Prevention of cerebrovasospasm following subarachnoid hemorrhage in rabbits by the platelet-activating factor antagonist, E5880

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Despite recent advances in the management of delayed cerebral ischemia using prophylactic calcium antagonists,23 intrathecal fibrinolytic therapy,7 and intravascular neurosurgical techniques such as transluminal angioplasty9 and intraarterial infusion of agents15,16 cerebral vasospasm remains an important cause of mortality and morbidity following subarachnoid hemorrhage (SAH). An incomplete understanding of the pathogenesis of this vasospasm has slowed progress toward more successful therapy.

Recently, an important role of platelet-activating factor (PAF), an inflammation mediator, has been demonstrated in the genesis of cerebral vasospasm following subarachnoid hemorrhage (SAH). In the current study, the authors examined whether intravenous administration of the novel PAF antagonist, E5880, can prevent vasospasm following SAH in rabbits.

A vasospasm model was produced in three groups of rabbits using two subarachnoid injections of autologous arterial blood, followed by intravenous administration of distilled water (control), a low dose of E5880 (0.1 mg/kg in distilled water), or a high dose of E5880 (0.5 mg/kg in distilled water). Neurological deterioration was largely prevented in the rabbits that received E5880. Basilar artery constriction was also reduced by both doses of E5880. Histological examination at autopsy predominantly showed ischemic changes in the brain. Animals in each E5880-treated group exhibited ischemic changes less frequently than those in the control group. Plasma thromboxane B2 concentrations were reduced in rabbits treated with E5880. Platelet-activating factor was immunolocalized in the intima and media of the basilar artery in the control group. The PAF immunoreactivity demonstrated in the basilar artery was decreased in the E5880 groups in a dose-dependent manner. Thus, this study provides evidence that PAF may play a role in the pathogenesis of vasospasm after SAH and that intravenous administration of E5880 is a promising approach in preventing vasospasm.

**Materials and Methods**

**Animal Preparation**

Female rabbits, each weighing 2.8 to 3.1 kg, were used for the study. All surgical and angiographic procedures were performed after the animals had been anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg body weight). The protocol was approved by the Animal Ethics Review Committee of the Toyama Medical and Pharmaceutical University, and all animals were cared for in accordance with the guidelines for animal treatment.
Platelet-activating factor in vasospasm following SAH

experiments proposed by the University. All animals were maintained on a standard diet of rabbit pellets and water. The procedures for subarachnoid blood injection and aortovertbral angiography have been described in detail previously.12 In brief, 2 weeks prior to the experimentally induced SAH, the bilateral carotid arteries of the rabbits were ligated. Only rabbits that were asymptomatic after carotid ligation received two subarachnoid injections of autologous arterial blood, 48 hours apart, with the time of the first injection designated as Day 0. During subarachnoid injection, spontaneous respiration was maintained and both PaCO2 and blood pressure were monitored. The animals’ PaCO2 was 35 to 45 mm Hg, and their mean arterial blood pressure prior to the induced SAH was 85 to 105 mm Hg in both the control group and E5880 groups. Following the subarachnoid injection, a dramatic increase in systemic blood pressure occurred. Within 5 minutes, however, the blood pressure gradually returned to the preinjection level. Using neurological status grades, examination was performed prior to induction of SAH and daily thereafter until death. Aortovertbral angiography was performed on Days 0 and 4 using manual injection of contrast material (Hexabrix, 7 ml) into a catheter inserted retrogradely through the carotid stump into the aortic arch. The diameter of the basilar arteries was measured on anteroposterior angiographic film at three points using a micrometer by two blinded investigators. The average of the three measurements was expressed as the percentage of the baseline diameter; the average of the data collected by the two investigators was calculated.6 Plasma 6-keto-prostaglandin F1α (6-ketoPGF1α) and thromboxane B2 (TXB2) concentrations were usually measured on days when the carotid arteries were ligated, that is, Days 0 and 4. Blood cell parameters, white and red blood cell, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet values also were compared on Day 4. Arterial blood was drawn from the neck common carotid artery both for use in the subarachnoid injection and for laboratory investigation.

