

## Antithrombotic Effects of Odorless Garlic Powder Both *in Vitro* and *in Vivo*

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**Antithrombotic activities of odorless garlic powder were demonstrated in blood fibrinolytic and coagulation systems. Though the odorless garlic preparation did not influence tissue-type plasminogen activator (t-PA) or its inhibitor secretions from human umbilical vein endothelial cells, it enhanced plasmin generation by t-PA on fibrin film and in chromogenic assays by 1.8-fold and 8.7-fold respectively. The coagulation system was considerably reduced after the administration of the garlic in a rat *in situ* loop model, indicating that increased levels of thrombin-antithrombin III (TAT) complex in the control group were significantly reduced to normal (sham) in the garlic group ( $p < 0.05$ ), which was associated with decreasing tendencies towards prolonged or increased values of coagulation parameters in the control group. These findings suggest that odorless garlic not only activates fibrinolytic activity by accelerating t-PA-mediated plasminogen activation, but also suppresses the coagulation system by downregulating thrombin formation, suggesting a beneficial role in preventing pathological thrombus formation in such cardiovascular disorders.**

**Key words:** antithrombotic activities; odorless garlic; fibrinolytic and coagulation system; thrombus; *in situ* loop model

Garlic is widely recognized as a functional foodstuff that possesses a variety of beneficial effects on human health.<sup>1)</sup> Since garlic especially possesses advantageous roles in blood circulation among its physiological effects on the human body, the prevention of cardiovascular disease and other metabolic syndromes by garlic has been well documented.<sup>2,3)</sup> Several studies have indicated that garlic and its preparation increased fibrinolytic activity<sup>4–6)</sup> but inhibited platelet aggregation<sup>7–9)</sup> as well

as lowering blood pressure<sup>10–12)</sup> and levels of cholesterol<sup>13–15)</sup> in humans. These effects are advantageous in preventing or ameliorating cardiovascular disorders such as acute myocardial infarction caused by occlusion of blood circulation due to damage to or dysfunction of vascular endothelial cells (VECs), resulting in the formation of blood clot called thrombus. Thrombus formation and degradation is regulated by a hemostatic mechanism consisting of the vascular system, the platelet system, the coagulation system, and the fibrinolytic system. A normal state of blood circulation is therefore maintained in such a way as to confer resistance to abnormal clot formation by the coagulation system and the platelet system and to promote an acceleration of clot degradation by the fibrinolytic system, called as an inclusive term, antithrombotic function. The blood fibrinolytic system is activated by tissue-type plasminogen activator (t-PA)<sup>16)</sup> which converts zymogen plasminogen to plasmin. Plasmin specifically degrades fibrin, the main component of thrombus, which in this case is called fibrinolysis or thrombolysis. This system is also inhibited by type-1 plasminogen activator inhibitor (PAI-1).<sup>17)</sup> Both t-PA and PAI-1 are secreted from VECs.<sup>18)</sup> Therefore, the fibrinolytic system in blood is mainly dependent on the balance between t-PA and PAI-1, the expression of which is potentially regulated by VECs. Since VECs also contribute to the regulation of other systems in hemostasis, including the coagulation system, antithrombotic regulation is mainly performed by VECs.<sup>19)</sup> Though the enhancement of fibrinolytic activity by garlic has been reported in several studies, the mechanism has not yet been analyzed in detail.

We investigated in this study whether odorless garlic exhibits antithrombotic activity, that is, hyper-fibrinolytic potential in VECs and the cell-free enzymatic

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**Abbreviations:** t-PA, tissue-type plasminogen activator; PAI-1, type 1 plasminogen activator inhibitor; HUVECs, human umbilical vein endothelial cells; PT, prothrombin time; APTT, activated partial thromboplastin time; TAT, thrombin-antithrombin III; FDP, fibrin degradation products; PIC, plasmin-plasmin inhibitor complex

system, as well as anticoagulation activity in rats. Data obtained from our study indicate that odorless garlic enhanced the fibrinolytic system by increasing enzymatic activity of t-PA *in vitro*, and reduced coagulation system by decreasing thrombin formation in a rat thrombus model *in vivo*.

