Effects of the Thromboxane A2 Receptor Antagonist on Platelet Deposition and Intimal Hyperplasia After Balloon Injury

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SUMMARY

Thromboxane A2 (TXA2) after vascular injury plays an important role in the process of restenosis. S-1452, a potent and selective TXA2 receptor antagonist, blocks the receptors of vascular smooth muscle cells (VSMC) as well as platelets. The purpose of this study was to determine whether S-1452 could reduce platelet deposition and intimal hyperplasia in vascular injury models. New Zealand White Rabbits (n = 41) were fed a 0.5% cholesterol diet. For the short-term study, eighteen rabbits after balloon injury of iliac artery were assigned to 3 groups; systemic administration of S-1452, single local administration of S-1452 using a local delivery balloon, and single local administration of saline solution. Platelet deposition in injured artery using 111In-labeled platelets was reduced by 50% in systemic administration and by 60% in local administration compared to saline infusion. For the long-term study, balloon injury of the iliac artery was performed 4 weeks after starting the 0.5% cholesterol diet. Twenty-three rabbits were classified into 4 groups; systemic administration of S-1452, oral placebo administration, single local administration of S-1452, and local administration of saline solution (control group). The platelet aggregation induced by U-46619 was significantly lower in the S-1452 group than in the control group. Systemic administration of S-1452 significantly reduced the intimal area (152 ± 33 vs 735 ± 135 μm², p < 0.001) and number of cells in the intima (513 ± 57 vs 993 ± 57, p < 0.01) compared to controls. In contrast, a single local administration failed to reduce neointimal thickness.

Systemic administration of S-1452 reduced intimal hyperplasia as well as platelet deposition in a rabbit injury model, but its single local administration inhibited only platelet deposition. (Jpn Heart J 1999; 40: 791–802)

Key words: Thromboxane A2 receptor antagonist, S-1452, Angioplasty, Platelet deposition, Restenosis, Smooth muscle cells, Local delivery
CORONARY angioplasty is a widely accepted and well-established intervention, but restenosis still remains as a major clinical problem. The exact mechanism of its process is not well understood, however, it is known that major components are related to thrombosis and cellular proliferation. Platelet deposition at the site of vascular injury plays an important role in thrombus formation and initiation of the restenotic process. Thromboxane A2 (TXA2), the prominent metabolite of arachidonic acid in platelet, is a potent inducer of platelets aggregation and vascular smooth muscle cell (VSMC) proliferation. Inhibition of TXA2 synthesis was reported to decrease thrombotic cardiovascular event associated with angioplasty. Thus, TXA2 receptor antagonists have been expected to prevent restenosis. Recently, two clinical studies examined the roles of TXA2 receptor antagonists on restenosis, however, they failed to demonstrate a beneficial effect.

TXA2 receptor antagonists possess different affinities for platelets and VSMC. Many TXA2 receptor antagonists have a high affinity for platelets but not for VSMC. However, S-1452, a potent and selective TXA2 receptor antagonist, has the highest affinity for VSMC among TXA2 receptor antagonists. Therefore, it was expected that S-1452 could reduce intimal hyperplasia as well as thrombus formation after vascular injury. The objectives of the present study were 1) to evaluate the effects of S-1452 on the substantial reduction of platelet deposition and intimal hyperplasia after balloon injury, and 2) to determine whether local administration of S-1452 could also reduce platelet deposition and intimal hyperplasia.

METHODS

Forty-one male New Zealand White Rabbits weighing 2.8 to 3.2 kg were used in this study. All animal experiments conformed to the guidelines of the Animal Research Committee of Juntendo University. The experimental protocol consisted of two portions, a short-term study and a long-term study, as shown in Figure 1. The short-term study was performed to evaluate the effects of systemic or local administration of S-1452 on platelet deposition immediately after balloon injury. The long-term study was conducted to determine whether systemic or a single local administration of S-1452 could reduce intimal hyperplasia 4 weeks after balloon injury.

SHORT-TERM STUDY

Animal models: Eighteen rabbits (average weight 3.2 kg, 14 weeks old) were acclimatized for a week before undergoing experimental manipulation. Animals
Figure 1. The experimental protocols for the short-term and long-term studies.

were assigned to one of three groups: systemic administration of S-1452 (50 mg/kg/day) for 2 weeks \( (n = 6) \), single local administration of S-1452 (3 mg/kg) using a local delivery balloon \( (n = 6) \), and single local administration of 0.9% saline solution \( (n = 6) \).