Experimental Design

Each rabbit received an intracisternal injection of autologous arterial blood (0.5 ml/kg body weight) on Days 0 and 2. Eight rabbits received injections of 1 ml distilled water into the aortic vein daily on Days 0 to 3 (control group). Fourteen animals received E5880. Seven of these were injected intravenously with a low dose of E5880 (0.1 mg/kg body weight; E5880-0.1 group) and the other seven with a high dose of E5880 (0.5 mg/kg body weight; E5880-0.5 group) daily for 4 days. The vehicle and drugs were administered immediately after the subarachnoid injection on Days 0 and 2. The injection was performed over a 2-minute period.

Method of Determining Plasma 6-ketoPGF1α and TXB2 Levels

Seven milliliters of blood was drawn from the carotid artery into a tube containing ethylenediamine tetraacetic acid, indomethacin, and Trasylol. The tube was centrifuged and the concentrations of 6-ketoPGF1α and TXB2 in plasma were measured using the [3H]6-ketoPGF1α and [3H]TXB2 assay systems.

Histological Evaluation

The rabbits were killed on Day 7 for histological examination of the brain. The brains were fixed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were cut into coronal slices and stained with hematoxylin and eosin. Ischemic lesions were evaluated in a blinded manner using light microscopy. The basilar artery was embedded in ornithine carbamyl transferase (OCT) compound. Transverse sections, 8 µm thick, were cut using a cryostat, and immunohistochemical analysis of PAF in the wall of the basilar artery was performed using the streptavidin–biotin peroxidase complex method and anti-PAF–IgG conjugated peroxidase.13

Sources of Supplies

Anti-PAF IgG was obtained as described12 and was conjugated to horseradish peroxidase using an IMMUNO-LINKTMHRP labeling kit provided by Cambridge Research Biochemicals, London, England. Streptavidin–biotin peroxidase complex was purchased from Dako Corp., Carpinteria, CA. The OCT compound was obtained from Tissue-Tek, Ekhart, IN. The [3H]6-ketoPGF1α and [3H]TXB2, assay systems were supplied by Amersham International, Buckinghamshire, England. Other chemicals and solvents used in this study were of reagent grade.

Statistical Analysis

The findings are reported as the mean ± standard deviation. Data were analyzed using Dunnett’s test to compare the diameter of basilar arteries, 6-ketoPGF1α and TXB2 concentrations, and the parameters of blood cells among the control, E5880-0.1, and E5880-0.5 groups. We also used Wilcoxon’s U-test to compare the neurological grades among these three groups. A probability value less than 0.05 was accepted as statistically significant.

Results

Neurological Evaluation

The rabbits were observed on a flat surface and their neurological deficits were graded using a four-point system:6 Grade 1 denoted no neurological deficit (normal); Grade 2, a minimal or suspected neurological deficit; Grade 3, a mild neurological deficit without abnormal movements; and Grade 4, a severe neurological deficit with abnormal movements. Of the eight control animals, six showed a progressive neurological deterioration that was most severe on Day 4 or 5 and then recovered gradually. Therefore, we compared neurological grades among control, E5880-0.1, and E5880-0.5 groups on Day 4. In both the E5880-0.1 and E5880-0.5 groups, one rabbit exhibited Grade 2 neurological deficits, whereas the other six animals showed no neurological deficits (Table 1). The neurological grades of animals in both groups treated with E5880 were better than those of the animals in the control group (p < 0.05, Wilcoxon’s U-test).

