Adjuvant Treatment With a Glycoprotein IIb/IIIa Receptor Inhibitor Increases the Therapeutic Window for Low-Dose Tissue Plasminogen Activator Administration in a Rat Model of Embolic Stroke

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Background—Platelet aggregation and fibrin deposition are key events leading to microvascular thrombosis and progressive impairment of downstream microvascular perfusion after stroke. We tested the hypothesis that inhibition of platelet function with a GP IIb/IIIa receptor antagonist would increase the efficacy and safety and increase the time window for thrombolytic therapy for stroke with full- and half-dose tissue plasminogen activator (tPA).

Methods and Results—Rats were subjected to embolic middle cerebral artery occlusion. Four hours after ischemia, rats were treated with 7E3 F(ab')2 (6 mg/kg) in combination with tPA at doses of 10 and 5 mg/kg, tPA alone at a dose of 10 or 5 mg/kg, 7E3 F(ab')2 (6 mg/kg) alone, or saline. Combination treatment with 7E3 F(ab')2 and tPA (full- or half-dose) significantly (P<0.05) reduced infarct volume and neurological deficits compared with saline-treated rats. However, treatment with 7E3 F(ab')2 or tPA (full- or half-dose) alone did not reduce infarct volume. Quantitative measurements of cerebral microvessels perfused by FITC-dextran revealed that combination treatment with 7E3 F(ab')2 and full-dose tPA significantly (P<0.05) increased the percentage of FITC-dextran–perfused vessels compared with saline and full-dose tPA–treated rats. In addition, treatment with 7E3 F(ab')2 in combination with full-dose tPA significantly (P<0.05) decreased microvascular platelet accumulation and matrix metalloproteinase 9 immunoreactivity and protected against loss of collagen IV immunoreactivity.

Conclusions—Combination treatment with 7E3 F(ab')2 with full- and half-dose tPA at 4 hours after ischemia significantly reduces infarct volume and improves neurological outcome. Enhancement of patency and integrity of cerebral microvessels most likely contributes to the benefits observed with this combination therapy. (Circulation. 2003;107: 2837-2843.)

Key Words: cerebral ischemia • plasminogen activators • platelets • microcirculation

Thrombolytic therapy has demonstrated efficacy in acute stroke when administered within 3 hours of stroke onset.1 It would be desirable, however, to improve the efficacy and safety of stroke therapy and to increase the time after stroke during which thrombolytic therapy is effective. The efficacy of thrombolytic therapy may be improved by increasing the rate of lysis of the occlusive thrombus or embolus, but even if this is achieved, it does not ensure perfusion of the microcirculation or freedom from reocclusion.2 Inhibition of platelet function with GP IIb/IIIa antagonists in animal models and in patients with ischemic cardiovascular disease has been shown to augment the efficacy of thrombolytic agents, even when administered at a reduced dose, in both speed of reperfusion and protection against reocclusion.3–6 Moreover, combination therapy in humans with myocardial infarction has been shown to result in improved cardiac microcirculatory function, as judged by ST-segment resolution.7 Our previous animal studies of stroke demonstrating platelet aggregation function in the ischemic microcirculation distal to an embolus of the middle cerebral artery (MCA) within 4 hours after initiation of the occlusion reinforce the theoretical benefit of antiplatelet therapy in protecting the microcirculation from damage that may prevent effective reperfusion even if the obstructing thrombus is lysed.8

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Hemorrhagic cerebrovascular complications of thrombolytic therapy are the major obstacles to extending the allowable time from the onset of symptoms to the initiation of
therapy. The mechanism of hemorrhagic transformation of thrombotic and thromboembolic cerebrovascular disease and its relationship to the time of initiation of thrombolytic therapy remain poorly understood. We previously demonstrated time-dependent immunohistochemical evidence of loss of integrity of microvascular collagen type IV in our experimental model. Moreover, there was a correlation between platelet deposition in the microcirculation and loss of collagen type IV integrity. Because platelets contain matrix metalloproteinases and other neutral proteases, it is possible that platelet aggregation may contribute to loss of microvascular integrity and hemorrhagic transformation. Thus, although admittedly paradoxical, it is possible that inhibition of platelet deposition and aggregation in the microcirculation by platelet GP Ib/IIa antagonists might actually protect against hemorrhagic transformation.

