Background and Purpose—We tested the hypothesis that treatment of embolic stroke with recombinant human tissue plasminogen activator (rhtPA) alters cerebral expression of adhesion molecules.

Methods—Male Wistar rats were subjected to middle cerebral artery occlusion by a single fibrin-rich clot. P-selectin, E-selectin, and intercellular adhesion molecule-1 (ICAM-1) immunoreactivity was measured at 6 or 24 hours after embolic stroke in control rats and in rats treated with rhtPA at 1 or 4 hours after stroke. To examine the therapeutic efficacy of combined rhtPA and anti–ICAM-1 antibody treatment at 4 hours after embolization, ischemic lesion volumes were measured in rats treated with rhtPA alone, rats treated with rhtPA and anti–ICAM-1 antibody, and nontreated rats.

Results—Administration of rhtPA at 1 hour after embolization resulted in a significant reduction of adhesion molecule vascular immunoreactivity after embolization in the ipsilateral hemisphere compared with corresponding control rats. However, when rhtPA was administered to rats at 4 hours after embolization, significant increases of adhesion molecule immunoreactivity in the ipsilateral hemisphere were detected. A significant increase of ICAM-1 immunoreactivity was also detected in the contralateral hemisphere at 24 hours after ischemia. A significant reduction in lesion volume was found in rats treated with the combination of rhtPA and anti–ICAM-1 antibody compared with rats treated only with rhtPA.

Conclusions—The present study suggests that the time of initiation of thrombolytic therapy alters vascular immunoreactivity of inflammatory adhesion molecules in the ischemic brain and that therapeutic benefit can be obtained by combining rhtPA and anti–ICAM-1 antibody treatment 4 hours after stroke.

Key Words: antibodies ■ cell adhesion molecules ■ embolism ■ plasminogen activator, tissue type ■ selectins ■ thrombolysis ■ rats

Administration of recombinant human tissue plasminogen activator (rhtPA) to stroke patients reduces functional deficits. However, at present, the time window for effective treatment is 3 hours. This window is narrow because longer durations of ischemia and subsequent reperfusion induced with rhtPA increase the likelihood of hemorrhagic transformation and reperfusion injury. In addition, late thrombolysis, beyond 3 hours, may increase cerebral edema, morbidity, and mortality. The mechanism of tissue injury after delayed treatment with rhtPA is not well understood.

Migration of leukocytes into the parenchyma of brain has been associated with secondary damage after cerebral ischemia. Interaction between β integrins and adhesion molecules is necessary for migration of leukocytes into tissue. E-selectin and P-selectin, which facilitate rolling and transient tethering of leukocytes to endothelial cells, are expressed within the first few hours after embolic stroke, peaking at 6 and 12 hours, respectively. Contrast, vascular expression of intercellular adhesion molecule-1 (ICAM-1), which is a ligand for β integrins, maximizes at 48 hours after onset of middle cerebral artery (MCA) occlusion in the rat. Treatment with antibodies against these adhesion molecules reduces ischemic cell damage and improves functional outcome in animal models of stroke. Likewise, increases of E-selectin and ICAM-1 have been detected in human brain and plasma after stroke.

rhtPA is a pluripotent serine protease and itself may promote ischemic cell damage. Treatment with rhtPA in combination with anti–CD18 antibody or with anti–ICAM-1 antibody within 2 hours after embolization significantly reduced neurological damage in a rabbit model of embolic cerebral ischemia. However, little information is available regarding whether thrombolysis with rhtPA alters adhesion molecule expression in the ischemic brain. In the present study, in a rat model of cerebral embolic ischemia, we...
test the hypotheses that administration of rhtPA affects the inflammatory response to ischemic cell damage through modulation of adhesion molecule expression and consequently that combined treatment of stroke with rhtPA and anti–ICAM-1 antibody enhances the therapeutic benefits of rhtPA treatment in rats.

