Abstract—We have investigated reproducible and quantitative thrombus formation in rat mesenteric arterioles using electrical stimulation under an inverted microscope. In vivo thrombi were formed in arterioles of 30–40 μm diameter by electrical stimulation using a tungsten electrode with a tip of 5 μm diameter. A "threshold" response was defined as one in which platelets adhered to the injured site of vascular wall and formed a white body which flowed away intermittently. The thrombus formations were classified into three stages: stage I, thrombi covering up less than 20% of the vessel lumen; stage II, when 20% to 40% of the lumen was covered; or stage III, when more than 40% was covered. This preparation was demonstrated to be valuable for testing the potency of anti-thrombotic drugs if we performed the experiment within 2 hr after removing the mesentery from the peritoneal cavity which exposed it to air. PG12, at a dose of 0.5 μg/100 g intravenously administered, inhibited the growth of the thrombus at stage I and III. PGE1, PGD2 and PGE2 had no effect at the same dose on these stages. The threshold response was significantly suppressed by both PGI2 and PGE1 at doses of 5 μg/100 g.

The present study is based on the idea that although platelet aggregation has an important role in the process of thrombus formation, the earliest change which initiates arterial thrombosis may occur in the wall of a vessel rather than on the platelets. Accordingly, the first step would be the adhesion of platelets to the damaged arteriole wall, and this is followed by aggregation.

Prostaglandin (PG) I2, PGE1 and PGD2 are potent inhibitors of platelet aggregation in vitro (1), while PGE2 has been shown to enhance aggregation in low concentrations and to inhibit aggregation at high ones (2). Emmons et al. first demonstrated that PGE1 inhibits platelet thrombus formation in vivo in injured rabbit-brain arterioles (3). Higgs et al. reported that PGI2 inhibited the formation of platelet thrombi in arterioles and venules of the hamster cheek pouch (2).

The paper describes some parameters in establishing a reproducible and quantitative method of in vivo thrombus formation in order to test the potency of anti-thrombotic agents. We studied some parameters such as vessel diameter, tip diameter of electrode, and the time after exposure of the mesentery which was removed from the peritoneal cavity which exposed it to air, and also studied the anti-thrombotic effects on thrombus formation of 4 kinds of PGs (PGI2, PGE1, PGD2 and PGE2) injected intravenously.

MATERIALS AND METHODS

Male rats of the Wistar strain, weighing
200 to 300 g, were anesthetized with pentobarbital sodium (40 mg/kg) intramuscularly, and if necessary 2 mg/kg was injected 50 or 60 min later. A midline incision was made on the shaved abdominal skin. The mesentery was pulled out of the peritoneal cavity onto a glass plate. To keep it moist, the tissue exposed over the glass plate was partly covered with pieces of cotton soaked with Tyrode's solution. The mesentery was displayed and held in place by clips at the periphery of the gut (4). The arterioles which are less branching and less tortuous were used for the experiment. PGs and their solvents (0.9% NaCl, 0.1 M phosphate buffer) were injected through a polyethylene tube into the jugular vein of the rat at 0.25 ml/200 g/min. PGs except PG12 were dissolved in saline containing 50% ethanol at 1 mg/ml, stored at −20°C and diluted with saline before use. PG12 was dissolved in absolute ethanol at 0.1 mg/ml, stocked at −80°C for less than 10 days and diluted with 0.1 M phosphate buffer (pH 9.0) immediately before use. Microscopic observations were done in a room maintained at 25°C.

The microelectrode was made of fine tungsten wire with a tip diameter of 5 μm. The electrode which was connected to the cathode was held in a micromanipulator (Narishige Co. Ltd.), and the electrode tip was placed just 25 to 50 μm away from the vessel wall at an angle of about 45 degrees. A square-wave stimulator (Nihon Kohden MSE-3R), set at a potential of 100V, was then used to deliver a single electrical pulse. The indifferent electrode was a silver plate under the gut and connected to the anode.