## Materials and Methods

**Materials.** The following materials were obtained from the commercial sources indicated: human glu-type plasminogen, Biopool AB (Umea, Sweden); human two-chain t-PA, American Diagnostica (Greenwich, CT); bovine thrombin, Mochida Pharmaceuticals (Tokyo); bovine fibrinogen, Itoham Foods (Nishinomiya, Japan); culture flask (T-25 cm<sup>2</sup>) and plates (24- and 96-well), Corning (Corning, NY); polystyrene dish ( $\phi = 90$  mm), Asahi Techno Glass (Funabashi, Japan). Sources of reagents and kits for diagnosis and specific items are described in the corresponding sections. All other reagents were of analytical grade or the highest quality commercially available.

**Garlic preparation.** Garlic powder as a commercial product, "Bizen odorless powder of garlic extracts," was provided by Bizen Chemical Co., Ltd. (Akaiwa, Japan). The garlic plant (*Allium sativum* L.), harvested at Shandong sheng in China, was processed to produce odorless powder as follows: The raw plants were initially heat-treated to inactivate alliinase, which converts alliin to allicin, the main odorous component in garlic. Then the odorless form of the garlic fraction was extracted by hot water, and the extract was filtrated by centrifugation and concentrated *in vacuo*. Finally, the odorless garlic powder was obtained by freeze-drying of the concentrated filtrate.

The garlic powder was dissolved in culture medium for garlic stimulation of endothelial cells, or dissolved in 0.01% Triton X-100 for fibrin film and chromogenic assays. The dried powder was directly mixed in the diet in the animal study. These garlic preparations are denominated simply as odorless garlic or garlic hereafter.

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo Industries (Osaka, Japan). HUVECs were maintained in T-25-cm<sup>2</sup> flasks and seeded on 24-well multiplates to form confluency for garlic stimulation experiments under 5% CO<sub>2</sub>, 100% humidity at 37 °C. The growth medium contained low concentration of fetal calf serum (FCS), several growth factors and antibiotics in RPMI-1640 (HuMedia-EG2, Kurabo Industries).

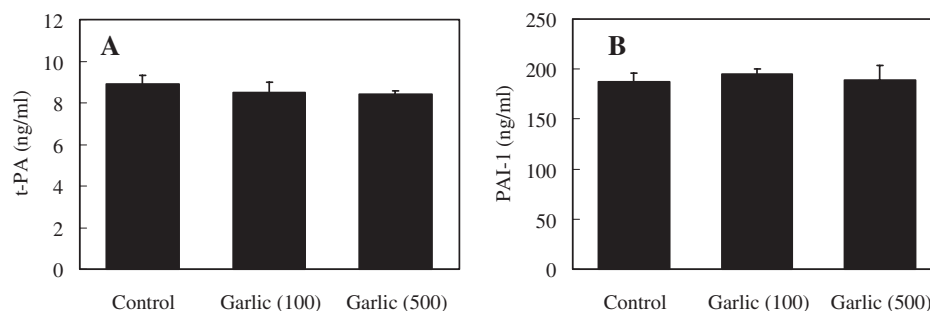
**Garlic stimulation of HUVECs.** The confluent HUVECs on the 24-well multiplate were briefly washed with FCS-free RPMI-1640, and subsequently cultured with fresh RPMI-1640 containing various concentrations of odorless garlic powder (0, 100, 500, and 1,000

$\mu\text{g/ml}$ ) at 37 °C for 18 h. The conditioned medium was harvested and added to Triton X-100 (final 0.01% v/v). An aliquot of the medium was then subjected to t-PA and PAI-1 ELISA (Imulyse t-PA and Imulyse PAI-1) (Biopool AB, Umea, Sweden). These ELISAs were performed according to the manufacture's manuals.

**Fibrin film assay.** Fibrin film was formed on a plastic dish ( $\phi = 90$  mm). Briefly, after mixing with 4.5 ml of bovine fibrinogen (1.5 mg/ml in Veronal buffer) and 4.5 ml of 50 mM CaCl<sub>2</sub>, 0.2 ml of 10 NIU/ml thrombin was added to form fibrin film, and the film was stabilized at room temperature for 3 h. Ten  $\mu\text{l}$  of t-PA (87.5 IU/ml) was supplemented with either 10  $\mu\text{l}$  of Triton X-100 (0.01%) or 10  $\mu\text{l}$  of odorless garlic solution (1 mg/ml in 0.01% Triton X-100), and the samples (20  $\mu\text{l}$ ) were spotted onto the surface of the fibrin film and incubated at 37 °C for 18 h. The fibrinogen preparation contained a small amount of plasminogen, so that t-PA in sample converted it to plasmin in the fibrin film, resulting in the degradation of fibrin (fibrinolysis). The area (mm<sup>2</sup>) of fibrinolysis was measured to express it as t-PA activity (IU/ml) estimated from the calibration curve (standard t-PA and lysed area).