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

Animal Model
Male Wistar rats (n=72) weighing 300 to 350 g were anesthetized with halothane (1% to 3.5% in a mixture of 70% N₂O and 30% O₂) with the use of a face mask. The rectal temperature was maintained at 37±1°C throughout the surgical procedure by means of a feedback-regulated water heating system. The MCA was occluded by placement of an embolus at the origin of the MCA.36 Briefly, a single intact, fibrin-rich, 24-hour-old homologous clot (~1 μL) was placed at the origin of the MCA via a 15-mm-length modified polyethylene catheter (PE-50).

rhtPA and Anti–ICAM-1 Antibody
rhtPA (Genentech, Inc) was intravenously infused at a dose of 10 mg/kg as a 10% bolus, and the remainder was continuously infused over a 30-minute interval with a Harvard pump (Harvard Apparatus). An equal volume of vehicle (Genentech, Inc) was infused as above. The anti–ICAM-1 antibody used in the present study is a mouse anti-ICAM-1 monoclonal antibody (clone: 1A29).37 This antibody inhibits leukocyte infiltration in rat heart and brain with ischemia/reperfusion.20,38,39 The anti–ICAM-1 antibody was infused (intravenously) over a 3-minute interval at a dose of 2 mg/kg. The doses used for rhtPA and anti–ICAM-1 antibody treatment were based on previous studies.40,41

Experimental Protocols
To measure expression of adhesion molecules in rat brain, the following protocols were used. (1) Rats were treated with rhtPA at 1 hour after onset of stroke. These rats were killed at either 6 (n=6) or 24 hours (n=4) after stroke onset. (2) Rats were treated with rhtPA at 4 hours after stroke onset. These rats were also killed at either 6 (n=4) or 24 hours (n=4) after stroke onset. (3) Control groups included naive rats treated with rhtPA and killed at 6 (n=2) or 24 hours (n=2) after treatment and nontreated rats subjected to embolic stroke and killed at 6 (n=5) or 24 hours (n=5) after stroke onset.

To measure the therapeutic efficacy of combined rhtPA and anti–ICAM-1 antibody treatment of embolic stroke, rats were subjected to embolic stroke, and the following treatment protocols were initiated at 4 hours after stroke onset: (1) rats (n=12) received rhtPA alone; (2) rats (n=12) received rhtPA and anti–ICAM-1 antibody; and (3) rats (n=16) did not receive any treatment. All rats were killed at 48 hours after embolization. Neurological deficits, body weight loss, brain myeloperoxidase (MPO), and ischemic lesion volumes were measured in all these rats.

Measurement of Neurological Deficits
Neurological examinations were performed at 4 and 48 hours after injection of a clot. The neurological findings were scored as described by Zea Longa et al42 with some modification: no neurological deficit (0); right Horner’s syndrome (1); failure to fully extend the left forepaw (2); turning to the left (3); and circling to the left (4).

Measurement of Body Weight Loss
Animals were weighed before and 48 hours after embolic ischemia. Body weight loss was presented as a percentage of preischemic body weight.

Measurement of Brain MPO
Inflammatory cells, primarily neutrophils, within the brain were quantified immunohistochemically at 48 hours after stroke with the use of an antibody against MPO.43 A 2-μm-thick paraffin-embedded coronal section from the block located within the center of the ischemic lesion at the level of the anterior commissure (coordinates: interaural 8.2 mm, bregma 0.8 mm)37 was cut and stained with a polyclonal rabbit anti-human MPO antibody (at 1:200 dilution; DAKO) for evaluation of MPO expression on inflammatory cells. MPO immunoreactive cells were counted throughout the whole hemisphere under a ×40 objective. Only morphologically intact MPO immunoreactive cells were included in the counts.

Measurement of Ischemic Lesion Volume
Animals were anesthetized (intramuscularly) with ketamine (44 mg/kg) and xylazine (13 mg/kg). Rats were transcardially perfused with heparinized saline and 10% buffered formalin, and brains were removed. Using a rat brain matrix, we cut each brain into 2-mm-thick coronal blocks, for a total of 7 blocks per animal. The brain tissue was processed and embedded, and 6-μm-thick paraffin coronal sections from each block were cut and stained with hematoxylin and eosin (H&E) for histopathological evaluation. The volume of the cerebral ischemic lesion was measured with the use of a Global Laboratory Image analysis program (Data Translation). Each H&E-stained coronal section was evaluated at ×2.5 magnification. The area of lesion and the area of the ipsilateral hemisphere (square millimeters) were calculated on H&E-stained sections by tracing the area on the computer screen, and the volumes (cubic millimeters) were determined by integrating the appropriate area with the section interval thickness. To reduce errors associated with processing of tissue for histological analysis, the area of lesion in each section was presented as the percentage of the lesion to the area of the contralateral hemisphere, and the lesion volume was also presented as the percentage of lesion area to the contralateral hemisphere.