Diameter of the Basilar Arteries

Sequential angiography of control rabbits showed maximum basilar artery constriction on Days 3 and 4 and subsequent dilation. Therefore, we compared the diameters of the basilar arteries in the control, E5880-0.1, and E5880-0.5 groups on Day 4. Significant vasoconstriction of the basilar artery was observed on Day 4 in control animals compared to Day 0 baselines (82.5% ± 13.1%, eight animals, p < 0.05, Dunnett’s test). This vasoconstriction was prevented by administration of E5880 (123.0% ± 13.1%, seven animals, p < 0.05, in the E5880-0.1 group;

<table>
<thead>
<tr>
<th>Neurological Deficit Grade*</th>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (8 animals)</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>E5880, 0.1 mg/kg body wt</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(7 animals)†</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E5880, 0.5 mg/kg body wt</td>
<td></td>
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<tr>
<td>(7 animals)†</td>
<td></td>
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</tbody>
</table>

* Neurological deficit grades are as follows: 1 = no deficit; 2 = minimal or suspected deficit; 3 = mild deficit without abnormal movements; and 4 = severe deficit with abnormal movements.

† p < 0.05 compared with control group (Wilcoxon’s U-test).
110.0% ± 12.3%, seven animals, p < 0.01, in the E5880-0.5 group; Dunnett’s test) (Fig. 1). No significant vasodilation of the basilar artery was observed in animals from either the E5880-0.1 or E5880-0.5 groups compared with their Day 0 baselines. We surmise from these data that E5880 prevented vasoconstriction on Day 4 in animals with SAH.

**Plasma 6-ketoPGF\textsubscript{1\alpha} and TXB\textsubscript{2} Levels**

There was no difference in plasma TXB\textsubscript{2} between the baseline values of animals before and after ligation of the carotid arteries. Thromboxane B\textsubscript{2} concentrations in the control group on Day 4 appeared to be higher than the two baseline values, although there was no significant difference. However, plasma TXB\textsubscript{2} concentrations in the E5880-0.1 and E5880-0.5 groups on Day 4 were lower than in the control group (p < 0.05, Dunnett’s test) (Fig. 2). Plasma 6-ketoPGF\textsubscript{1\alpha} did not show any differences in concentration among baseline, control, and E5580-treated values (Fig. 2).

**Histological Evaluation**

Two types of ischemic change—selective neuronal degeneration and total necrosis (infarction)—were the major histological findings in the rabbit brains. Selective neuronal degeneration was observed in the CA1 region of the hippocampus, whereas infarction usually affected the cerebrum and the brainstem. Six (75%) of eight control animals showed ischemic changes histologically. However, only one (14.3%) of seven rabbits in both the E5880-0.1 and E5880-0.5 groups showed ischemic changes (Table 2). Mild hydrocephalus was observed in one (12.5%) of eight control animals, two (28.6%) of seven E5880-0.1 animals, and one (14.3%) of seven of E5880-0.5 animals.

Regions of PAF immunoreactivity were located in the intima and media of the basilar artery in rabbits treated with vehicle, and PAF immunoreactivity was diminished in rabbits treated with E5880 in a dose-dependent manner (Fig. 3).

**Parameters of Blood Cells**

Blood cell parameters, including white and red blood cell, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet values measured on Day 4 did not differ among the control, E5580-0.1, and E5580-0.5 groups (data not shown).

**Discussion**

Delayed ischemic neurological deficits and/or cerebral infarction due to vasospasm following SAH may be a consequence of the following: 1) a decrease in cerebral blood flow caused by constriction of the cerebral main trunk arteries, 2) cerebral microcirculatory dysfunction, or 3) direct damage to neuronal cells by injurious substances produced during vasospasm. Previously we reported that intrathecal administration of PAF constricts the basilar artery in rabbits with SAH.\textsuperscript{12} Systemic administration of PAF provokes a reduction of blood flow in the spinal cord microcirculation.\textsuperscript{18} An elevation in blood TXB\textsubscript{2}, level and coagulability, resulting in cerebral microcirculatory dysfunction, has been reported in patients who experienced cerebral vasospasm after SAH,\textsuperscript{22} an effect that may be attributed to platelet activation by PAF.\textsuperscript{4} Platelet-activating

