Cilostazol Reduces the Risk of Hemorrhagic Infarction After Administration of Tissue-Type Plasminogen Activator in a Murine Stroke Model

Yukiko Kasahara; Takayuki Nakagomi, MD; Tomohiro Matsuyama, MD; David Stern, MD; Akihiko Taguchi, MD

Background and Purpose—Prior use of antiplatelet agents improves stroke outcome in patients undergoing thrombolytic therapy as shown by reduced arterial reocclusion, although the risk of cerebral hemorrhage can be increased.

Methods—The effect of cilostazol, an antiplatelet drug that improves endothelial function through upregulation of intracellular cAMP, on cerebral hemorrhage after thrombolytic therapy was investigated using a highly reproducible transient ischemia model.

Results—Treatment with cilostazol for 7 days before ischemia significantly suppressed the risk and severity of cerebral hemorrhage after injection of tissue-type plasminogen activator, although treatment with aspirin had no such protective effect compared with nontreated mice. Immunohistological analysis revealed that treatment with cilostazol suppressed disruption of the microvasculature in the ischemic area associated with reduced matrix metalloproteinase-9 activity.

Conclusions—Our results suggest that patients treated with cilostazol before onset of stroke could have a lower risk of cerebral hemorrhage after thrombolytic therapy and might also have a longer therapeutic time window for thrombolysis. Furthermore, the risk of cerebral hemorrhage can be significantly altered by prestroke therapies, and analysis of the effects of multiple drugs on tissue-type plasminogen activator-induced cerebral hemorrhage in animal models is essential for extending safe and effective thrombolytic therapy to a wider group of patients. (Stroke. 2012;43:499-506.)

Key Words: antiplatelet drugs ▪ brain ischemia ▪ ICH ▪ murine model ▪ thrombolysis

Intravenous thrombolysis with tissue-type plasminogen activator (tPA) has been shown to improve functional outcomes of patients with stroke when given within 3 hours from the onset of stroke.1,2 However, treatment with tPA significantly increases the risk of bleeding events, including hemorrhagic infarction.3,4 Although a number of clinical studies indicate that prior use of antiplatelet agents increases the risk of cerebral hemorrhage after thrombolytic therapy,5-7 the use of antiplatelet agents had been shown to improve stroke outcome compared with patients without such therapy.8,9 The beneficial effects of antiplatelet drugs may be attributed to improved microcirculatory function and diminished reocclusion after tPA treatment, the latter observed in 20% to 34% of patients after initially successful recanalization.10,11 Reocclusion after tPA treatment is induced, at least in part, by the activation of the coagulation cascade by tPA.12,13 These findings indicate that the use of antiplatelet drugs is potentially a double-edged sword in the context of tPA treatment; that is, use of antiplatelet agents is likely to be beneficial for stroke outcome despite increased risk of hemorrhagic infarction. Consistent with these findings, a randomized controlled trial in the Netherlands has shown that thrombolysis in combination with antiplatelet drugs prevented reocclusion and improved the clinical outcome.14

Recently, cilostazol, an antiplatelet drug that inhibits the activity of cAMP phosphodiesterase Type 3, has been shown to be superior to aspirin for secondary prevention of stroke with fewer hemorrhagic events.15 Compared with other antiplatelet drugs, cilostazol is known to have milder hemorrhagic side effects16 and prevent the increase in bleeding time.17 In this study, we focused on cilostazol and investigated its effect on hemorrhagic infarction after treatment with tPA using a murine ischemia–reperfusion model.

Materials and Methods

All procedures were performed under the auspices of an approved protocol of the National Cerebral and Cardiovascular Center Animal Care and Use Committee.

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Induction of Focal Cerebral Ischemia

To evaluate the effect of cilostazol on tPA-induced hemorrhagic infarction, we developed a highly reproducible murine transient cerebral ischemia model based on modification of our previous method. In brief, the left middle cerebral artery (MCA) was isolated in male 7-week-old CB17/cr mice (Clea, Tokyo, Japan) under halothane inhalation (3%) anesthesia and transient focal cerebral ischemia was induced under direct vision by transiently occluding the distal portion of the left MCA with a monofilament nylon suture (7-0 in size; Tyco) for 90, 120, 180, or 240 minutes. During surgical procedures, rectal temperature was monitored and controlled at 37.0±0.2°C by a feedback-regulated heating pad. Cerebral blood flow in the MCA area was monitored as described. 

