treated control groups. The neuroprotective effect of the combined treatments was superior to the additive effects from each treatment of rhtPA or UK-279,276 alone.

Conclusions—These data suggest that the combination treatment with UK-279,276 and rhtPA may extend the window of thrombolytic therapy for the acute treatment of stroke. (Stroke. 2003;34:1790-1795.)

Key Words: neuroprotection ■ reperfusion injury ■ stroke ■ tissue plasminogen activator ■ rats

Administration of recombinant human tissue plasminogen activator (rhtPA) within 3 hours of the onset of stroke is beneficial for patients with ischemic stroke.1–3 However, thrombolysis with rhtPA beyond the 3-hour therapeutic window fails to provide therapeutic benefit and increases hemorrhagic transformation.4 Preclinical data suggest that reperfusion initiated by delayed thrombolysis can precipitate further cerebral injury.4–6

Neutrophils exacerbate reperfusion injury after ischemic insult.7,8 Neutrophil adhesion and migration after stroke are mediated by interaction of CD11b/CD18 β2 integrins on neutrophils with intercellular adhesion molecules expressed on the vascular endothelial cell surface.9 Treatment with antibodies against CD11b/CD18 or intercellular adhesion molecule 1 (ICAM-1) reduces parenchymal neutrophil accumulation and infarct volume in experimental transient focal cerebral ischemia.10,11 However, inhibition of neutrophil adhesion and migration does not reduce infarct volume in permanent experimental stroke and does not show clinical benefit for stroke patients.12

UK-279,276 (also referred to as recombinant neutrophil inhibitory factor [rNIF]) is a glycoprotein isolated from the canine hookworm.13 This 41-kd protein specifically binds to the I-domain CD11b/CD18 β2 integrin and thereby inhibits neutrophil adhesion to the endothelium and the subsequent release of hydrogen peroxide.14 UK-279,276 potently inhibits...
human neutrophil function in vitro. Moreover, treatment of rats subjected to transient middle cerebral artery (MCA) occlusion with UK-279,276 significantly reduces infarct volume and neutrophil infiltration.

Delayed thrombolytic therapy with rhtPA significantly increases adhesion molecule expression and exacerbates cerebral injury after focal cerebral ischemia, whereas treatment with rhtPA in combination with antiadhesion molecules that inhibit neutrophil adhesion and migration extends the therapeutic window of thrombolysis. These data suggest that adjunctive thrombolysis with inhibition of neutrophil adhesion has a synergistic effect on thrombolytic therapy. In the present study we evaluated the neuroprotective efficacy of UK-279,276 in combination with delayed thrombolysis with rhtPA in a model of embolic focal cerebral ischemia in rats. Our data indicate that coadministration of UK-279,276 and rhtPA extends the therapeutic window of thrombolysis and that the therapeutic benefit is superadditive.

Materials and Methods
All experimental procedures were approved by the Care of Experimental Animals Committee of Henry Ford Hospital. All measurements were performed blindly.

Animals
Male Wistar rats (n = 88) weighing 320 to 400 g were used in the experiments.

Embolic Model of Stroke
The MCA was occluded by placement of a single fibrin-rich clot at the origin of the MCA.

Experimental Protocols
UK-279,276 (Pfizer) was intravenously injected as a bolus dose of 3.2 mg/kg over a 2-minute period through the externalized femoral vein catheter, which was followed by infusion at a dose of 0.2 mg/kg for 7 days with a Harvard pump (Harvard Apparatus). The animals were allowed complete freedom of movement and access to food and water while maintaining the infusion. rhtPA (Genentech) was infused intravenously at a dose of 10 mg/kg as a 10% bolus, and the remainder was infused continuously over a 30-minute interval with a Harvard pump (Harvard Apparatus). After embolization, animals were randomly divided into the following 8 groups. (1) To examine the effect of UK-279,276 alone on ischemia, UK-279,276 and rhtPA vehicle were administered to ischemic rats at 2 (n = 11) or 4 hours (n = 11) after MCA occlusion. (2) To examine the effect of rhtPA alone on ischemia, rhtPA and saline were administered to ischemic rats at 2 (n = 11) or 4 hours (n = 11) after MCA occlusion. (3) To examine the effect of combination therapy of UK-279,276 and rhtPA on ischemia, UK-279,276 and rhtPA were administered at 2 (n = 11) or 4 hours (n = 11) after MCA occlusion. Control groups consisted of ischemic rats treated with same volumes of saline and rhtPA vehicle at 2 (n = 11) or 4 hours (n = 11) after MCA occlusion.