To test these hypotheses, in the present study we treated rats given stroke with a GP Ib/IIa antagonist, 7E3 F(ab’)_2 in combination with tissue plasminogen activator (tPA) and examined the integrity and patency of cerebral microvessels. We demonstrate that adjunctive fibrinolysis with tPA, even at half-dose, and 7E3 F(ab’)_2 significantly improves the integrity and patency of cerebral microvessels after stroke, in addition to extending the therapeutic window of effective thrombolysis without increasing hemorrhagic transformation.

Methods

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

Animal Model

Male Wistar rats (n=62; Charles River Laboratories, Wilmington, Mass) weighing 300 to 350 g were subjected to embolic MCA occlusion.

Experimental Protocols

The F(ab’)_2 fragment of the murine monoclonal antibody 7E3, which reacts with GP Ib/IIa and α,β, (Centocor), was given intravenously at a bolus dose of 6.0 mg/kg 4 hours after embolic MCA occlusion and was followed by a second intraperitoneal dose of 6.0 mg/kg at 12 hours after the first dose. This dosing protocol produces marked inhibition of platelet aggregation for up to 4 days. Recombinant human tPA (Genentech) was infused intravenously at a total dose of 5 or 10 mg/kg (10% bolus 4 hours after ischemia, and the remainder at a continuous infusion with a syringe infusion pump over a 30-minute interval; Harvard Apparatus). The doses of tPA were selected on the basis of previous studies of tPA in rats, which demonstrate that the specific fibrinolytic effect of tPA is at least 10 times less in the rat than in the human. After the embolus was placed in the MCA, animals were randomly assigned to treatment with 7E3 F(ab’)_2 alone (n=6), tPA alone [10 (n=14) or 5 (n=6) mg/kg], 7E3 F(ab’)_2 in combination with tPA [10 (n=14) or 5 (n=8) mg/kg], or saline (n=14). Rats were killed at 1 or 7 days after MCA occlusion.

Measurements of Infarct Volume

Seven days after MCA occlusion, infarct volume was measured on 7 coronal sections by use of a Global Laboratory Image analysis program (Data Translation). The ischemic volume was expressed as the percentage of infarct volume of the contralateral hemisphere (indirect volume calculation).

Measurements of Hemorrhage

Gross hemorrhage, defined as blood evident to the unaided eye on the hematoxylin and eosin–stained coronal sections, was evaluated on 7 coronal sections for each animal at 7 days after MCA occlusion. Evidence of gross hemorrhage on any section was considered to show the presence of hemorrhage in that animal; data are presented as percentage of animals per group.

Measurements of Microvascular Patency

To examine the patency of cerebral microvessels, fluorescein isothiocyanate (FITC) dextran (2×10^4 molecular weight, Sigma; 1 mL of 50 mg/mL) was administered intravenously to the rats 1 day after stroke. Three coronal sections (100 mm) from each rat at bregma −0.2, −0.8, and −2.8 mm were analyzed with a MicroComputer Imaging Device (MCID) system (Imaging Research). Briefly, fluorescence was digitized with a 5× objective (Zeiss Plan Neoflura) attached to a fluorescent microscope (Zeiss, Axioplan 2) via a digital CCD camera (Hamamatsu) connected to the MCID system. Ten fields of view (4.4 mm^2) from each coronal section were acquired in the territory supplied by the right MCA. A threshold was applied to each digitized image to ensure that the numbers of FITC pixels reflected the original FITC-dextran-perfused patterns. Data are presented as the numbers of FITC pixels divided by the total numbers of pixels within the field of view, expressed as a percentage.