Assessment of Hemorrhage and Infarction

Cerebral blood flow in the MCA area was monitored as described. During surgical procedures, rectal temperature was monitored and controlled at 37.0±0.2°C by a feedback-regulated heating pad. Cerebral blood flow in the MCA area was monitored as described.

Drug Administration

Mice were fed cilostazol (0.3% in the diet; Otsuka, Tokushima, Japan), aspirin (0.1% in the diet; Eizai, Tokyo, Japan), or a normal diet for 7 days before induction of ischemia. Doses of cilostazol and aspirin were determined according to previous reports.

Assessment of Hemorrhage and Infarction

Hemorrhagic infarction was evaluated at 24 hours after induction of ischemia as described previously. Briefly, coronal forebrain sections (1 mm thick) were stained with 1% 2,3,5-triphenyltetrazolium (Sigma-Aldrich, St. Louis, MO) for 20 minutes at 37°C and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS; pH 7.4). Infarct volume was measured using a microscopic digital camera system (Olympus, Tokyo, Japan) as described previously. Briefly, the 2,3,5-triphenyltetrazolium-positive area of each hemisphere was estimated using National Institutes of Health Image software (Version 1.62), and volume of the surviving/viable tissue was calculated by integrating the overall coronally oriented area. Percent stroke volume was evaluated by [(contralateral hemisphere volume−infarcted hemisphere volume)/(contralateral hemisphere volume)]×100%.

The severity of cerebral hemorrhage was quantified by investigators who were not informed regarding the experimental protocol and identity of samples under study, as described previously: non-hemorrhage (Score 0); hemorrhagic infarction Type 1 (HI-1), defined as homogeneous small petechiae, generally along the boundary of the infarct (Score 1); hemorrhagic infarction Type 2 (HI-2), with more confluent petechiae within the infarcted area (Score 2); parenchymal hemorrhage Type 1 (PH-1), characterized by hematoma covering <30% of the injured parenchyma (Score 3); and parenchymal hemorrhage Type 2 (PH-2) with dense hematoma in >30% of the infarct (Score 4). Examples of each score are demonstrated in Figure 1B (no mouse showed PH-2 in our experiment).

Immunohistochemistry

Twenty-four hours after reperfusion, mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde. Forebrain coronal sections (20 μm) were prepared using a vibroslicer (Leica, Wetzlar, Germany) and immunostained with antibodies to platelet endothelial cell adhesion molecule 1 (PECAM-1; BD Pharmingen, San Jose, CA; dilution 1:500), lectin (Invitrogen, Carlsbad, CA; 1:50), and matrix metalloproteinase (MMP)-9 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:100) using standard immunohistochemical procedures. Anti-PECAM-1 and lectin were visualized by the 3,3'-diaminobenzidine method. Alexa488 or Alexa555 antibody was used as the secondary antibody for anti-MMP-9 and PECAM-1. Vascular density was evaluated using anti-PECAM-1 antibody as described previously.

Briefly, the number of PECAM-1-positive vascular structures in the anterior cerebral artery area (border of cerebral ischemia; approximately 0.5 mm from the border of infarction), MCA area (stroke area), and contralateral cortex at the exact center of the forebrain section was counted by investigators who were not informed regarding the experimental protocol and identity of samples under study (3 random fields in each section were scored and the area of each field was 0.12 mm²). Sections stained with antilectin...
were counterstained with Mayer hematoxylin solution (Wako, Osaka, Japan).

