Experimental Model of Lower Limb Ischemia in Rats and the Effect of YM466, an Oral Direct Factor Xa Inhibitor

Yoshiyuki IWATSUKI,* Chinatsu SAKATA, Tomihisa KAWASAKI, and Masamichi OKADA

Pharmacology Research Laboratories, Drug Discovery Research, Astellas Pharma Inc.; 21 Miyukigaoka, Tsukuba, Ibaraki 305–8585, Japan. Received November 10, 2006; accepted July 27, 2007; published online August 7, 2007

A simple, quantitative, and reproducible model of lower limb ischemia was developed. Vascular injury was induced by ferric chloride (FeCl₃) solution to the rat iliac artery, after which blood flow in all of the lower limbs was continuously monitored using a scanning laser Doppler flowmeter. After FeCl₃ injury, a distinct decrease in blood flow in the ischemic lower limb was observed and blood flow did not recover during the 30 min after vascular injury. YM466, an oral direct factor Xa inhibitor, dose-dependently inhibited the reduction of peripheral blood flow. The area under the blood flow–time curve during 30 min after vascular injury improved dose-dependently, with significance at doses of 3 and 10 mg/kg. These results suggest that factor Xa inhibitors are effective in patients with peripheral arterial disease, and that this vascular injury model is a useful tool for the screening and evaluation of the efficacy of new antithrombotic agents.

Key words peripheral arterial disease; factor Xa inhibitor; lower limb ischemia; thrombosis; YM466

Activated factor X (FXa) is a pivotal blood coagulation factor that works at the point where the extrinsic and intrinsic coagulation cascades cross.¹ For this reason, the inhibition of FXa may be more effective than the inhibition of thrombin.² YM466 is a direct oral FXa inhibitor³ that has been shown to have a strong antithrombotic effect in rat,⁴ guinea pig,⁵ and squirrel monkey⁶ arterial and venous thrombosis models. Furthermore, it is clear that YM466’s antithrombotic effect is distinct from its effect on bleeding time when the effects are compared with those of warfarin⁷ and low molecular weight heparin.⁸

Major surgery on the lower limbs or abdomen markedly increases the risk of venous thromboembolism. When newly developed FXa inhibitors are tested in clinical trials, many are first evaluated as thromboprophylaxis agents in high-risk orthopedic patients. Recent phase II trials have shown that the efficacy and safety of the FXa inhibitor rivaroxaban (BAY59-7939) is similar to that of subcutaneous enoxaparin (BA 9689) in patients who underwent elective total knee or hip replacement surgery.⁹,¹⁰

In addition to venous thromboembolic diseases, the use of FXa inhibitors for arterial thromboembolic diseases such as acute coronary syndrome (ACS) has been suggested in several clinical trials.¹¹,¹² Peripheral arterial disease (PAD) of the lower extremities is a common manifestation of the atherosclerotic process that may also be treatable with FXa inhibitors. A heightened awareness of the symptomatic and asymptomatic forms of the disease is imperative since patients with PAD have an increased risk of cardiovascular disease, cerebrovascular disease, and even death. Although FXa inhibitors may also be effective in patients with PAD as well as ACS, few papers on this subject (not even animal studies) have been published so far.

In this study, a useful PAD animal (rat) model that can be used for the pharmacological evaluation of antithrombotic agents was established. This model was then used to evaluate the effect of the orally active FXa inhibitor YM466.

MATERIALS AND METHODS

Agents YM466 was synthesized at Astellas Pharma Inc. The compound was dissolved in 0.05 N HCl just prior to use.