Immunohistochemistry

To examine the integrity of cerebral microvessels, a mouse anti-rat type IV collagen monoclonal antibody (M3F7, Developmental Studies Hybridoma Bank, University of Iowa) was used at a titer of 1:200 to detect collagen type IV, one of the major basal lamina components of cerebral microvessels. An increase of matrix metalloproteinase 9 (MMP9) activity contributes to disruption of the blood-brain barrier (BBB). To detect the presence of MMP9 in the microvessels, a rabbit polyclonal antibody against mouse MMP9 (a gift from Dr. R. Senior, Washington University, St Louis, MO) and a mouse anti-rat MMP9 (95-kDa) antibody (Chemicon International) were used at a titer of 1:200. To detect platelets and platelet aggregates, a rabbit polyclonal antibody against rat thromboxy (Inter-Cell Technologies) was used at a titer of 1:200. A monoclonal antibody against von Willebrand factor (1:400, DAKO) was used as a marker for endothelial cells. The specificity of these antibodies has been demonstrated in rats.

Functional Outcome

Neurological severity scores (NSSs) and foot-fault tests were measured at 1 and 7 days after MCA occlusion.

Neurological Severity Score

The NSS is a composite of motor, sensory, reflex, and balance tests. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18).

Foot-Fault Test

Rats were tested for placement dysfunctions of forelimbs with the modified foot-fault test. The total number of steps (movement of each forelimb) that the rat used to cross the grid and the total numbers of foot faults for each forelimb were recorded.

Body Weight Loss

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

Statistics

All values are presented as mean±SD. Two-way ANOVA was used to test overall treatment effects of ordinal data between groups. Fisher’s exact test was used to test the gross hemorrhagic rates among the groups. Statistical significance was set at P<0.05.
Results

Infarct Volume and Hemorrhage

Monotherapy with either tPA or 7E3 F(ab’)2 at 4 hours after stroke did not significantly reduce infarct volume (Figure 1). However, combination treatment with tPA, both full-dose and half-dose, and 7E3 F(ab’)2 significantly ($P<0.05$) reduced infarct volume compared with saline-treated rats (Figure 1).

Gross hemorrhage was identified in 13% of saline-treated rats, 17% of 7E3 F(ab’)2–treated rats, 25% of full-dose tPA–treated rats, and 13% of half-dose tPA–treated rats. In contrast, rats treated with tPA at full-dose and half-dose in combination with 7E3 F(ab’)2 exhibited 13% and 0% gross hemorrhage, respectively. No significant differences were detected among the groups.

Functional Outcome

Rats in all groups exhibited severe deficits on NSS and the foot-fault test at 1 day after MCA occlusion, and no significant differences were detected among the groups. Rats treated with 7E3 F(ab’)2 alone and tPA alone at 4 hours after MCA occlusion failed to demonstrate improved recovery of neurological function as assayed by the NSS and foot-fault test compared with saline-treated rats at 7 days (Table). However, rats treated with combination therapy showed significant ($P<0.05$) improvements in neurological function compared with saline-treated rats at 7 days after MCA occlusion (Table). In addition, combination treatment with tPA and 7E3 F(ab’)2 significantly ($P<0.05$) reduced body weight loss compared with saline-treated rats (Table). No significant reduction in body weight loss was detected in rats treated with 7E3 F(ab’)2 alone or tPA alone (Table).

Patency and Integrity of Microvessels

To test the effect of treatment on the patency of downstream cerebral microvessels, microvessels perfused with FITC-dextran were examined in rats treated at 4 hours after ischemia with full-dose tPA alone, with 7E3 F(ab’)2 alone,
and with combination therapy of full-dose tPA and 7E3 F(ab')2. A significant (P<0.05) reduction of the percent microvascular plasma area in the ipsilateral hemisphere relative to the contralateral hemisphere 24 hours after stroke was detected in saline-treated, full-dose tPA–treated (n=3) and 7E3 F(ab')2-treated rats (n=3) (Figure 2), which is consistent with our previous findings that occlusion of the MCA results in progressive impairment of cerebral microcirculation in the territory supplied by the MCA.20 Combination treatment with full-dose tPA and 7E3 F(ab')2 (n=3) was associated with a significantly (P<0.05) higher percentage of FITC-dextran–perfused vascular area in the territory supplied by the MCA compared with saline-treated rats (Figure 2). Rats treated with full-dose tPA alone or saline also exhibited significant increases in the number of ipsilateral hemisphere microvessels containing platelet aggregation compared with the number in the contralateral hemisphere (59.3±10.9 and 54.2±10.1 versus 5.6±3.9 and 4.6±3.2). Combination treatment with full-dose tPA and 7E3 F(ab')2 significantly (P<0.05) reduced the number of microvessels with platelet aggregation in the ipsilateral hemisphere (37.7±9.8) compared with full-dose tPA–treated (59.3±10.9) and saline-treated rats (54.2±10.1). The significant reduction of platelet aggregation in microvessels and improvement of microcirculation after combination treatment is consistent with the platelet aggregates being the cause of the impaired patency of the microcirculation.

