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EFFECT OF A POTENT THROMBIN INHIBITOR, NO. 407, ON NOVEL EXPERIMENTAL THROMBOSIS GENERATED BY ACETIC ACID#

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INDEXING WORDS

synthetic thrombin-inhibitor; arginine derivative; animal model; thrombosis; acetic acid

SYNOPSIS

By means of injuring the vessel wall with acetic acid, which has strong penetration and stimulation to tissue, we developed a novel animal model of the arterial thrombosis as indicated below, and investigated the antithrombotic effect of No. 407 in vivo by this model. The exposed carotid artery of rabbit anesthetized by pentobarbital was surrounded with absorbent cotton in a capsule, and then absorbent cotton was soaked sufficiently with acetic acid. Three hours after soak, the arterial segment was cut out, and the degree of the thrombus generation was observed under microscope. The optimal concentration of acetic acid for the thrombus generation under the treatment time of 3 hours was 90%. It was found that the thrombus generated by such method was the "white thrombus" at low magnification, and under the observation

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by scanning electron microscope, this thrombus was almost composed of aggregated platelets with entrapped erythrocytes and leukocytes. No. 407 administered intravenously and via continuous infusion prevented significantly the thrombus generation.

INTRODUCTION

Although heparin, oral anticoagulants, and inhibitors of platelet aggregation etc. are used clinically for the prevention and therapy on thrombotic diseases at present, there is the earnest need for the development of more effective and nontoxic antithrombotic agent. The numerous models for experimental thrombosis in many species of animals have been designed for such studies, but none appears to be without some disadvantages such as poor reproducibility of lesion of the vessel wall,[2,5,6,19] necessity for the special instruments,[2,3,11] difficulty of the operation,[10,15] and complicatedness of experimental conditions.[1,9,12] Therefore, we developed a novel animal model of the arterial thrombosis. Acetic acid, which is used in animal gastric ulcer model because of its strong penetration and stimulation to tissue,[4] was used as the means to injure the vessel wall.

The present studies deal with the preparation of an animal model for the study of thrombus generation and the potential usefulness of the model for evaluation of the effects of No. 407, a highly selective and potent thrombin inhibitor in vitro,[7,18] as a possible antithrombotic compound in vivo. The preventive effect of No. 407 on the thrombus generation was also confirmed by scanning electron microscopical observation.

MATERIALS AND METHODS

Preparation of Animal Model of Thrombosis.

Male rabbits weighing from 2.0 to 3.0 kg were anesthetized by intraperitoneal administration of pentobarbital (40 mg/kg body weight). Bilateral carotid artery was exposed carefully from the surrounding tissue in the length of at least 20 mm. The exposed artery was surrounded with absorbent cotton in a capsule as illustrated in Fig. 1, and then absorbent cotton was soaked suffi-

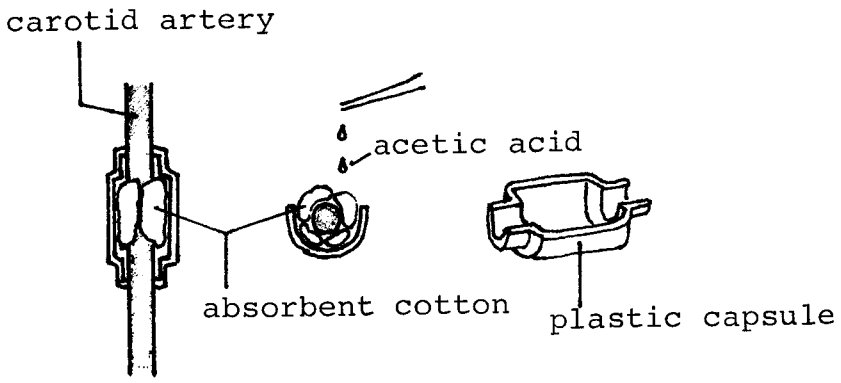

Fig. 1

ciently with an aqueous solution of acetic acid. Three hours after soak, the arterial segment was cut out at at least 5 mm from both ends of capsule, longitudinally opened, and then were pinned on a small plate. The degree of the thrombus generation was observed under microscope.