Neurological Severity Scores
The neurological severity scores (NSS) test is a composite of motor, sensory, reflex, and balance tests. NSS was measured at 1 hour and again at 7 days after MCA occlusion.

Foot-Fault Test
Rats were tested for placement dysfunction of forelimbs with the modified foot-fault test at 1 hour and again at 7 days after MCA occlusion.

Body Weight
Animals were weighed before and 7 days after embolic ischemia. Body weight loss is presented as a percentage of preischemic body weight.

Histopathologic Studies
All the animals were killed 7 days after MCA occlusion. Lesion volume, the primary outcome, was measured on the hematoxylin and eosin (H&E)-stained coronal sections with the use of a Global Laboratory Image Analysis program (Data Translation). The ischemic volume is presented as the percentage of infarct volume of the contralateral hemisphere (indirect volume calculation).

Measurement of Hemorrhage
Gross hemorrhage, defined as blood evident to the unaided eye on the H&E-stained coronal sections, was evaluated on 7 H&E-stained coronal sections for each animal. The percentage of gross hemorrhage in each experimental group was calculated. Petechial hemorrhage, defined as a cluster of red blood cells outside the lumen of blood vessels, was measured on 7 H&E-stained coronal sections with the use of a Global Laboratory Image Analysis program. The area of hemorrhage (µm²) was calculated by tracing the areas on the computer screen; the sum of hemorrhage areas from each section was calculated as the total area of hemorrhage.

Measurement of Myeloperoxidase-Immunoreactive Cells
Inflammatory cells within the brain were measured immunohistochemically. A 6-µm-thick paraffin-embedded coronal section from the center of the ischemic lesion at the level of the anterior commissure (coordinates: interaural, 8.2 mm; bregma, 0.8 mm) was stained with a polyclonal antibody against human myeloperoxidase (MPO) (1:200 dilution; DAKO) for evaluation of neutrophils. Numbers of MPO-immunoreactive cells were counted throughout the whole hemisphere at ×400 magnification. Only morphologically intact MPO-immunoreactive cells were included in the counts. Data are presented as the number of MPO-positive cells relative to the intact area (µm²).

Measurement of Fibrin Deposition
A coronal section from the center of the ischemic lesion was incubated with an anti-mouse fibrinogen/fibrin antibody at a titer of 1:1000 (Accurate Chemical & Scientific) for 1 hour at room temperature and then with the corresponding secondary antibody for 1 hour. Immunoreactivity was visualized with diamobenzidine. Fibrin deposition in the parenchyma was determined by counting the number of microvessels with fibrin deposition outside the vessel.

Statistical Analysis
All data are presented as mean±SD. We first evaluated normality of each measure of interest. Data transformation and nonparametric approach would be considered if the data were ill-behaved. Two-way ANOVA was used to test the overall treatment effects of ordinal data between groups. The interaction between rhtPA and UK-279,276 was tested with the critical level 0.05. If interaction (multiplicative effect) was significant at the 0.05 level, the effect of combination treatment would be further studied for superadditivity (the combined rhtPA and UK-279,276 effect is superior to the combined effect of each treatment alone) or subadditive effect (vice versa). We estimated the coefficient of superadditive/subadditive effect and its 95% CI. The CI excluding zero indicates a superadditive/subadditive effect of the combined rhtPA and UK-279,276 treatment.

Results
Neurological Severity Scores
All rats exhibited severe deficits 1 hour after MCA occlusion. Treatment with rhtPA alone at 2 but not at 4 hours after MCA occlusion significantly (P<0.05) improved neurological...
functional recovery (7.0±1.2) 7 days after ischemia compared with the control groups (8.1±1.1). Treatment with UK-279,276 alone at 2 but not 4 hours after MCA occlusion significantly (P<0.05) improved neurological functional deficit (7.5±1.5) on the NSS test compared with control groups (8.1±1.1). Combination treatment with UK-279,276 and rhtPA 2 and 4 hours after MCA occlusion significantly (P<0.05) reduced neurological functional deficits (6.3±1.3 at 2 hours and 6.5±1.3 at 4 hours) compared with the control groups (8.1±1.1).

Foot-Fault Test
One hour after MCA occlusion, there were no significant differences among the groups on the percentage of foot faults. Treatment with rhtPA alone at 2 but not at 4 hours after MCA occlusion significantly (P<0.05) reduced the percentage of foot faults (9.1±1.2) 7 days after ischemia compared with the control groups (11.5±1.1). Rats treated with UK-279,276 and rhtPA at 2 or 4 hours had a foot-fault test score (7.3±1.3 at 2 hours or 9.1±1.4 at 4 hours) significantly lower than that of the controls (11.5±1.1) 7 days after MCA occlusion (P<0.01). However, no significant reduction in foot-fault test score was detected in rats treated with UK-279,276 at 2 or 4 hours after ischemia.