**Chromogenic assay.** A synthetic chromogenic substrate, H-D-Val-L-Leu-L-Lys-p-nitroanilide, S-2251 (Daichi Pure Chemicals, Tokyo) was used to measure plasmin activity. Plasminogen and S-2251 were dissolved in 50 mM Tris/HCl (pH 7.4) containing 0.1 M NaCl, and t-PA and garlic was diluted in 0.01% Triton X-100. Plasminogen (10  $\mu\text{g/ml}$ , 20  $\mu\text{l}$ ), t-PA (10 IU/ml, 50  $\mu\text{l}$ ) and S-2251 (2.5 mM, 50  $\mu\text{l}$ ) were mixed, and odorless garlic (1, 10, 100, 500, and 1,000  $\mu\text{g/ml}$ , 20  $\mu\text{l}$ ) or Tris buffer (20  $\mu\text{l}$ ) was further added on 96-well plates for incubation at 37 °C for 18 h. In order to analyze the kinetic parameters of t-PA, plasminogen (12.5, 25, 50, and 100  $\mu\text{g/ml}$ , 20  $\mu\text{l}$ ), t-PA (20 IU/ml, 50  $\mu\text{l}$ ) and S-2251 (2.5 mM, 50  $\mu\text{l}$ ) were mixed, and garlic (1,000  $\mu\text{g/ml}$ , 20  $\mu\text{l}$ ) or Tris buffer (20  $\mu\text{l}$ ) was added on 96-well plates for incubation at 37 °C for 240 min. Released p-nitroanilin, by amidolysis of S-2251, was colorimetrically measured at O.D. 405 nm as plasmin activity. Kinetic constants were obtained by Lineweaver–Burk plots.

**Thrombosis model in the rat.** Male 8-week-old Crl:CD (SD) rats were obtained from Charles River Laboratories (Yokohama, Japan). and were acclimatized for 1 week with a normal diet (CRF-1, Oriental Yeast, Tokyo). The rats were maintained in a room (22  $\pm$  3 °C, 60  $\pm$  15% humidity) under a 12-h dark/12-h light (from 7:00 to 19:00) cycle and ventilation (12–15 times/h), and free to access to the diet and tap water *ad libitum*. Diets with or without odorless garlic powder were given to the rats (1 g/kg/d) for 2 weeks. Body weight and diet intake were checked during the breeding period. These rats were divided into three groups: rats that had been



**Fig. 1.** Release of Antigens of t-PA (A) and PAI-1 (B) from HUVECs.

After HUVECs were cultured in FCS-free medium with or without odorless garlic for 18 h, the conditioned media were harvested to measure the secreted antigens of t-PA and PAI-1. No differences in the levels of the proteins were found in the presence or absence of garlic. Control, no garlic; garlic (100), 100  $\mu$ g/ml of odorless garlic; garlic (500), 500  $\mu$ g/ml of odorless garlic. Each column represents the mean with S.E. bar.

fed the normal diet were given a sham operation (sham,  $n = 4$ ), or an *in situ* loop operation (control,  $n = 6$ ), and rats that had been fed the diet containing the garlic powder were given an *in situ* loop implantation (garlic,  $n = 7$ ). The *in situ* loop model for the induction of thrombosis was performed according to methods described elsewhere<sup>20</sup>). Briefly, this model was constructed as follows: a looped polyethylene cannula (10 cm long) filled with heparin (50 U/ml in saline) was inserted in the abdominal aorta by clipping and sewing the artery. The center portion of the cannula (about 2 cm) was exposed from the abdomen to check the recurrence of blood circulation after the clips were removed. Thrombosis was induced inside the cannula due to the lack of endothelium and the disturbance of blood flow through the loop. The cannula was left in the artery for 6 h in the anesthetized rats. All procedures were conducted according to the Guiding Principles for the "Care and Use of Animals in the field of Physiological Science and of the Physiological Society of Japan."

**Measurements of coagulation and fibrinolytic systems.** Blood collection was done from the vena cava of anesthetized rats at the end of the experiment in the presence of sodium citrate (0.32% w/v, final), and then plasma was obtained from the whole blood by centrifugation (3,000 rpm for 10 min at 4°C) within 1 h. Plasma samples were assayed by diagnostic reagents for coagulation and fibrinolytic systems, prothrombin time (PT) (Sanko Junyaku, Tokyo), activated partial thromboplastin time (APTT) (Sanko Junyaku), thrombin-antithrombin III (TAT) complex (SRL, Tokyo), fibrin(ogen) degradation products (FDP) (Daiichi Pure Chemicals, Tokyo) and plasmin-plasmin inhibitor complex (PIC) (Mitsubishi Kagaku Iatron, Tokyo).