The degree of the thrombi was classified by the percentage of the area occupied with thrombi to the total area of vessel wall injured by acetic acid as follows: 1) 100% occlusion, occlusion thrombus; 2) 70-99% occlusion, large thrombus; 3) 30-69% occlusion, middle thrombus; 4) 1-29% occlusion, small thrombus; 5) no occlusion, none.

One shot intravenous administration of No. 407 was performed at 5 minutes before soak of acetic acid, and in case of two shot intravenous administration the second shot was performed at 90 minutes after the initiation of soak. Continuous infusion was started 5 minutes before the initiation of soak.

Scanning Electron Microscopical Studies.

The arterial segment cut out was immersed immediately in 0.1 M phosphate buffer (pH 7.2), fixed in 2% glutaraldehyde, and post-fixed in 1% osmium tetroxide for a few hours. After dehydration by graded ethanol, the segment was immersed in isoamyl acetate, dried by the critical point method, coated with gold, and examined in a scanning electron microscope.

RESULTS

A) Preparation of Experimental Thrombosis by Acetic Acid.

A series of experiments were performed in order to determine the optimal concentration of acetic acid for the generation of thrombus under the treatment time of 3 hours. As shown in Table 1, it was found that various sizes of the thrombi were generated by using various concentrations of acetic acid. Namely, at 100% concentration the generated thrombi were too large, loose at 80%, and too small at 70%.

Table 1 The generation of thrombus by using various concentrations of acetic acid.

Conc. of CH_3COOH (%)	No. of rabbit	Thrombus				
		Occlusion	Large	Middle	Small	None
100	3	3	3	0	0	0
90	5	1	6	3	0	0
80	3	0	2	2	2	0
70	3	0	0	1	4	1

From these results, an optimal concentration of acetic acid for the formation of thrombus was thought to be 90% and the following experiments were performed under this condition.

The non-occluded thrombus at 1 hour or 3 hours after the soak of 90% acetic acid and the occluded thrombus at 3 hours after the soak were observed under scanning electron microscope.

The thrombus after 1 hour was small at the low magnification. Under the scanning electron microscopical observation it was found that the platelets adhered dispersedly on the surface of vessel wall (Fig. 2). It was recognized that the shape of adhered platelets was almost discoidal but with some pseudopods. The endothelium had been detached from the subendothelial tissue. Sometimes platelets were observed to be adhered to leukocytes.

The thrombus after 3 hours was almost white colored at the low magnification (Fig. 3). Under the scanning electron microscopical observation, it was shown that this thrombus was composed of aggregated platelets with entrapped erythrocytes and leuko-

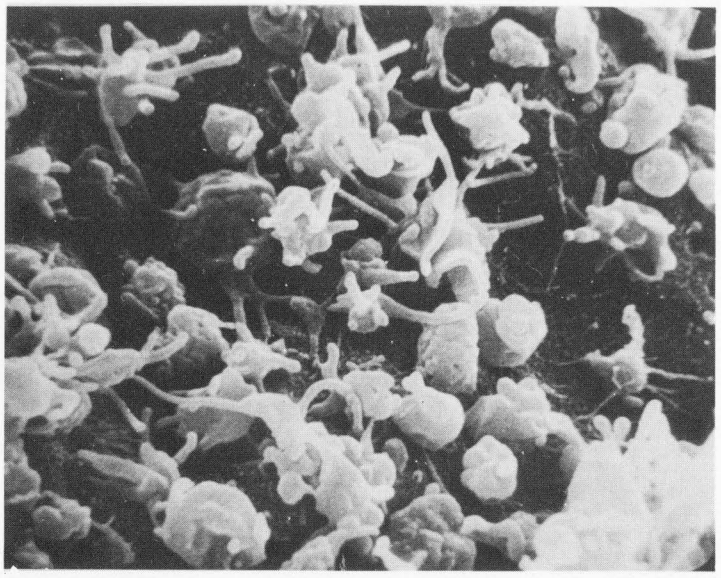

Fig. 2 Surface of the vessel wall after acetic acid treatment for 1 hour. (x 8,800) Numerous platelets adhere on the subendothelial tissue.