Immunohistochemistry for P-Selectin, E-Selectin, and ICAM-1 Expression
Rats were anesthetized with intraperitoneal administration of ketamine (44 mg/kg) and xylazine (13 mg/kg) and were transcardially perfused with heparinized 0.9% sodium chloride. The brains were rapidly removed, embedded in Tissue-Tek OCT compound (Miles, Inc), frozen in 2-methylbutane (Fisher Scientific), and cooled on dry ice. Coronal brain sections (8 μm thick) were cut on a cryostat and thaw-mounted onto gelatin-coated slides.

A rabbit polyclonal anti-human P-selectin antibody (Pharmingen) was used to detect P-selectin. The specificity of this antibody has been demonstrated.46 A monoclonal mouse anti-human E-selectin antibody (clone ENA1; Caltag Laboratories) was used to detect ICAM-1.48 To localize P-selectin immunoreactivity, tissue sections were incubated with a P-selectin antibody at 1:50 dilution for 1 hour at room temperature. Biotinylated rat–absorbed anti-rabbit IgG (Vector) was used as a secondary antibody. Immunohistochemical staining for the E-selectin antibody was performed according to the manufacturer’s instructions. Brain sections were incubated with an E-selectin antibody at 1:20 dilution in 0.5% bovine serum albumin for 1 hour at room temperature. Biotinylated rat–absorbed anti-mouse IgG (Vector) was used as a secondary antibody. Nonimmune mouse IgG1 (Becton Dickinson), used at the same concentration as the primary antibodies, or deletion of primary antibody was performed as a control. To quantify P-selectin, E-selectin, and ICAM-1 immunoreactive vessels, a coronal section located at interaural 8.2 mm, bregma 0.8 mm was selected from each animal.44 An adjacent section was stained with H&E for histopathological evaluation. The numbers of P-selectin, E-selectin, and ICAM-1 immunoreactive microvessels were counted throughout the ipsilateral hemisphere with a light microscope under a ×40 objective.
Statistical Analysis
Student’s t tests were performed to determine differences of infarct volume and body weight loss between groups. The Mann-Whitney rank sum test was performed for analysis of neurological score. All data are presented as mean±SE.

Results

Physiological Parameters
The arterial blood gas values and mean arterial blood pressure values were within the normal physiological range for all animals (data not shown).

Immunoreactivity of P-Selectin, E-Selectin, and ICAM-1
Immunoreactivity of P-selectin, E-selectin, and ICAM-1 was detected on a few scattered vessels in brain tissue of nonsurgical rats killed at 6 and 24 hours after infusion of rhtPA. Administration of rhtPA at 1 hour after embolization resulted in a significant (P<0.05) redution of immunoreactivity for P-selectin and E-selectin at 6 hours and for ICAM-1 at 24 hours after embolization in the ipsilateral hemisphere compared with animals without rhtPA treatment and killed at the corresponding time points (Figure 1). However, when rhtPA was administered to rats at 4 hours after embolization, E-selectin immunoreactive vessels significantly (P<0.05) increased at 6 hours after embolization, and significant (P<0.01) increases of P-selectin, E-selectin, and ICAM-1 immunoreactivity were detected at 24 hours after embolization compared with nontreated animals with embolic stroke (Figure 1). Increased P-selectin and E-selectin immunoreactive vessels were limited to the ipsilateral hemisphere. However, when rhtPA treatment was initiated at 4 hours after onset of embolization, a significant increase of ICAM-1 immunoreactivity was also detected in the contralateral hemisphere at 24 hours after ischemia (89±16.7 versus nontreated 36±4.3).

Treatment With rhtPA and Anti–ICAM-1 Antibody
Two rats from the rhtPA-treated group died within 24 hours after embolic ischemia. Autopsy showed that these rats had massive brain edema. These rats were excluded from data analysis.

Neurological Deficits
Severe neurological deficits were evident in all groups at 4 hours after onset of stroke: control nontreated (3.2±0.2), rhtPA treated (3.3±0.3), and combined rhtPA and anti–ICAM-1 treated (3.3±0.2). At 48 hours after onset of stroke, rats treated with rhtPA and anti–ICAM-1 antibody showed a trend toward a reduced neurological deficit compared with rhtPA-treated rats (0.8±0.2 versus 1.4±0.2, respectively; P=0.07). A significant reduction in neurological deficit was not detected between rats treated with the combination of rhtPA and anti–ICAM-1 antibody and nontreated rats (1.2±0.2; P=0.16).