**Balloon injury:** Balloon denudation was conducted two hours after reinjection of labeled platelets. The right superficial femoral artery was surgically exposed and ligated at its distal site. Denudation was induced three times by gently pulling a 2 Fr. balloon embolectomy catheter (Baxter Fogarty) from the aortic bifurcation to the right femoral artery.

For the local delivery group, a local drug-delivery balloon was advanced to the right iliac artery after removal of the Fogarty catheter. S1452 (3 mg/kg) or
saline vehicle was locally infused for 60 seconds at a pressure of 4 atm through the local delivery balloon catheter. The local drug delivery system used in this study was a 3.0-mm Transport™ coronary angioplasty catheter (Cardio Vascular Dynamics, Irvine, CA, USA). This catheter consists of an inner balloon for lesion dilatation and an outer porous balloon with a separate infusion channel for drug infusion.

**Preparation of ¹¹¹In-platelets:** Autologous platelets were isolated and labeled according to the method of Johnstone et al. using ¹¹¹In-labeled platelets.¹⁵ Briefly, a plastic tube was first inserted (PE-50) through the left femoral vein to the inferior vena cava after administering 30 mg/kg of intravenous sodium pentobarbital. Whole blood (10 ml) was withdrawn via the tube into a plastic syringe that contained 1.5 ml of acid citrate dextrose (ACD) solution. The blood was centrifuged at 350 g for 10 minutes at room temperature. The platelet-rich plasma (PRP) was removed and centrifuged at 2000 g for 10 minutes. The platelet pellet was then suspended with ¹¹¹In-tropolone labeling solution and centrifuged at 2000 g for 10 minutes. The labeled platelet pellet was resuspended and incubated with platelet-poor plasma (PPP) for another 5 minutes to remove any free unbound ¹¹¹In-tropolone and centrifuged at 2000 g for 10 minutes. This step was repeated twice. The pellet was finally suspended in PPP, and reinjected intravenously via the tube.

**Quantitation of platelet deposition:** One hour after balloon injury, the rabbits were sacrificed with an overdose of sodium pentobarbital, and the blood and non-adherent postmortem clot were completely removed by rapidly washing with saline. The femoral arteries were removed and prepared for analysis. Platelet deposition was evaluated by ¹¹¹In activity in the injured artery. The radioactivity per unit weight of each arterial sample was obtained by a well counter. The platelet deposition at the site of vascular injury was calculated by dividing the radioactivity (cpm) of the injured artery sample by the contralateral non-injured one.

**Long-term study**

**Animal models:** Twenty-three rabbits were randomized into four groups; systemic administration of S-1452 (n = 6), oral placebo administration (n = 4), single local administration of S-1452 (3 mg/kg) (n = 7), and local administration of 0.9% saline solution (n = 6). The rabbits in each group had been fed a 0.5% cholesterol diet from 2 weeks before balloon injury until euthanasia 4 weeks after the procedure. Oral drug was administered once daily in capsule form at doses of 500 mg. Therapy was started 2 weeks before the procedure and continued for 4 weeks until euthanasia.
Balloon injury and local drug delivery were carried out as mentioned above. The platelet aggregation induced by U-46619 was measured in all animals using the blood samples obtained with 38% sodium citrate at 4 weeks after balloon injury. The effect of treatment on U-46619 induced aggregation was expressed as percent inhibition, with the baseline, pretreatment aggregation response used as 100%.

The rabbits were euthanized at four weeks after injury by an intravenous injection of sodium pentobarbital. The injured segments of the common iliac artery were removed after perfusion with saline, fixed for 24 hours in 95% ethanol and 1% acetate in phosphate-saline buffer, and sectioned at 3 to 4 mm intervals from the proximal end. All cross sections, embedded in paraffin and sectioned, were stained with hematoxylin eosin and elastica van Gieson. These sections were measured with quantitative high power light microscopy where digital planimetry was utilized for making length and area measurements in the plane of microscopic view. Intimal area (IA), intimal/medial area (IA/MA), and maximal intimal thickness (MIT) were calculated. The observer was blinded with regard to treatment or control group.