**Statistics.** Values are expressed as means  $\pm$  standard error (S.E.). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the Tukey-Kramer test. Differences at  $p < 0.05$  were considered to be significant.

## Results

### *Secretion of t-PA and PAI-1 from HUVECs stimulated by odorless garlic*

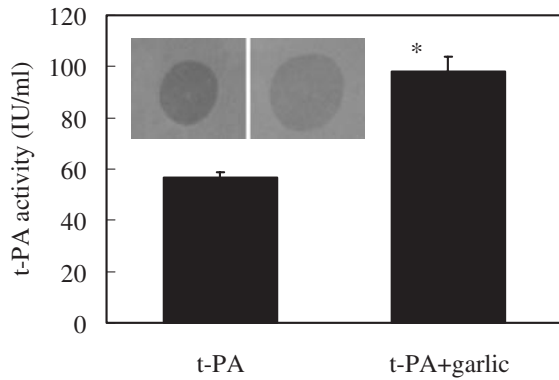
After HUVECs were cultured in the garlic-containing medium for 18 h, no significant changes were found in the secretion of t-PA or PAI-1 into the conditioned medium. Figure 1 shows representative data with the concentration of garlic at 100 and 500  $\mu$ g/ml. When HUVECs were also cultured in the presence of garlic at higher concentrations up to 1 mg/ml, and lower concentrations down to 0.4  $\mu$ g/ml. No differences in t-PA or PAI-1 release were found (data not shown). Hence, it was concluded that the odorless garlic employed in this study did not have any effect on the release of t-PA or PAI-1 from HUVECs.

### *Plasminogen activation activity by t-PA in the presence of odorless garlic*

When t-PA was incubated with garlic on fibrin film, the lysis area of fibrin increased (Fig. 2, inset) as a result of increased degradation of fibrin by t-PA-mediated activation of plasminogen. Based on calibration of t-PA and the lysis zone, the t-PA activity in the presence of garlic increased by 180% as compared with that in the absence of garlic (Fig. 2). Increased plasmin generation by t-PA was also observed in the presence of garlic in a dose-dependent manner by chromogenic assay using plasmin substrate S-2251 (Fig. 3). Kinetic analysis by Lineweaver-Burk plot showed that  $V_{\max}$  did not change but that  $K_m$  decreased in the presence of garlic (Fig. 4), indicating an increase in the affinity of t-PA with plasminogen. The efficacy of enzyme reaction in the presence of garlic was found also to be enhanced as  $k_{\text{cat}}$  increased, resulting in a significant increase in total reactivity, by over 8-fold (Table 1).

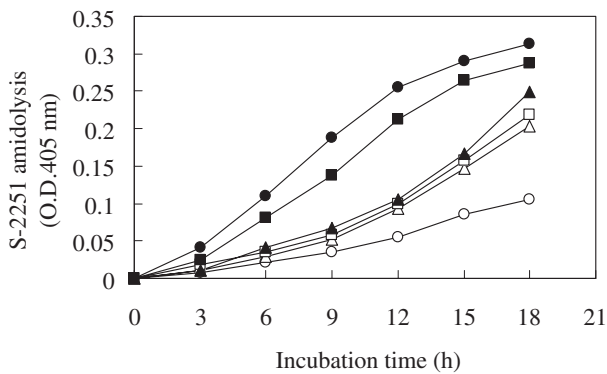
### *Body weight and diet intake of rats administered odorless garlic*

Every rat administered garlic in the diet (in the control and garlic groups) showed a steady gain in body weight



**Fig. 2.** Fibrinolytic Activity of t-PA Evaluated by Fibrin Film.

Inset is a representative set of lysis appearances. The lysis area ( $\text{mm}^2$ ) was converted to t-PA activity (IU/ml). Data were obtained from quadruplicate samples on the same fibrin film. t-PA, t-PA (87.5 IU/ml); t-PA + garlic, t-PA (87.5 IU/ml) + odorless garlic (1 mg/ml). Each column represents the mean with S.E. bar. \* $p < 0.01$ .