Body Weight
Animals treated with rhtPA and anti–ICAM-1 antibody exhibited a significant (P<0.05) reduction of weight loss compared with animals treated with rhtPA alone (11.9±1.1% versus 17.9±1.5%) and with nontreated animals (11.9±1.1% versus 16.6±1.5%) at 48 hours after embolization.

Ischemic Lesion Volume
Rats treated with rhtPA and anti–ICAM-1 antibody at 4 hours after embolization showed a significant (P<0.05) reduction in ischemic lesion volume (28.9±2.7%) compared with rats treated with rhtPA treatment alone (39.1±3.9%) (Figure 2). A trend toward a reduction (P=0.055) of ischemic lesion volume was observed between the group treated with rhtPA and anti–ICAM-1 antibody and the nontreated group.
but an effect of the pharmacological intervention that alters response with rhtPA intervention 4 hours after embolic stroke that it is not the lesion size that dictates the inflammatory response between treatment and nontreatment implies nontreated groups, a significant difference in the inflammatory activity to adhesion molecules within the lesion implies that treatment with rhtPA at 4 hours after onset of stroke significantly increases vascular immunoreactivity to E-selectin, P-selectin, and ICAM-1.

Our data demonstrate that treatment of embolic stroke in the rat with rhtPA modulates vascular immunoreactivity of adhesion molecules as a function of time of initiation of treatment. Treatment within the therapeutic window with rhtPA, eg, at 1 hour after onset of embolic stroke, significantly reduces vascular immunoreactivity to E-selectin, P-selectin, and ICAM-1 within the ischemic lesion; in contrast, treatment outside of the therapeutic window, eg, at 4 hours after onset of stroke, significantly increases vascular immunoreactivity to these adhesion molecules. Increased vascular immunoreactivity to E-selectin, P-selectin, and ICAM-1 is indicative of compromised and inflamed vessels. Thus, these vessels may be primed and vulnerable to reperfusion injury and eventual disruption. Our data suggest that modulation of the inflammatory response by rhtPA may be a mechanism by which rhtPA affects outcome from stroke.

Treatment of embolic stroke within the therapeutic window reduces the ischemic lesion; subsequent to the reduction of the lesion, inflammation is reduced. However, the observation that treatment with rhtPA at 4 hours after onset of embolic stroke significantly increases vascular immunoreactivity to adhesion molecules within the lesion implies that rhtPA has a direct effect on adhesion molecule expression and is not secondary to the alteration of the lesion. The volume of the cerebral ischemic lesion is not significantly different between treatment with rhtPA at 4 hours and nontreated conditions. Thus, for a similar lesion in treated and nontreated groups, a significant difference in the inflammatory response between treatment and nontreatment implies that it is not the lesion size that dictates the inflammatory response with rhtPA intervention 4 hours after embolic stroke but an effect of the pharmacological intervention that alters expression of adhesion molecules. We note that adhesion molecule expression is not increased in naïve animals treated with rhtPA. If rhtPA alone was the sole factor in mediating increased expression of adhesion molecules, then we would expect that administration of rhtPA to nonsurgical animals would yield increased expression of these adhesion molecules in the brain. rhtPA lyses clots by converting plasminogen into plasmin. Plasmin increases P-selectin expression on endothelial cells. In addition, thrombin generated by thrombolysis increases vascular immunoreactivity to E-selectin, P-selectin, and ICAM-1. Whether the proinflammatory effect of delayed administration of rhtPA is mediated by rhtPA, plasmin, thrombin, and other factors is unknown. The interpretation of our data should be tempered by the possibility that the doses of rhtPA used in the present study are very high relative to those used in humans.

The increased expression of adhesion molecules with rhtPA treatment initiated at 4 hours after embolization supports the need for a combined therapeutic approach, ie, thrombolysis and a therapy that reduces the posts ischemic inflammatory response.

MPO Immunoreactive Cells

Rats treated with rhtPA and anti–ICAM-1 antibody at 4 hours after embolization showed a significant reduction (P<0.05) in the number of MPO immunoreactive cells (491±145) accumulated in the ischemic lesion compared with administration of rhtPA alone at 4 hours after embolization (959±107).