**Immunohistochemistry:** Vessel segments were examined to evaluate the effect of S-1452 on VSMC proliferation by immunohistochemical staining for \( \alpha \)-smooth muscle actin (1A4 clone; DAKO, A/S, Denmark) and proliferating cell nuclear antigen (PCNA) and the expression of macrophage by RAM 11 (DAKO). The primary antibodies used were as follows; mouse monoclonal antibody to mouse monoclonal antibody \( \alpha \)-actin (1/50 dilution), and to PCNA (1/250 dilution) and rabbit polyclonal antibody to RAM11 (1/2 dilution). The slides were incubated overnight at 43°C with the primary antibody at the indicated dilution. The sections were blotted with biotinylated secondary antisera cocktail (including goat anti mouse IgG or goat anti rabbit) diluted 1/400 for 30 minutes at room temperature. Streptavidin-horseradish peroxidase was incubated for 30 minutes at room temperature. Immunoreactivity was then visualized using a 3-amino-9-ethylcarbazole substrate solution. Negative controls were incubated with normal mouse IgG (DAKO) or with PBS alone in a similar fashion. The total number of cells and number of \( \alpha \)-actin, PCNA, and RAM11 positive cells per high-power magnification (×150) were counted in five microscopic fields per section.

**Statistical analysis:** All data are expressed as mean ± SEM. N values indicate the number of animals. Differences between groups were evaluated using two tailed, unpaired Student's t test. The Mann-Whitney U test was used to analyze normally distributed platelet deposition data. Statistical significance was defined as \( p < 0.05 \).
RESULTS

Each group of animals was well matched for age and weight at the start of the experiment. The weight gain and final weight did not differ significantly among the groups. There were no differences in lipid levels at baseline among the groups. The absolute change in serum total cholesterol during the treatment period was not significantly different among the groups. Cholesterol levels increased from 32.4 ± 6.4 mg/dl to 1025.4 ± 274.3 mg/dl after 4 weeks on this diet.

Figure 2. Bar graphs showing %\textsuperscript{111}In platelet deposition. Platelet deposition in local and systemic administration of S-1452 was significantly lower than control (*\textit{p} < 0.05 versus control). S = systemic; L = local delivery.

Figure 3. Bar graph showing the maximum platelet aggregation induced by U-46619 at 28 days after balloon injury. Platelet aggregation was significantly reduced in the S-1452 group compared with the control (*\textit{p} < 0.05 versus control). S = systemic.
**Figure 4.** Representative histological cross sections of left common iliac arteries 28 days after balloon injury. Intimal thickening is significantly reduced in the S-1452 group (top) compared with the control (bottom). (elastica-van Gieson-stained, original magnification × 40)

**111In platelet deposition in vascular injury site (Short-term Study):** Platelet deposition in injured artery using 111In-labeled platelets was significantly reduced by 50% with systemic administration and by 60% with local administration compared to saline infusion (Figure 2). Local delivery of S-1452 was more effective at inhibiting platelet deposition than systemic administration.

**Platelet aggregation (Long-term Study):** The maximum platelet aggregation induced by U-46619 at 4 weeks was significantly inhibited in the S-1452 group compared to the control group (Figure 3).

**Intimal hyperplasia:** To observe the effect of systemic therapy on intimal hyperplasia, we compared the response to injury between systemic S-1452 and placebo control (Figure 4). In the treated group, the neointimal response decreased significantly compared to the untreated group 28 days after balloon injury (Figure 5). Furthermore, intimal lesions in control vessels showed a marked increase in cell numbers compared to treated arteries (Figure 6). The difference in number of α-smooth muscle actin positive cells between the treated and control groups was statistically significant. The percentage of PCNA positive cells
Figure 5. Inhibition of intimal and medial thickening by S-1452 at 28 days after balloon injury. Intimal area, intimal/medial area and maximal intimal thickness were significantly reduced in the systemic administration of S-1452 compared with the control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ versus control). On the contrary, no difference was apparent between local administration of S-1452 and control. S = systemic; L = local delivery.

Figure 6. Inhibition of vascular smooth muscle cell proliferation by S-1452 at 28 days after balloon injury. Number of cells, $\alpha$-actin and PGNA positive cells were significantly reduced in the systemic administration of S-1452 compared with the control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, versus control). S = systemic.
was lower in the treated arteries than in untreated arteries. Most of the cells with replication activity were α-actin positive. However, there was no significant difference in the number of RAM11 positive cells.

In vessels with single local treatment, the neointimal area and the number of α-SM actin positive cells were similar to those in control vessels (303 ± 42 vs 358 ± 491 m², 558 ± 34 vs 622 ± 53 SMC/mm², respectively). These results suggest that systemic continuous therapy is necessary to reduce the number of replicating cells in the vessel wall, resulting in a reduction of SMC number and intimal hyperplasia. A single local treatment appears to have limitations with respect to inhibiting intimal hyperplasia and SMC proliferation.