Body Weight Loss
Treatment with rhtPA at 2 hours and combination treatment with UK-279,276 and rhtPA at 2 or 4 hours after the onset of ischemia significantly (P<0.05) reduced body weight loss compared with control groups (Figure 1).

Lesion Volume
Administration of rhtPA at 2 but not at 4 hours after MCA occlusion significantly (P<0.05) reduced lesion volume compared with control groups (Figure 2). Combination treatment with rhtPA and UK-279,276 when given at 2 and 4 hours after MCA occlusion significantly (P<0.01) reduced lesion volume compared with controls (Figure 2).

Hemorrhage
Gross hemorrhage in the ipsilateral lesion was detected in 27% of the 4-hour rhtPA-treated group, 18% in the 4-hour vehicle-treated group, and 9% in the remaining groups, with no differences detected among groups (P>0.58) (Table).

MPO-Immunoreactive Cells
Treatment with UK-279,276 alone at 2 but not 4 hours after ischemia significantly reduced the MPO-immunoreactive cells compared with the control group (Figure 3A). However, combination treatment with UK-279,276 and rhtPA at 2 and 4 hours after MCA occlusion significantly (P<0.05) reduced the density of MPO-immunoreactive cells in the ipsilateral hemisphere compared with the control group (Figure 3A).

Fibrin Deposition
Treatment with rhtPA 2 hours after MCA occlusion significantly (P<0.01) reduced numbers of vessels with fibrin leakage in the parenchyma compared with control animals (Figure 3B). In contrast, a significant (P<0.05) increase in the numbers of vessels with fibrin leakage was detected in the 4-hour rhtPA-treated group compared with control groups. Treatment with UK-279,276 alone at 4 hours after MCA occlusion significantly (P<0.05) reduced the numbers of vessels with fibrin leakage (Figure 3B). Combination treat-
ischemia significantly reduced infarct volume and improved neurological function without increasing hemorrhagic transformation, whereas treatment with neither UK-279,276 or rhtPA alone at 4 hours after ischemia reduced infarct volume. Furthermore, treatment with UK-279,276 in combination with rhtPA 4 hours after ischemia significantly reduced parenchymal neutrophil accumulation and blood-brain barrier (BBB) leakage in rats. Therefore, our data suggest that inhibition of neutrophil activity with UK-279,276 and concomitant reduction of BBB leakage extends the therapeutic window for thrombolysis.

We and others have demonstrated that treatment with UK-279,276 and other antiadhesion molecule compounds that inhibit neutrophil activity is effective only in a model of transient but not permanent MCA occlusion.12,15,23 In a model of embolic MCA occlusion, intravascular fibrin deposition and platelet aggregation secondary to MCA occlusion result in a progressive impairment of downstream microvascular plasma perfusion during the early stages of MCA occlusion, which resembles permanent ischemia.22 However, spontaneous thrombolysis occurs after embolic MCA occlusion. In parallel with clinical and experimental studies, the present data demonstrated that treatment with rhtPA alone 2 hours after embolic ischemia significantly reduced infarct volume and improved neurological outcome, whereas treatment with rhtPA alone 4 hours after embolic ischemia increased hemorrhagic transformation and exacerbated ischemic cell damage.16 Treatment with UK-279,276 alone at 2 hours after MCA occlusion did not significantly reduce infarct volume. In contrast, combination treatment of rhtPA with UK-279,276 at 4 hours after embolic ischemia significantly reduced infarct volume and improved neurological function deficits without increasing hemorrhagic transformation. Taken together, our result supports the hypothesis that inhibition of neutrophil activity improves ischemic outcome. The failure of a reduction in infarct volume after treatment with UK-279,276 alone may be attributed to the fact that the spontaneous thrombolysis occurs relatively late after embolic stroke at a time when ischemic cell damage is irreversible. In viewing recent failures of clinical trials of neuroprotective drugs and antineutrophil trafficking agents, the present study strongly supports the premise that thrombolysis-effected reperfusion is essential for neuroprotective drug treatment after embolic stroke.12,24