Induction of Lower Limb Ischemia All experiments were performed in accordance with the regulations of the Animal Ethics Committee of Astellas Pharma Inc. A vascular injury-induced lower limb ischemia model was designed by modifying the method used by Kurz et al.¹³ Vascular injury was produced in fasted male Sprague–Dawley rats weighing 260—315 g (CLEA Japan, Inc., Tokyo, Japan) as follows: The surgical operation was performed at random. Rats were anesthetized by i.p. injection of urethane (1.3 g/kg). A minimum of 10 mm of the left iliac artery was carefully exposed and separated from the surrounding tissue after the midline abdominal incision was made. A scanning laser Doppler blood flowmeter (Laser Doppler Perfusion Imager System, Perimed AB, Järfalla, Sweden) was used to evaluate the perfusion of both the left (ischemic) and right (non-ischemic) hindlimbs. After the blood flow equilibrated, vascular injury was induced by applying filter paper saturated with 35% ferric chloride (FeCl₃) to the left iliac artery for 3 min. The perfusion signal was subdivided into 6 different intervals, each displayed as a separate color (low or no perfusion: dark blue, highest perfusion interval: red). The perfusion values corresponding to the color-coded pixels remained available for use in data analysis. Before and during scanning, the animals were placed on a heating plate set at 37 °C to minimize variations in temperature. After the laser Doppler images were recorded, the average perfusion values for both the ischemic and non-ischemic limbs were calculated based on the colored histogram pixels. To minimize the variables, including ambient light and temperature, the perfusion value calculated for each animal was expressed as the ratio of the left (ischemic) to the right (non-ischemic) hindlimb perfusion.

Experimental Protocols The experimental protocols are shown in Fig. 1. Either YM466 or the vehicle was intraduodenally administered using a gastric tube 12 min before the start of vascular injury. Perfusion of the hindlimbs was con-
continuously recorded by a scanning laser Doppler flowmeter starting before vascular injury and ending 30 min afterward.

**Histological Examination** The histological characteristics of the injured arteries were observed in normal, vehicle-treated, and YM466 (10 mg/kg)-treated rats. Fifteen minutes after injuring the iliac artery with FeCl3, 10% formalin in phosphate buffered saline was used for perfusion fixation, which was performed under physiological pressure for 30 min after left ventricular cardiac puncture. The injured segment of the left iliac artery, which can be easily distinguished because of discoloration due to FeCl3 injury, was carefully dissected, postfixed overnight at room temperature, and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin to prepare them for light microscopy.

**Statistical Analysis** The experiments were performed on groups of 6 animals. Changes in the blood flow ratio of the hindlimb perfusion in each group are presented as the mean±S.E.M. The area under the blood flow ratio–time curve (AUC) was calculated for each animal, and the data were presented as the mean±S.E.M. Statistical analyses were performed using Dunnett's multiple comparison test, with comparison to the vehicle group. A p value of less than 0.05 was considered significant.

**RESULTS**

**Visualization of Blood Flow in the Lower Limbs** Figure 2 shows representative perfusion images after administration of the vehicle and 10 mg/kg of YM466. In the vehicle group, a distinct decrease in blood flow in the ischemic hindlimb (arrow) was observed 10 min after FeCl3, application and blood flow did not recover during the 30 min after vascular injury. In contrast, 10 mg/kg of YM466 prevented the blood flow from decreasing in the ischemic hindlimb (arrow). Blood flow in the right non-ischemic hindlimb did not change in either group during the period of measurement.

**Histological Characteristics of Injured Vessels** Figure 3 shows representative hematoxylin–eosin-stained histological sections from normal vessels as well as the FeCl3-injured arteries in the vehicle and YM466 groups. In the vehicle-treated vessels, the lumen was almost completely occluded by a thrombus, in which some erythrocytes and a few leukocytes were seen. At the site of FeCl3 injury, endothelial cells were detached from the basal lamina. The thrombus was composed of thick fibrin nets, and the fibrin-rich portion was seen at the surface of the thrombus as well as at the thrombus-basal lamina contact site. However, in the vessel from the YM466-treated group, only a small amount of fibrin thrombus was observed, even though endothelial cells were injured.

**Quantitation of Blood Flow** Figure 4A shows the time course of changes in ischemic hindlimb perfusion in each group from before vascular injury up to 30 min afterward. In the vehicle-treated group, the blood flow decreased gradually and reached nadir within 8 min after the FeCl3 injury, which suggests the formation of an occlusive thrombus. YM466 dose-dependently inhibited the decrease in blood flow after vascular injury to the iliac artery. No decrease in blood flow was observed in any animals after administration of YM466 at 10 mg/kg. The AUC during the first 30 min after vascular injury increased dose-dependently, with significance at 3 and 10 mg/kg of YM466 (Fig. 4B). These data show that the FXa inhibitor YM466 can potently prevent lower limb ischemia induced by vascular injury.