Discussion

Our data demonstrate that treatment of embolic stroke in the rat with rhtPA modulates vascular immunoreactivity of adhesion molecules as a function of time of initiation of treatment. Treatment within the therapeutic window with rhtPA, eg, at 1 hour after onset of embolic stroke, significantly reduces vascular immunoreactivity to E-selectin, P-selectin, and ICAM-1 within the ischemic lesion; in contrast, treatment outside of the therapeutic window, eg, at 4 hours after onset of stroke, significantly increases vascular immunoreactivity to these adhesion molecules. Increased vascular immunoreactivity to E-selectin, P-selectin, and ICAM-1 is indicative of compromised and inflamed vessels. Thus, these vessels may be primed and vulnerable to reperfusion injury and eventual disruption. Our data suggest that modulation of the inflammatory response by rhtPA may be a mechanism by which rhtPA affects outcome from stroke.

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In summary, early (1 hour) thrombolysis with rhtPA reduces vascular immunoreactivity to P-selectin, E-selectin, and ICAM-1, and delayed (4 hours) thrombolysis increases vascular immunoreactivity to these adhesion molecules. Combination treatment at 4 hours after embolic stroke in the rat with rhtPA and anti–ICAM-1 antibody significantly reduces ischemic lesion volume and neutrophil accumulation compared with rhtPA treatment alone.

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References


Editorial Comment

Although tPA was approved for acute ischemic stroke therapy in the United States in the summer of 1996, relatively few patients are receiving this treatment. There are a variety of reasons for the delayed implementation of this treatment strategy by the medical community. Perhaps the most important is that the time window for effective therapy is 3 hours from symptom onset. The results of the European Cooperative Acute Stroke Study (ECASS II)\(^1\) showed that tPA can be safely administered up to 6 hours after the stroke begins, but it is not clearly effective at the later time points. Thus, supplying tPA to a large fraction of the population likely to benefit from it will require a revolution in the medical care system. This is beginning to happen, but it is a slow and difficult process.

Acceptance of medical management for acute stroke will be facilitated by adjunctive therapies that improve efficacy or increase the length of the treatment window. A number of neuroprotective agents have been tested in acute stroke therapy trials, and thus far, all have failed to provide unequivocal benefit. All of the trials to date have used a neuroprotective agent as monotherapy. Some recent trials have permitted administration of the experimental drug after tPA, but the number of patients who have received such combinations has been too small to provide meaningful information. However, there is experimental evidence suggesting that some combinations of thrombolytics and neuroprotective agents will act synergistically.

In the accompanying article, Zhang et al have studied the combination of tPA and an antibody to ICAM-1, the endothelial receptor for adhesion molecules expressed on the surface of leukocytes, in a rat embolic stroke model. The idea is that this combination might prevent reperfusion injury after effective thrombolysis. This article suggests that the time of initiation of therapy is critical. A beneficial effect is shown if treatment is initiated rapidly, but delayed coadministration may be harmful. The authors briefly mention that a controlled clinical trial of an ICAM-1 antibody (enlimomab), administered as monotherapy, produced an adverse outcome.\(^2\) The precise causes of this devastating result are unclear, but Zhang et al give us some insight as to one possible reason. Delayed therapy can produce vascular inflammation that may exacerbate the ischemic injury.

Any clinical trial protocol entails many arbitrary decisions, particularly in the initial stages, and some of these choices may have been responsible for the unfortunate outcome of the enlimomab stroke trial. It is rare for the first phase III trial of a new form of treatment to result in a successful conclusion. It will be regrettable if the strategy of combined use of thrombolytics and adhesion molecule antibodies is prematurely abandoned because of the enlimomab clinical trial. There are many lessons that can be derived from the initial efforts, and if we are to progress, we will need to adequately analyze our failures and use the information so obtained in an iterative fashion. Furthermore, the results of the study by Zhang et al suggest that even if 2 drugs are independently able to reduce ischemic damage, combining them will not necessarily produce a positive result. Many factors interact to produce ischemic injury, and it will require painstaking investigation to develop truly effective acute stroke treatments.

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Thrombolysis With Tissue Plasminogen Activator Alters Adhesion Molecule Expression in the Ischemic Rat Brain
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