UK279,276 is a foreign glycoprotein that may develop an immune response, as has been demonstrated in the Enlimomab clinical trial.12 However, in blinded, placebo-controlled phase II trials of UK279,276, there was no evidence for the presence of neutralizing antibodies early enough after treatment to interfere with a potential acute treatment effect (M. Krams, PhD, unpublished data, 2002). Augmented hemorrhagic transformation is related to disruption of the BBB, which is exacerbated by delayed treatment with rhtPA.25 In the present study, using parenchymal fibrin deposition as a marker for disruption of the BBB, we demonstrated that the trend toward increased hemorrhagic transformation was coincident with a significant increase of BBB leakage in rats treated with rhtPA 4 hours after ischemia. Treatment with UK-279,276 at 4 hours after MCA occlusion significantly reduced BBB leakage. Moreover,
combination of rhtPA with UK-279,276 at 4 hours after the onset of ischemia significantly reduced BBB leakage and did not exacerbate hemorrhagic transformation, suggesting that UK-279,276 protects BBB integrity and consequently reduces hemorrhagic transformation.

Although the present study did not directly investigate pathways by which administration of UK-279,276 in combination with rhtPA reduces infarct volume and hemorrhagic transformation, mechanisms can be deduced from data in the present and previous studies. Evidence exists that reperfusion after thrombolysis increases the likelihood of neutrophil-mediated thrombosis in the microvasculature and neutrophil infiltration into the brain parenchyma after stroke, which may contribute to secondary brain damage. However, it is not obvious that neutrophils within the parenchyma contribute to ischemic cell damage. Administration of rhtPA 4 hours after MCA occlusion significantly increases the expression of I-CAM, E-selectin, and P-selectin, which exacerbate reperfusion injury after stroke. The combination of rhtPA with antibodies against ICAM-1 or CD18 enhances the efficacy of thrombolytic therapy, which is associated with the reduction of neutrophil–endothelial cell adhesion and parenchyma infiltration when evaluated 48 hours after MCA occlusion.

UK-279,276 is a selective inhibitor of the CD11b/CD18 β2 integrin, which blocks neutrophil adhesion to endothelium and does not bind to other members of the β2 integrin subfamily (CD11c and CD11d). Consistent with these studies, the present data revealed that combination treatment with UK-279,276 and rhtPA significantly reduced neutrophil accumulation assayed by MPO-immunoreactive cells, indicating that inflammatory response limits the efficiency of thrombolytic therapy. Furthermore, neutrophils play a role in the regulation of vascular permeability. The engagement of β integrins on neutrophils triggers the release of neutrophil-derived heparin-binding protein and further increases the vascular permeability.

Combination treatment enhances neuroprotection. Although a trend of increased hemorrhagic transformation was found among animals treated with rhtPA at 4 hours after the onset of ischemia, this treatment did not significantly exacerbate neurological impairments compared with the control groups. This discrepancy suggests that areas with parenchymal hemorrhage observed in the present study are relatively small compared with hematomata. Our observations are comparable to clinical data from the European Cooperative Acute Stroke Study (ECASS) II that demonstrate that only parenchymal hemorrhage exceeding 30% of ischemic lesion volume significantly deteriorates prognosis. Moreover, the National Institute of Neurological Disorders and Stroke rhtPA trial showed that symptomatic intercerebral hemorrhages increased from 0.6% in untreated stroke patients to 6.4% in rhtPA-treated stroke patients, but stroke patients exhibited functional improvements when rhtPA was given within 3 hours of the onset of stroke.

Our statistical analysis revealed that the neuroprotective effect of the combined treatments is superior to the additive effects from each treatment of rhtPA or UK-279,276 alone, especially when the single treatment of rhtPA or UK-279,276 did not have a significant effect on infarct volume. The superadditive effects of combined rhtPA and UK-279,276 treatment were manifest as reductions of lesion volume, body weight loss, NSS, and foot-fault test score at 7 days. Our results suggest that UK-279,276 and rhtPA may interact to improve the effectiveness of neutrophil inhibition and thrombolysis. Neutrophils play a role in fibrin formation. Under venous flow conditions, neutrophils promote fibrin formation and deposition. Thus, the superiority of combination treatment over single treatment may be due to inhibition of neutrophil-mediated fibrin deposition, which enhances the thrombolytic efficacy.

In summary, our study demonstrates that combination treatment with UK-279,276 and rhtPA at 4 hours after ischemia significantly reduces ischemic cell damage and improves neurological function without increasing hemorrhagic transformation. The combination of a selective CD11b/CD18 integrin inhibitor and thrombolytic therapy may extend the therapeutic window for the treatment of stroke without sacrificing safety.

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References

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UK-279,276 Plus rhtPA Reduces Cerebral Infarction

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