To examine the treatment efficacy on the integrity of the cerebral microvessels, the numbers of collagen type IV immunoreactive vessels in the MCA territory were measured. In saline-treated rats, there was a significant (P<0.05) reduction in collagen type IV immunoreactive vessels throughout the occluded MCA territory relative to the contralateral unoccluded MCA territory (Figure 3). Treatment with full-dose tPA alone at 4 hours after occlusion was associated with an even greater loss (P<0.05) of collagen type IV–immunoreactive vessels compared with saline-treated rats (Figure 3). In contrast, combined treatment with full-dose tPA and 7E3 F(ab')2 resulted in a smaller reduction in collagen type IV immunoreactive vessels in the ipsilateral cortex relative to the contralateral cortex (Figure 3).
To test whether MMP9 and/or platelet aggregates are associated with degradation of collagen type IV, double immunostaining was performed. Double immunostaining of platelets and MMP9 revealed colocalization of platelets with MMP9 immunoreactivity (Figure 4). MMP9 immunoreactivity was also observed on adjacent microvessels without obvious platelet aggregates (Figure 4). Cerebral microvessels with platelet accumulation exhibited diffuse collagen type IV immunoreactivity, suggesting a correlation between platelet deposition and loss of microvascular integrity (Figure 3). Quantitative analysis shows that combination treatment with full-dose tPA and 7E3 F(ab’)_2 significantly reduced the numbers of vessels (43.3±9.1) with MMP9 immunoreactivity compared with the numbers in saline-treated (56.0±9.7) and full-dose tPA–treated groups (66.7±8.7).

Discussion

Our data indicate that combination treatment at 4 hours after stroke with 7E3 F(ab’)_2 and tPA even at half-dose significantly reduces infarct volume and improves neurological function without increasing the incidence of hemorrhagic transformation. Enhancement of microvascular patency and integrity probably contributes to the benefits observed in the combination therapy.

7E3 F(ab’)_2 is a potent GP IIb/IIIa antagonist that effectively inhibits GP IIb/IIIa–mediated platelet aggregation in rats. In a rat model of focal cerebral ischemia, administration of 7E3 F(ab’)_2 effectively reduced brain infarction when treatment was initiated within a 3-hour therapeutic window. However, in the present study, delayed treatment with 7E3 F(ab’)_2 at 4 hours after ischemia neither reduced ischemic lesion volume nor improved neurological function after MCA occlusion, suggesting that preventing platelet aggregation may not be sufficient to achieve effective reperfusion. This hypothesis is supported by our results showing that combination treatment with 7E3 F(ab’)_2 and tPA at 4 hours after ischemia significantly reduces infarct volume and enhances functional recovery. Our results are consistent with previous studies showing that intravascular fibrin deposition, platelet aggregation, and leukocyte accumulation contribute to progressive ischemic cell damage.