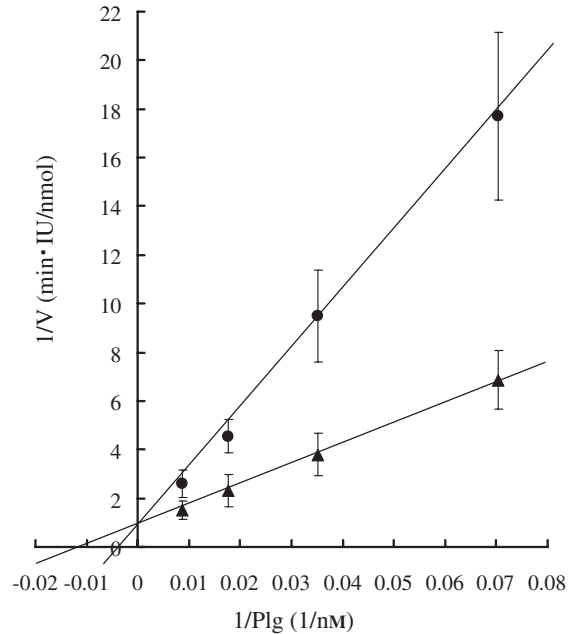
**Fig. 3.** Time Course of S-2251 Amidolysis by t-PA-Mediated Plasminogen Activation.

Plasminogen activation by t-PA with varying concentrations of odorless garlic ( $\bigcirc$ , 0  $\mu\text{g/ml}$ ;  $\triangle$ , 1  $\mu\text{g/ml}$ ;  $\square$ , 10  $\mu\text{g/ml}$ ;  $\blacktriangle$ , 100  $\mu\text{g/ml}$ ;  $\blacksquare$ , 500  $\mu\text{g/ml}$ ;  $\bullet$ , 1,000  $\mu\text{g/ml}$ ) was measured as S-2251 amidolysis. One representative result is shown.

from day 1 to day 15, and no significant differences in body weight were found among the three groups at each day (Table 2). In addition, consumption of the diet with garlic was not significantly different from that of the diet without garlic in the periods of days 1–8 and days 8–15, and the intake of these diets did not change between the former and latter periods (Table 3).

#### Effects of odorless garlic in rat thrombosis model by in situ loop method

Thrombotic states were induced after the recurrence of blood flow in the loop by initiating a blood coagulation system. However, activation of the coagulation system tended to be suppressed in the garlic group, as evaluated by both PT and APTT (Fig. 5A, B). In addition, TAT complex as a result of the generation of active thrombin in the garlic group was found to decrease significantly (Fig. 5C). The levels of FDP,



**Fig. 4.** Kinetics of t-PA in the Presence ( $\blacktriangle$ ) and Absence ( $\bullet$ ) of Odorless Garlic.

Values are expressed as mean  $\pm$  S.E. in triplicate samples in duplicate measurements. Data were analyzed by Lineweaver–Burk plots.

**Table 1.** Kinetic Analysis of Plasminogen Activation by t-PA in the Presence and Absence of Odorless Garlic

	$K_m$ (nM)	$k_{cat}$ ( $\text{s}^{-1}$ )	$k_{cat}/K_m$ ( $\text{s}^{-1} \cdot \text{nM}^{-1}$ )	Increase (fold)
t-PA	222.2	0.217	$9.77 \times 10^{-4}$	1
t-PA + odorless garlic	76.9	0.652	$84.8 \times 10^{-4}$	8.7

The kinetic constants were calculated from Lineweaver–Burk plots (Fig. 4).