**DISCUSSION**

In the present study, a useful PAD model that is sensitive to antithrombotic agents was established by modifying a FeCl3-induced vascular injury method.13) The iliac artery was injured by FeCl3 solution, and then blood flow in all lower limbs was continuously monitored using a scanning laser Doppler blood flow meter. Although FeCl3 is widely used to induce vascular injury, there are three points in the revised method that differ from the conventional PAD or thrombosis models.

First, this model is a simple and quantitative way to evaluate the effects of antithrombotic agents on PAD. Laurate and lactic acid-induced PAD models have been used in previous pharmacological studies to evaluate antithrombotics such as antiplatelet agents and anticoagulant agents. These PAD models, however, are not sufficient to evaluate drug effects. This is because subjective lesion scores are used as an efficacy index, and the severity of the lesions in these models makes them resistant to the compounds to be evaluated.14) On the other hand, in our model, the efficacy of the drug can be easily and quantitatively evaluated by monitoring blood flow in the lower limbs using a scanning laser Doppler flowmeter. Since this model is quantitative and highly reproducible, it can serve as a useful tool for the screening and evaluation of the efficacy of newly developed antithrombotic agents. Second, the revised method allows blood flow to be monitored over a large area. In methods using a conventional Doppler flowmeter, a probe should be placed on an artery. Thus, the measurement of blood flow is restricted to great vessels such as the carotid artery, and blood flow is measured at just one spot where a probe is settled.15,16) Whereas, using a scanning laser Doppler flowmeter, blood flow in all of the lower limbs can be monitored directly and simultaneously in our model. Third, blood flow in the lower limbs is visualized with a color display in our method. In previous methods, blood flow was expressed only numerically. Using a scanning laser Doppler flowmeter, the blood flow in each region of the lower limbs can be seen at a glance. For these reasons, this method allows drug effects to be easily detected.

Although the mechanism by which FeCl3 induces injury is not well defined, it is known that iron ions enhance the conversion of O2 and H2O2 to oxidizing species, such as hydroxyl radicals, which injure endothelial cells.13) In this model, the injured iliac artery was completely occluded, although blood flow of the lower limb did not reach the zero level. Histological examination showed that the injured por-
tion of the iliac artery was filled with fibrin thrombus. Moreover, blood flow did not decrease further when the iliac artery was ligated by a cotton thread at the end of the experiment (data not shown). This continued blood flow suggests that collateral routes of circulation that bypass the occluded site exist. Occasionally, blood flow that had diminished after FeCl₃ injury subsequently recovered, which implied thrombus dissolution. However, this recovery was transient, blood flow decreased again within 30 min. This suggests that the iliac artery was sufficiently injured by FeCl₃, and procoagulant activity persisted during the experiment. Consequently, this may be a good PAD model for evaluation of the efficacy of antithrombotic agents. Injury to the femoral artery was attempted in a preliminary examination, but the reduction in peripheral blood flow was not enough to evaluate the efficacy of drugs.

In the present study, the FXa inhibitor YM466 inhibited the reduction of peripheral blood flow induced by FeCl₃ injury. Histological analysis showed that endothelial injury and thrombus formation were observed in the vehicle group. However, in the YM466 group, endothelial injury was observed, but the amount of fibrin deposition was small. These results suggest that improvement of peripheral blood flow by YM466 is due to its antithrombotic effect rather than a direct
effect on endothelial cells.

In conclusion, we established a simple, quantitative, and reproducible PAD model in rats. In this model, the FXa inhibitor YM466 prevented blood flow from decreasing in an ischemic lower limb. YM466 may be a useful oral antithrombotic agent for patients with PAD. Furthermore, this model may be suitable for evaluation of the effects of other antithrombotic agents on PAD.

Acknowledgements We gratefully acknowledge Dr. Hajime Takamatsu and Dr. Seiji Kaku for their interest and encouragement. We also thank Dr. Fukushima Hirohama, Dr. Hirono Koshio, and Dr. Yuho Matsumoto for the synthesis of YM466.

REFERENCES