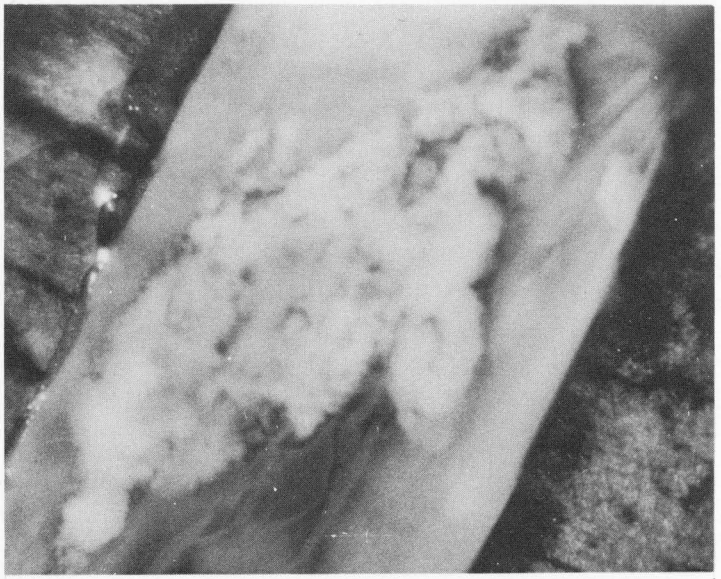

Fig. 3 White thrombus generated by 90% acetic acid treatment for 3 hours. (x 25)

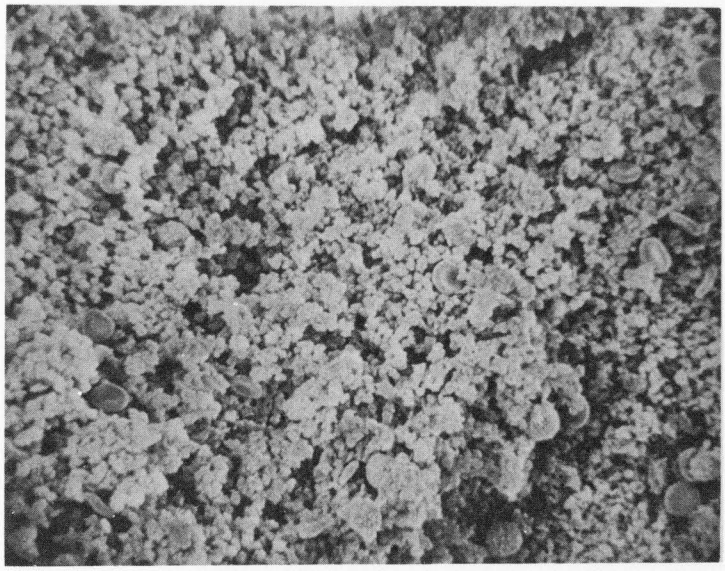

Fig. 4 Surface of a large thrombus generated by acetic acid treatment for 3 hours. The thrombus is composed of aggregating platelets plus erythrocytes and leukocytes. Fibrin is not observed in this area. (x 1,000)

cytes (Fig. 4). It was noted that the surfaces of these platelet aggregates were composed of platelets without pseudopods. From these observations the blood vessel was found to have the "white thrombus"

At the low magnification the thrombus occluded after 3 hours consisted of two kinds of materials which stained differently: a "white head" attached to the vessel wall, and a "red tail" which streamed away from the "white head" in the direction of blood flow. Under the electron microscopical observation this heavy thrombus was composed of networks of fibrin fibers with numerous aggregated platelets and entrapped erythrocytes (Fig. 5). In the interstices of the fibrin networks numerous erythrocytes were observed to be trapped and aggregated (Fig. 6).

From above observations, an optimal time of acetic acid treatment was thought to be 3 hours and the following experiments were performed under this condition.