**Gelatin Zymography**

The level of MMP-9 in the ischemic brain was evaluated by gelatin zymography, as described previously.23 Briefly, at 24 hours after reperfusion with injection of tPA, brain tissue from the ipsilateral ischemic and contralateral nonischemic hemispheres was removed and homogenized in lysis buffer (CelLytic MT; Sigma). After centrifugation at 500 g for 10 minutes, supernatant was collected and protein concentrations were measured by the Bradford assay (Bio-Rad). Protein samples (35 μg/mL) were mixed with 2× zymogram sample buffer (TEFCO, Tokyo, Japan) and loaded onto 10% Zymogram-PAGE mini (TEFCO). After electrophoresis, the gel was stained with Coomassie blue R-250 according to the manufacturer’s protocol (ZYMOGRAM buffer kit; TEFCO). Gel images were captured using a digital camera (Olympus, Tokyo, Japan) with reversed brightness, and the intensity of each band was quantified with National Institutes of Health Image.

**Data Analysis**

Statistical comparisons among groups were determined using the Kruskal-Wallis test to compare with controls. Data are expressed as mean±SE.

**Results**

**Administration of tPA Increases the Risk of Cerebral Hemorrhage After Transient Ischemia**

To confirm the increased risk of cerebral hemorrhage after administration of tPA, transient ischemia was induced and tPA or PBS was injected just before reperfusion. The incidence and degree of hemorrhagic infarction were evaluated at 24 hours after reperfusion. As shown in Figure 1A, the incidence of hemorrhagic infarction was significantly increased with tPA injection after 90, 120, and 180 minutes of transient ischemia, although no significant increase was observed after 240 minutes ischemia. To investigate the severity of hemorrhagic infarction, degrees of hemorrhage were scored according to 5 subtypes, as described previously.28,29 In our experimental groups, no mice showed PH-2 (Grade 4). Representative photographs of hemorrhage subtypes are shown in Figure 1B. It is notable that none of the mice that received PBS before reperfusion showed parenchymal hematoma (Grade ≥2) after 90, 120, and 180 minutes transient ischemia (Figure 1C). In contrast, mice receiving tPA showed PH even after 90 minutes ischemia. In both treatment groups, more than half of the mice showed PH after 240 minutes ischemia. Quantitative analysis revealed a significant increase in hemorrhagic score in mice treated with tPA at 90, 120, and 180 minutes after transient ischemia compared with PBS-treated groups (Figure 1D). The volume of infarcted tissue at 24 hours, consequent to 90 minutes ischemia to induce stroke, was evaluated. There was no significant difference in percent stroke volume between treatment with tPA and PBS (13.8%±1.1% and 13.7%±0.8%, respectively; P=0.96).

**Cilostazol Reduced the Risk of tPA-Induced Cerebral Hemorrhage**

To evaluate the risk of cilostazol on tPA-induced cerebral hemorrhage, mice were fed cilostazol for 7 days and transient ischemia was induced followed by injection of tPA. Contrary
to our initial expectation, the incidence of cerebral hemorrhage was significantly reduced on administration of cilostazol in mice after 90 and 120 minutes of transient ischemia, although no significant difference was observed after 180 and 240 minutes (Figure 2A). Figure 2B shows the distribution of severity in each group. It is notable that all of the mice fed cilostazol before injection of tPA showed no or mild hemorrhage (score 0 or 1) after 90 or 120 minutes ischemia. Quantitative analysis using the hemorrhagic score revealed a significant reduction of severity in mice pretreated with cilostazol at 90 and 120 minutes after transient ischemia compared with the nontreated group (Figure 2C). However, no statistical difference in the severity was observed between groups after 180 or 240 minutes of transient ischemia.

Aspirin Did Not Reduce the Risk of tPA-Induced Cerebral Hemorrhage

Aspirin is known to increase the risk of cerebral hemorrhage. To investigate its effect on tPA-induced cerebral hemorrhage, mice were treated with aspirin for 1 week and transient ischemia was induced (90 minutes). The results displayed no significant reduction or increase in the incidence of cerebral hemorrhage on treatment with aspirin compared with normal controls (Figure 3A). Analysis of severity using the hemorrhagic score also revealed no significant change between the aspirin-treated and normal diet groups (Figure 3B–C). Although the incidence of hemorrhage was dependent on the time to reperfusion, these results indicate that aspirin has a nonsignificant effect on reduction of tPA-induced cerebral hemorrhage after 90 minutes of transient ischemia, whereas cilostazol had significant protective effects.