**Discussion**

This study has demonstrated that S-1452 reduces platelet deposition immediately after balloon injury and inhibits SMC proliferation 4 weeks after balloon injury in a cholesterol diet rabbit model. However, a single local dose immediately after injury had no beneficial effect at reducing neointimal formation.

**Effect of S-1452 in platelet deposition and intimal hyperplasia:** Thrombus has been implicated in the formation of intimal hyperplasia. Schwartz *et al.* demonstrated that thrombus volume determined the formation of neointima after angioplasty.15 Similarly, Banters *et al.* showed the importance of association between thrombus and restenosis by means of angioscopic study.16 Moreover, a IIb/IIIa receptor inhibitor reduced clinical restenosis after angioplasty in a randomized trial, which emphasized the role of the platelet-thrombus in the restenosis process.17

The importance of platelets in restenosis had been observed in a thrombocytopenic animal study which showed that prolonged and severe thrombocytopenia reduced intimal thickening after vascular injury.18 Theoretically, antiplatelet therapy appears to be one strategy for preventing intimal hyperplasia. However, clinical trials using aspirin have failed to show any benefit in reducing restenosis.19,20 Furthermore, the effect of selective TXA2 receptor antagonists on the prevention of restenosis were tested in randomized clinical studies.15,16 However, these studies failed to demonstrate any beneficial effects on restenosis. These results may imply that these TXA2 receptor antagonists could reduce platelet deposition but not inhibit VSMC proliferation. In the present study, however, S-1452, which also has specific TXA2 receptor antagonist activity, inhibited intimal hyperplasia after balloon injury in the rabbit model. This discrepancy can be explained as follows.

Platelet activation leads to the release of TXA2 from platelets, the vascular wall, and mitogens such as PDGF.21 Recently, through the use of their stable
mimetics, TXA2 has been shown to be mitogenic or even to act as a progression factor for VSMC proliferation. Furthermore, a specific TXA2 receptor antagonist has been shown to inhibit the potentiating effect of U46619 and I-BOP on mitogenesis induced by PDGF-BB. Therefore, inhibiting TXA2 action may reduce VSMC proliferation and finally contribute to a reduction in intimal hyperplasia. Moreover, S-1452 has a high affinity for VSMC. Hanasaki showed that TXA2 receptor antagonists have different affinities for platelet and VSMC by means of binding studies. They also showed that S-1452 had the highest affinity for VSMC and platelets among the several TXA2 receptor antagonists studied. This marked reduction of mitogenic action of TXA2 on VSMC through its specific receptor may be important in preventing the development of restenosis.

Limitations of the study: Local drug delivery to the vascular wall has the potential to achieve suprasystemic drug concentrations and decreases in adverse side effects. The present study showed that local administration of S-1452 at a smaller dose significantly reduced platelet deposition compared to systemic administration. However, a single local dose immediately after injury had no beneficial effect at reducing neointimal formation. In previous studies, local delivery of an antithrombotic agent did not reduce intimal hyperplasia after balloon angioplasty, since high tissue concentrations could not be achieved over a long period of time. Restenosis is not transient, but chronic due to three distinct and well defined stages: a thrombotic phase, recruitment phase, and proliferation phase. Thus, it is unlikely that one treatment will limit neointimal hyperplasia. Therefore, continuous systemic treatment may be necessary to protect the tissue from vascular injury.

Another limitation is that there is no complicated preexisting appearance such as human atheromatous plaque in this cholesterol diet rabbit model. The process of human restenosis is complex and involves many factors such as remodeling, thrombus formation, and VSMC proliferation, in contrast to the rabbit atherosclerotic model which is primarily a VSMC and macrophage proliferation model. Despite this limitation, this model may be an important first step for evaluating TXA2 receptor antagonist treatment which appears to be valuable in the prevention of restenosis.

In conclusion, the potent and selective TXA2 receptor antagonist S-1452 reduced acute platelet deposition and intimal hyperplasia after balloon injury in a cholesterol diet rabbit model. This agent may represent a new possibility to reduce restenosis after angioplasty in humans. Further studies in larger animal models of restenosis are required to determine its potential application in preventing restenosis after angioplasty in humans.
REFERENCES