Graded responses could be produced by varying the duration of electrical stimulation. A "threshold" response was defined as when several platelets adhere to form a white body at the injured site and the white body detached and was carried into blood stream intermittently. Increasing the duration of electrical stimulation produced larger platelet thrombi at the site. The thrombi formed after stimulation were then classified into three stages as follows: stage I, thrombi covering up less than 20% of the vessel lumen; stage II, when 20% to 40% of the lumen was covered; or stage III, when more than 40% was covered. The judgement of the stage was made 2 min after the stimulation. When we examined effects of PGs on the duration of electrical stimulation to form thrombi using these preparations, the stimulation was begun a minute after the injection of 0.9% NaCl or phosphate buffer. The duration of stimulation for producing each stage of thrombus formation was measured (control experiment). One of the PGs was injected 25 min after the control, and another arteriole of similar diameter was stimulated electrically until development of each stage. We compared the duration of electrical stimulation required to form each stage of platelet thrombus after injection of PGs with that required in the control experiment. Statistically analysis was made by the Student’s t-test.

RESULTS

Studies of some parameters which influence thrombus formation: First of all, we examined the effect of electrode tip diameter, 5 and 150 μm, on the duration of electrical stimulation to form each step of platelet thrombi. Figure 1 shows the duration-related formation of thrombi using both 5 and 150 μm electrode tip diameters. We found that there was a small amount of electrolytic gas bubbling around the tip of the electrode and at the injured site of the arteriole wall for 30 sec when we stimulated using the 5 μm electrode. However, when the vessel was stimulated with the 150 μm electrode, a number of large gas bubbles developed and lasted a few minutes. We used, therefore, the 5 μm electrode in the
Fig. 1. An electrode with a 5 or 150 μm tip diameter produced duration-dependent thrombus formation. Five μm tip diameter electrode: 100V, 0.16±0.01 mA. 150 μm tip diameter electrode: 50V, 1.6±0.1 mA. Ordinate: Duration (msec) of single pulse of electrical stimulation. Abscissa: Stages in thrombus formation. Thr: Threshold, I, II and III: as described in the text. The arabic numbers in the parentheses show the number of preparations examined. Each value represents the mean with the standard error (error bars).

In the following experiments, Figure 2 shows the duration-related formation of platelet thrombi in arterioles with 3 ranges of diameters. With a 20 to 30 μm diameter of vessel lumen, the duration of electrical stimulation which was required to form stage II was close to that to form stage III. As there were a few arterioles of 40 to 50 μm in diameter, we used 30 to 40 μm arterioles for the present study of thrombus formation.

In the following experiments, effects of PGs on thrombus formation were studied using different vessels from the same rat. Therefore, we determined whether similar thrombi were formed by electrical stimulation in different vessels of the same rat. Furthermore, the air exposure time after removal of the mesentery from the peritoneal cavity is likely to influence thrombus formation. In Fig. 3, the comparison of duration of stimuli required to form each stage of thrombus was made in two vessel lumens of similar diameter at about a 2 hr interval. As there was no significant difference in the duration of stimuli for thrombus formation between these two different vessels, we could examine the effect of PGs on thrombus formation using arterioles which were similar in diameter, which were less branching and less tortuous. The experiments were per-
formed within 2 hr.

Effects of PGs on thrombus formation:
Effects of intravenous injection of 0.5 μg/100 g of 4 kinds of PGs (PGI₂, PGE₁, PGD₂, and PGE₂), are reported to have potent inhibitory effect on platelet aggregation with the exception of PGE₂, were examined. Of the 4 kinds of PGs, only PGI₂ injection resulted in a requirement for a longer duration

Fig. 4. Significant inhibition by PGI₂ (0.5 μg/100 g) was seen at stages II and III of thrombus formation and there was no inhibition of "threshold" response and stage I. Each value represents the mean with standard error of rats. *Significantly different from the control at P<0.05. See details in Fig. 1.

Fig. 5. PGE₁ (0.5 μg/100 g) tended to inhibit thrombus formation at the "threshold" response and at all stages. Each value represents the mean with standard error of 6 rats. See details in Fig. 1.

Fig. 6. PGD₂ (0.5 μg/100 g) did not show any effect on thrombus formation compared with the control. Each value represents the mean with standard error of 6 rats. See details in Fig. 1.