Cilostazol Prevented the Degradation of Cerebrovasculature After Transient Ischemia and Administration of tPA

To investigate mechanisms underlying the protective effect of cilostazol pretreatment on cerebral hemorrhage, morphological changes in cerebromicrovasculature were investigated at 24 hours after induction of transient ischemia (90 minutes). Immunohistological analysis revealed a decrease in PECAM-1-positive microvasculature at the border of cerebral ischemia after transient ischemia with tPA injection compared with the contralateral cortex (Figure 4A, contralateral; Figure 4B; ipsilateral). In contrast, pretreatment with cilostazol prevented the reduction in PECAM-1-positive microvasculature (Figure 4C). These impressions were confirmed by quantitative analysis of PECAM-1-positive vascular density (Figure 4D). PECAM-1 is known to be important for survival, migration, and functional organization of endothelial cells, and our data indicate a beneficial effect of cilostazol on the preservation of these endothelial functions at the border of the stroke. In contrast, pretreatment with aspirin had no effect on the preservation of microvasculature (Figure 4E–F).

Next, we investigated possible degradation of cerebrovasculature in the stroke area with antilectin antibody, a marker of vascular morphology. At 24 hours after transient ischemia (90 minutes) with tPA injection, a marked dissociation of microvasculature was observed in the ischemic brain in mice.
fed a normal diet (Figure 4G, contralateral; Figure 4H, ipsilateral). In contrast, preservation of vascular structure in the stroke area was observed in mice pretreated with cilostazol (Figure 4I). Similar to the results obtained with anti-PECAM-1 antibody, the pretreatment with aspirin had no protective effect on degradation of cerebrovasculature at the poststroke area (Figure 4J).

Cilostazol Prevented Activation of MMP-9 in the Poststroke Cortex

Activation of MMP-9 is well known to cause the deterioration of tight junctions and basement membranes. To investigate activation of MMP-9 in the vasculature in the poststroke cortex, brain sections were costained with anti-PECAM-1 and anti-MMP-9 antibodies. Although no MMP-9-positive vascular structures were observed in the contralateral cortex (Figure 5A–C), MMP-9-positive vasculature was observed in the poststroke cortex in control mice (Figure 5D–F). In contrast, no MMP-9-positive vasculature was observed in mice pretreated with cilostazol (Figure 5G–I). In contrast, pretreatment with aspirin did not prevent activation of MMP-9 in the poststroke cortex (Figure 5J–L). To confirm these results, protein samples were extracted from each brain and MMP-9 activity was investigated by zymography. Consistent with results obtained by immunohistologic analysis, suppressed expression of MMP-9 activity was observed with pretreatment with cilostazol compared with pretreatment with aspirin (Figure 5M–N).

Discussion

In this study, we have demonstrated that treatment with cilostazol for 7 days before induction of cerebral ischemia significantly reduced the hemorrhagic risk accompanying tPA injection and was associated with suppressed MMP-9 activity in stroke vasculature (and its endothelium).

Thrombolysis with tPA after stroke is associated with an increased risk of hemorrhagic transformation. In addition to endothelial cell injury caused by reperfusion after transient ischemia, tPA is known to induce disruption of the blood–brain barrier. Consistent with these reports, administration of tPA after 90, 120, or 180 minutes of transient ischemia significantly increased the risk of cerebral hemorrhage, compared with PBS-injected mice, in our experimental model. Because of the homogeneity of cerebral vascular structure/organization between animals in CB-17 mice, the ischemia induced in this strain by transient occlusion of the MCA under direct visualization produced a highly reproducible ischemic area. Although thrombolytic effects of tPA cannot be addressed in this model, these findings indicate the model in CB-17 mice is suitable to evaluate the effect of drugs on hemorrhagic transformation caused by tPA injection with high reproducibility.