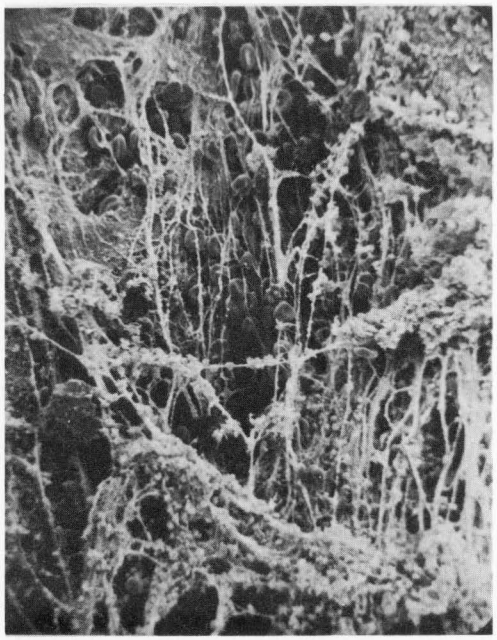

Fig. 5 Occluded thrombus generated after 3 hours. (x 1,000)
Networks of fibrin fibers with numerous aggregated platelets and entrapped erythrocytes are observed.

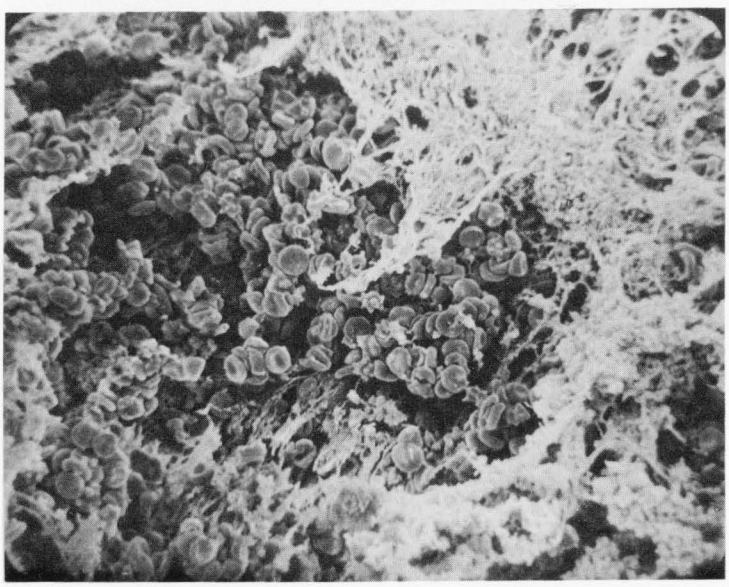

Fig. 6 Occluded thrombus generated after 3 hours. (x 1,000)
It is recognized that numerous erythrocytes are entrapped in the interstices of the fibrin networks.

B) Effects of No. 407 and Heparin on This Model.

No. 407 was administered via various routes. The formation of thrombosis was prevented as shown in Table 2. Two shot intravenous administrations of 20 or 40 mg/kg induced almost equal effects. Two shot intravenous administrations of No. 407 were more effective than one shot administration. The greater effect in two shot administrations would be due to the fast disappearance of the plasma concentration after intravenous injection of No. 407.

The continuous infusion at a rate of 0.174 μmole/kg/min showed a significant effect while more potent effect was shown at 0.348 μmole/kg/min , the presumed plasma concentrations being 5-10 μM and 10-20 μM respectively.[17)]

Heparin was administered at 5 minutes before soak of acetic acid and showed the dose-dependent inhibitory effects at the dose of 300 u/kg or more of intravenous administration.

Under scanning electron microscope in the sample with two shots of intravenous administration of No. 407 the subendothelial tissue with little adherence of platelets was observed, as shown in Fig. 7. Differing from the normal blood vessel wall, No. 407

Fig. 7 Surface of the vessel wall injured by acetic acid of No. 407 treatment. (20 mg/kg x 2) The rupture and the detachment from the subendothelial tissue of endothelial cells are observed. The adherence and aggregation of platelets in this area may appear without No. 409 treatment.

Table 2 The effect of No. 407 and heparin on thrombosis by acetic acid.