**TABLE 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Selective Neuronal Degeneration (no. of animals)</th>
<th>Cerebral Infarction (no. of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (8 animals)*</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>E5880, 0.1 mg/kg body wt (7 animals)†</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>E5880, 0.5 mg/kg body wt (7 animals)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Six animals exhibited ischemic changes. One of these showed both selective neuronal degeneration and cerebral infarction.
† The same animal exhibited both selective neuronal degeneration and cerebral infarction.
factor is a potent chemoattractant, increasing the permeability of the endothelial layer and activating neutrophils. Activated neutrophils may damage endothelial cells and promote subsequent thrombus formation at the interface between blood and vessel walls. Increased amounts of interleukin-6 (IL-6) have been found in the CSF following SAH. Cytokines and PAF increase each other’s production. They induce procoagulant factors through their action on endothelial inflammatory cells and thus contribute to circulatory dysfunction. Prehn and Kriegstein have demonstrated a neuroprotective effect of PAF antagonists in both glutamate neurotoxicity in primary cell culture and in animal ischemic models. They have proposed that PAF plays an important role in the pathophysiology of ischemic–excitotoxic neuronal injury via a direct action on neurons.

A novel analog-type antagonist of PAF, E5880 inhibits both PAF binding to human platelet PAF receptors and PAF-induced platelet aggregation. In vivo, E5880 has proven to be effective in preventing passive anaphylactic death in mice, lipopolysaccharide-induced shock in rats, and intestinal mucosal damage following intestinal ischemia–reperfusion injury in rabbits.

In this study, the intravenous administration of E5880 reduced the incidence and severity of vasospasm following SAH in rabbits. The PAF antagonist reduced basilar artery spasm significantly (Fig. 1) and prevented treated animals from suffering most neurological deficits (Table 1). Histological findings from the dissected brains generally correlated with the neurological findings (Tables 1 and 2). Thus, PAF may cause constriction of the basilar artery and E5880 may reverse this effect. The improved neurological status and decreased ischemic brain lesions observed in rabbits that received E5880 may be attributed to either brain microcirculatory improvement or the reduction of direct neuronal damage. Plasma TXB concentrations were reduced in rabbits treated with E5880 compared to those treated with vehicle alone. Thromboxane synthetase inhibitor has been reported to be useful in treating cerebral ischemic symptoms accompanied by cerebral vasospasm. Therefore, the present results demonstrate that PAF metabolically stimulates the blood TXB concentrations at the upper stream in subjects with vasospasm following SAH.

Interestingly, PAF was immunolocalized in the intima and media of basilar arteries that were infused with distilled water alone. This PAF immunoreactivity in the basilar artery was decreased in rabbits treated with E5880 in a dose-dependent manner. Streptavidin–biotin peroxidase complex method using anti-PAF immunoglobulin G–conjugated peroxidase. Original magnification × 100.
vessels. It appears that E5880 may break a vicious circle of mutual cytokine and PAF induction and subsequently reduce cytokine concentrations and PAF production in vessel walls. Recently it has been reported that PAF receptor activation induces cytosolic phospholipase A_2 activation via mitogen-activated protein kinase activation. Therefore, another possibility of decreased immunoreactivity of PAF in the walls of the basilar artery is that E5880 may inhibit phospholipase A_2 in endothelial cells, which is a rate-limiting enzyme in PAF synthesis. Not only PAF depletion in the vessel walls, but also other beneficial changes, such as ischemic change of brain and plasma TXB_2 and 6-ketoPGF_1 concentrations, were observed with the higher dose of E5880 without statistical significance.

Another analog-type antagonist of PAF has been reported to have a detergent effect that induces hemolytic anemia. However, E5880 caused no changes in blood cell parameters compared to controls. These results indicate that a novel PAF receptor antagonist, E5880, is a promising drug for preventing cerebral vasospasm following SAH.

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References

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