Intracerebral hemorrhage is associated with worse clinical outcomes in the context of stroke. Prior use of antiplatelet drugs remains a concern in terms of increasing the risk of hemorrhage after tPA treatment. However, patients who received aspirin for prevention of stroke showed better
clinical outcomes after treatment with tPA, although some studies reported increased risk of cerebral hemorrhage in patients with aspirin compared with patients who did not receive it.\(^5,9,41,42\) This discrepancy can be attributed to reocclusion of the artery after initial successful recanalization by tPA,\(^10,11\) which can be suppressed by antiplatelet drugs, thereby improving outcome.\(^14\) Cilostazol is an antiplatelet drug with additional effects, including improvement in function of vascular endothelium.\(^43\) It is known to be superior to aspirin in terms of reduction of the risk of cerebral hemorrhage.\(^16\) Consistent with these previous reports, pretreatment with cilostazol for 7 days before ischemia and subsequent tPA administration significantly suppressed the occurrence/extent of cerebral hemorrhage. In contrast, pretreatment with aspirin had no effect on the risk of bleeding compared with nontreated control mice. These findings suggest that patients treated with cilostazol for prevention of ischemic diseases would have a lower risk of hemorrhagic transformation after thrombolytic therapy compared with nontreated or aspirin-treated patients. Cilostazol-treated patients might be expected to have a reduced risk of reoccluding the recanalized cerebral artery compared with nontreated patients.

To extend the therapeutic time window for effective thrombolytic therapy, the risk of cerebral hemorrhage must be evaluated in individual cases. Our current study demonstrates that the risk of cerebral hemorrhage can be significantly modified by treatments administered before the onset of stroke. However, the effects of other commonly used drugs for patients with a high risk of stroke such as calcium channel blockers, angiotensin receptor blockers, and statins are still controversial.\(^44,45\) We believe that analysis of the effects of multiple drugs on tPA-induced cerebral hemorrhage in animal models is essential for extending safe and effective thrombolytic therapy to a wider group of patients, especially for those beyond the current 3-hour window for treatment.

Figure 5. Pretreatment with cilostazol suppressed activation of MMP-9 in vasculature in the poststroke cortex. A–L, Representative micrographs of the contralateral (A–C) and ipsilateral cortex (D–L) at 24 hours after ischemia with tPA treatment (PECAM-1 [A, D, G, red]; MMP-9 [B, E, K, green]; and merged image [C, F, L, yellow]). Although no expression of MMP-9 was observed in the microvasculature of the contralateral cortex (A–C), expression of MMP-9 was observed in the ipsilateral cortex in mice pretreated with a normal diet (D–F). In contrast, reduced expression of MMP-9 was observed in the stroke-affected cortex in mice pretreated with cilostazol (G–I). Pretreatment with aspirin did not change the expression of MMP-9 in the microvasculature in the stroke-affected cortex compared with the normal diet (J–L). M–N, Representative photograph of zymogram. Suppressed activity of MMP-9 (105 kDa) was observed with pretreatment with cilostazol, although no change was observed with aspirin (M). Reverse images were obtained and the ratio of activity between cilostazol or aspirin vs the normal diet was quantified. Significant reduction of MMP-9 activity was observed in mice pretreated with cilostazol compared with mice pretreated with aspirin (N). \(P<0.05\) vs aspirin. \(n=3\), in each group. Scale bar, 80 \(\mu m\) (A). MMP indicates matrix metalloproteinase; tPA, tissue-type plasminogen activator; PECAM-1, platelet endothelial cell adhesion molecule 1.
Activation of MMP-9 in injured endothelial cells has been suggested as a mechanism for tPA-induced cerebral hemorrhage in addition to direct injury due to ischemia-reperfusion. MMP-9 activation enhances the permeability and decreases structural integrity of the blood–brain barrier in postischemic brain. Our studies have shown that pretreatment with cilostazol markedly reduced the expression of MMP-9 in endothelial cells after injection of tPA and suppressed degradation of cerebral vasculature in the ischemic brain. Our findings are consistent with a previous study demonstrating that cilostazol decreased MMP-9 expression in balloon-injured vasculature. Cilostazol is known to raise the intracellular cAMP concentration in endothelial cells. In this context, cAMP promotes functional integrity of tight junctions between endothelial cells in the blood–brain barrier.