Drug	Dose	Route	No. of rabbit	Thrombus				
				Occlusion	Large	Middle	Small	None
No. 407	40 mg/kg x 2	i.v.	5	0	0	1	7	2
	20 mg/kg x 2	i.v.	4	0	0	1	6	1
	20 mg/kg x 1	i.v.	4	0	3	2	3	0
	0.174 μmole/kg/min.	infusion	5	0	4	2	4	0
	0.348 μmole/kg/min.	infusion	4	0	0	0	6	2
Heparin	5000 u/kg	i.v.	4	0	1	1	5	1
	1000 u/kg	i.v.	4	0	1	2	5	0
	300 u/kg	i.v.	4	0	2	2	4	0
	100 u/kg	i.v.	4	1	3	3	1	0
Control	--------	---------	39	7	51	16	3	1

treated vessel showed the rupture and detachment of the endothelial cells and the exposure of the subendothelial tissue, and the surface of subendothelial tissue was wavelike and rugged. These observations suggest that No. 407 has the effect to prevent platelet adherence and aggregation on the surfaces of injured blood vessels.

DISCUSSION

Although numerous experimental animal models of arterial thrombosis have been reported, these models present methodologically many difficult problems. Therefore, acetic acid having strong penetration and stimulation to tissue being used, the animal model which has such advantages as simplicity of operation, good reproducibility of lesion of the vessel wall, good objectivity, and no necessity for the special instruments was devised by the authors. The thrombus generated by this method was the "white thrombus" as observed under scanning electron microscope to consist of masses of platelet aggregates with a little entrapped erythrocytes and leukocytes, and the thrombus formation was considered to be triggered by endothelial injury by acetic acid.

No. 407, a potent thrombin inhibitor, was examined for the antithrombotic effect on this new animal model and found to be effective at the plasma concentration above 5-10 μM.[17]

Platelet thrombus generation triggered by the injury of vessel wall has been generally supposed to progress according to the following three step mechanisms. 1) Platelets adhere to the exposed subendothelial tissue by their pseudopods. 2) The adhered platelets undergo the release reaction and the released substances such as ADP and 5-HT make platelet aggregated. 3) The platelet aggregate masses are made irreversible by a little thrombin produced by the response between factor X_a, which is generated by the action of tissue thrombo-plastin released from the injured tissue, and the blood at the neighborhood of the platelet aggregate masses.

It has been known that the compounds which inhibit the platelet aggregation induced by collagen, ADP, or arachidonic acid are effective to prevent the platelet thrombosis while anticoagulants are not effective.[13,14,16] No. 407 has been shown not to inhibit platelet aggregation induced by collagen, ADP or arach-

idonic acid, but to inhibit potently the platelet aggregation induced by thrombin in vitro at the low concentration.[7] In the present experiment, No. 407 showed the remarkable inhibitory effect on this model. This effect of No. 407 would be due to its potent inhibitory effect on thrombin induced platelet aggregation demonstrated in in vitro studies. On the other hand, at the dose of 300 u/kg, nearly that of clinical use, heparin prevented significantly the platelets thrombus generation, suggesting again that thrombin plays an important role in the platelet thrombus generation in acetic acid method.

Virchow suggested the trial of causative factors leading to thrombosis:[20] 1) alterations in the vessel wall; 2) alterations in blood flow; and 3) alterations in blood content. Many an experimental thrombosis has been formed as a result of a combination of these factors. In the present study, under the observation by scanning electron microscope in the sample of No. 407 treatment, surface of endothelial structure was noted to be wave-like and uneven. Therefore, it was suggested that the alteration in blood flow induced by the physical changes of the subendothelial surface also influenced the aggregation of platelets, in addition to the direct action of the exposed basement membrane and microfibrile of the subendothelium to platelet aggregation.

It has been reported that the thrombus is generated in several minutes after injury of the vessel wall.[8] On the present model the adherence and/or aggregation of platelets were observed after 1 hour soak of acetic acid. Therefore, it was suggested that the detachment of endothelium induced by acetic acid treatment might be produced within 1 hour.

Although it is too early to discuss availability of No. 407 in clinical use, results obtained seem to support the possibility that No. 407 may be useful in preventing arterial thrombosis in patients.

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