Fibrinolysis with tPA activates platelets via the release of thrombin, and aggregation of activated platelets obstructs microcirculation. Moreover, activated platelets retract clots and release plasminogen activator inhibitor 1, α2-antiplasmin, and factor XIII, all of which are known to contribute to resistance to thrombolysis. Inhibition of platelet aggregation could amplify the efficacy of fibrinolytic therapy with tPA via increased exposure of fibrin to tPA, and thereby, the dose of tPA could be reduced. The incidence of hemorrhagic transformation increases with increased dose of tPA. Therefore, the effectiveness of combination of 7E3 F(ab’)_2 and half-dose tPA (5 mg/kg) was investigated in the present study. Administration of tPA at a dose of 5 mg/kg along with 7E3 F(ab’)_2 significantly reduced ischemic lesion volume and reduced the incidence of hemorrhagic transformation com-

Figure 4. Platelet deposition and MMP9 immunoreactivity in cerebral microvessels. Double immunostaining shows that platelet deposition (A) and MMP9 immunoreactivity (B) colocalized to ipsilateral microvessels (C, merged image from A and B), although some MMP9-immunoreactive vessels did not exhibit platelet deposition (C, arrows). MMP9-positive vessels (D, red) were von Willebrand factor–immunoreactive (D, green). Quantitative data (E) show that combination treatment with full-dose tPA and 7E3 F(ab’)_2 significantly reduced number of MMP9-immunoreactive vessels assessed 1 day after MCA occlusion. *P<0.05 vs saline and full-dose tPA alone. Bar=10 μm.
pared with control and full-dose tPA alone (10 mg/kg). Although tPA doses used in the present study are not directly comparable to the doses used in humans, our results are consistent with previous studies in myocardial infarction. \(^4,^5\) A combination of abciximab and half-dose alteplase is effective in restoring flow in the infarct-related artery and achieving microvascular reperfusion. \(^6\) Patients with acute myocardial infarction show greater reperfusion and improved ST-segment resolution after combination treatment with abciximab and low-dose alteplase. \(^4,^7\)

The increased risk of hemorrhagic transformation limits the clinical use of tPA. \(^1\) Disruption of the BBB causes hemorrhagic transformation. \(^20\) In addition, rethrombosis in microvessels is one of main reasons for failure of fibrinolytic therapy. \(^30\) However, the effects of combination treatment with 7E3 F(ab\(^\prime\))\(_2\) and tPA on the integrity and patency of cerebral microvessels have not been examined. Our data show that combination treatment with 7E3 F(ab\(^\prime\))\(_2\) and full-dose tPA significantly increased downstream cerebral microvascular patency and reduced the microvascular platelet deposition, indicating that the combination therapy reduces platelet-mediated thrombosis and improves cerebral microvascular patency. In addition, the combination treatment significantly reduced degradation of collagen type IV. Increases in microvascular patency and reduction in degradation of collagen type IV were detected primarily in the cortical penumbra after the combination treatment, which is consistent with our previous findings that impairment of cortical microcirculation develops by 6 hours after embolic stroke. \(^30\) Furthermore, the combination therapy significantly reduced the number of MMP9-immunoreactive vessels. Double immunostaining indicated that platelets and endothelial cells are a source of MMP9, which is consistent with previous reports. \(^9,^10\) Collagen type IV is one of the major basal lamina components of cerebral microvessels. \(^14\) Embolic stroke induces acute degradation of collagen type IV, which increases disruption of the BBB and hemorrhagic transformation after stroke. \(^8\) MMP9 cleaves collagen type IV, and inhibition of MMP9 activity reduces the incidence of hemorrhagic transformation. \(^31\) Collectively, our data suggest that the combination therapy inhibits platelet aggregation, enhances thrombolysis, and consequently improves cerebral microcirculation. Because platelets are one of the cellular sources of MMP9, reduction of platelet deposition in microvessels results in diminishing of MMP9 activity and thereby decreases the degradation of collagen type IV. Enhanced integrity and patency of cerebral microvessels therefore contribute to reductions of ischemic cell damage and of the incidence of hemorrhagic transformation observed after the combination therapy.

In summary, the data demonstrate that administration of 7E3 F(ab\(^\prime\))\(_2\) in combination with tPA at 4 hours after MCA occlusion significantly enhanced the integrity and patency of downstream microvessels, reduced infarct volume, and improved functional outcome without increasing the incidence of hemorrhagic transformation. Thus, fibrinolytic therapy with tPA in conjunction with 7E3 F(ab\(^\prime\))\(_2\) may provide a safe and effective therapeutic strategy that extends the therapeutic window for thrombolytic treatment of stroke.

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