The vasoprotective effect of cilostazol was also shown in other studies in which cilostazol suppressed endothelial hyperpermeability by inhibiting redistribution of the actin-based cytoskeleton and protected endothelial cells against lipopolysaccharide-induced apoptosis by the activation of MAP kinase. These findings indicate that the beneficial effect of cilostazol on cerebral hemorrhage might be achieved, at least in part, through suppression of endothelial injury after thrombolysis with tPA injection. Consistent with these findings, cilostazol-treated mice displayed retention of vascular density in ischemic cerebral cortex after tPA treatment, whereas aspirin did not prevent reduction in the number of cerebral microvessels. In the current study, we used 1 dose of cilostazol. Because both antiplatelet and vasoprotective activity of cilostazol are known to be dose-dependent, further study will be necessary to determine the optimal dose of cilostazol to suppress cerebral hemorrhage after tPA treatment.

In conclusion, our results suggest that treatment of patients with cilostazol for prevention of stroke may have significant merit with regard to suppressing the risk of hemorrhagic transformation after thrombolytic therapy as well as reducing the risk of cerebral hemorrhage compared with treatment with aspirin. Furthermore, our data suggest that the therapeutic time window of thrombolytic therapy using tPA might be extended in patients treated with cilostazol. Furthermore, antithrombotic treatment might be safely started with cilostazol soon after injection of tPA to reduce the incidence of reoclusion of the artery after initial successful recanalization.

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Disclosures

None.

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シロスタゾールはマウス脳卒中モデルにおける組織プラスミノゲン活性化因子投与後の出血性梗塞リスクを低減する
Cilostazol Reduces the Risk of Hemorrhagic Infarction After Administration of Tissue-Type Plasminogen Activator in a Murine Stroke Model

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Abstract

背景および目的: 血栓溶解療法を行う患者に対し事前に抗血小板薬を投与すると, 動脈再閉塞が減少し, 患者の脳卒中転帰が改善するが, その一方で脳出血リスクが上昇する可能性がある。

方法: シロスタゾールは, 細胞内cAMPのアップレギュレーションによって内皮機能を改善する抗血小板薬である。本研究では, 再現性の高い一過性虚血モデルを用いて, 血栓溶解療法後の脳出血に対するシロスタゾールの効果を検討した。

結果: 虚血前7日間のシロスタゾール投与により, 組織プラスミノゲン活性化因子注入後の脳出血リスクおよび重症度が有意に抑制された。一方, アスピリンを投与した場合には, 非投与マウスに比べてこうした保護作用は認められなかった。免疫組織学的分析では, シロスタゾール投与によって, マトリックスメタプロテアーゼ-9の活性低下に伴う虚血領域の微小血管系の破綻が抑制されることが示された。

結論: 本研究結果が示唆するように, 脳卒中発症前にシロスタゾール投与した患者は, 血栓溶解療法後の脳出血リスクが低くなり, 血栓溶解の治療適応時間を延長する可能性がある。さらに, 脳卒中前の治療によって脳出血リスクは大きく変化すると考えられる。より幅広い患者群に安全かつ有効な血栓溶解療法を行うには, 動物モデルを用い, 組織プラスミノゲン活性化因子誘発性の脳出血に対する各種薬剤の効果を分析することが不可欠である。

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図2
シロスタゾール投与により, t-PA投与後の脳出血リスクが低下した。A: 通常食を与えたマウスに比べ, 虚血作製前7日間にわたってシロスタゾール含有食を与えたマウスでは, 90分間および120分間の虚血後のt-PA注入に伴う脳出血リスクが有意に低下した。

B-C: 各出血の重症度分布をBに示す。定量的分析の結果, 通常食を与えたマウスに比べ, シロスタゾール含有食を与えたマウスでは, 90分間および120分間の虚血後のt-PA注入に伴う脳出血の重症度が低下したことが示された(C)。*p<0.05。通常食対照群との比較(A, C), 各群Bを示す。t-PA: 組織プラスミノゲン活性化因子, HI-1: 出血性梗塞1型, HI-2: 出血性梗塞2型, PH-1: 脳実質内出血1型。