Fig. 7. PGI₂ and PGE₁ at 5 μg/100 g significantly inhibited the "threshold" response. Ordinate: Duration (msec) of single pulse of electrical stimulation to produce "threshold" response. Abscissa: Dose of PGs/100 g. Zero on the abscissa shows injection of 0.9% NaCl solution instead of PGE₁ and PGD₂ and phosphate buffer solution instead of PGI₂ into the jugular vein of the rat (control experiment). **Significantly different from the control at P<0.01. See details in Fig. 1.
in electrical stimulation to form platelet thrombi at stages II and III than that in the control experiment (Fig. 4). No significant effect was seen after the injection of PGE₁, PGD₂ (Figs. 5 and 6) and PGE₂ (not shown in figure). In comparing the effects of the 3 PGs (PG₁₂, PGE₁ and PGD₂) on the "threshold" response, PG₁₂ and PGE₁ at 5 μg/100 g significantly delayed the onset of the "threshold" response (Fig. 7).

**DISCUSSION**

Several kinds of small blood vessels from different species including hamster cheek pouchs have been examined for thrombus formation (2-4). Rat mesenteric arterioles were used in the present study.

In the basic studies of thrombus formation, when the electrical current was passed through the electrode, visible gas bubbles were produced around the microelectrode tip and the injured site of vascular tissue. The gas bubbles dispersed within 30 sec when we used the 5 μm electrode. The 150 μm tip diameter electrode evolved much gas which lasted for a few minutes. In this respect, the 5 μm electrode is more suitable for this experiment than the 150 μm electrode.

Each stage in thrombus formation in arterioles of 20 to 50 μm in diameter was dependent on the duration of electrical stimulation. In the rat mesentery, the distribution of arterioles of 40 to 50 μm in diameter was less than those of the 30 to 40 μm diameter. Therefore, it is convenient to use the vessels of 30 to 40 μm, and furthermore these vessels seemed to be more resistant to exposure to air than the arterioles of 40 to 50 μm. There were 4 or 5 arterioles of 30-40 μm in the same rat.

The distance between the electrode tip and the vessel wall was important for development of platelet thrombi as Gordon et al. reported (5). When the electrode tip was in direct contact with the vessel, the duration of electrical stimulation required to produce a thrombus was less than 2 msec. If the electrode was moved 50 to 75 μm away from a vessel wall, much greater duration was necessary to produce the same response than required in the present experiments.

We could confirm that the preparation was useful for testing the potency of anti-thrombotic agents in vivo since the duration of electrical stimuli developing the thrombus into the same stage did not vary in the mesenteric vessels of the same rat. Furthermore, it must be considered that the time of exposure of mesenteric arterioles to the air may influence the blood flow in microcirculations. Figure 3 indicates that there are no problems if we can perform the experiment within 2 hr and if we can choose arterioles of similar diameters and from a similar location.

Development of thrombus in vivo is not only based on the adhesiveness of blood platelets to the injured site of the vascular wall, but also on aggregation with other platelets. Stimulation of much greater duration will produce platelet thrombi large enough to occupy the vessel lumen and may cause complete stasis. If there was such complete occupation, we would not be able to examine the effect of PG on the thrombus formation in other aspects of circulation. Therefore in our investigations, development of thrombus formation was usually limited to stage III. The stimulus which caused the "threshold" response was always sufficient for the adhesion of platelets. Development of stage I to the III was manifested by platelets aggregating at the injured site induced by electrical stimuli to the arteriole wall. Accordingly, PG₁₂ suppression on the growth of stages II and III of thrombus formation may suggest the inhibition of platelet aggregation at the vessel wall caused (Fig. 4) and delay of the onset of the "threshold" response by
PGI₂ and PGE₁ may indicate inhibition of platelet adhesion to the sites of vascular injury. In the present study, a higher dose of PGI₂ (5 μg/100 g) was required for inhibition of platelet adhesion than for inhibition of aggregation (0.5 μg/100 g). Higgs et al. (6) showed that inhibition of platelet adhesion by PGI₂ required a higher concentration (20 ng/ml) than that of platelet aggregation (0.1 ng/ml). PGE₁ is reported by Kloeze (7) to inhibit the formation and growth of platelet thrombi induced by an electric stimulus in rat cortical veins. PGI₂ (0.5 μg/100 g, i.v.) significantly inhibited the thrombus formation, while PGE₁ tended to inhibit it in the present study. This may be due to PGI₂ having a more potent platelet aggregation preventing effect than PGE₁. PGD₂ showed no significant effect on thrombus formation in this study. This may be due to the low sensitivity of rat platelets to PGD₂ compared with PGI₂ and PGE₁ as Whittle et al. reported (8).

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