**Table 2.** Growth of Rats with and without Odorless Garlic in Diet

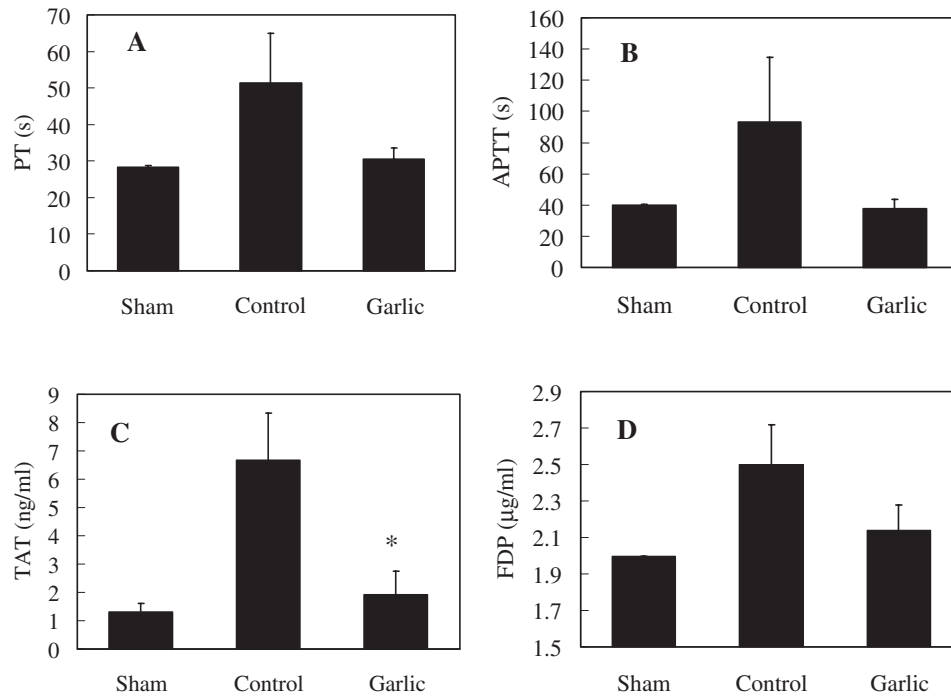
Group	Body weight (g)		
	Day 1	Day 8	Day 15
Sham	$335 \pm 5$	$376 \pm 7$	$416 \pm 11$
Control	$340 \pm 4$	$388 \pm 3$	$433 \pm 8$
Odorless garlic	$340 \pm 4$	$385 \pm 5$	$427 \pm 9$

Values are expressed as mean  $\pm$  S.E. No significant differences were found among the three groups for each day.

**Table 3.** Daily Diet Intake

Group	Consumption (g/d)	
	Days 1–8	Days 8–15
Sham	$26.0 \pm 0.5$	$26.4 \pm 0.6$
Control	$28.4 \pm 0.6$	$28.3 \pm 0.6$
Odorless garlic	$26.9 \pm 0.5$	$27.4 \pm 0.9$

Values are expressed as the mean  $\pm$  S.E. No significant differences were found among the three groups for each period of feeding.



**Fig. 5.** Parameters and Factors in Coagulation System by PT (A), APTT (B), TAT Complex (C), and FDP (D) in Rat Plasma in the Thrombosis Model.

Thrombosis was caused in the loop implanted in the abdominal arteries of rats. Plasmas from sham ( $n = 4$ ), control ( $n = 6$ ), and garlic ( $n = 7$ ) groups were subjected to the various assays. "Garlic" represents odorless garlic. Each column represents the mean with S.E. bar. Significant difference from control and garlic: \* $p < 0.05$ .

which was generated as the thrombus was being degraded, decreased in the garlic group, though not to a statistically significant extent (Fig. 5D).

## Discussion

Several studies have shown that garlic oil or its powder enhanced fibrinolytic activity in humans. This effect appears not to be due to an increase in t-PA secretion or a decrease in PAI-1 secretion from vascular endothelial cells, but rather an enhancement of t-PA activity, since we found that odorless garlic did not stimulate t-PA or PAI-1 secretion in HUVECs (Fig. 1), and that t-PA exhibited increased plasminogen activation in the presence of garlic in both fibrin film and chromogenic substrate assays (Figs. 2, 3, and 4). A similar increase in plasmin generation was observed under low doses of garlic (1, 10, and 100  $\mu\text{g/ml}$ ), but a dose-dependent increase of plasmin was found under high doses of garlic (500 and 1,000  $\mu\text{g/ml}$ ). This unique increase in t-PA activity due to garlic can be attributed to the nature of garlic, which should be revealed in further studies. Kinetic analysis showed a decrease in  $K_m$  and an increase in  $k_{cat}$  in the presence of garlic (Table 1), suggesting that garlic increased the affinity between t-PA and plasminogen for their molecular interaction. Previously, we found that t-PA specifically binds to t-PA receptor (t-PAR) expressed on HUVECs, resulting in considerable enhancement of plasminogen

activation by t-PA, and that t-PAR-bound t-PA increased its affinity for plasminogen. Therefore, t-PAR might induce conformational changes in the t-PA molecule, which perhaps exposes the binding sites for plasminogen more effectively.<sup>21)</sup> Thus, it seems that the active components of garlic might also act as cofactors that make t-PA more accessible to plasminogen by exposing its affinity site for plasminogen more efficiently. The mechanism by which garlic promotes plasminogen activation by t-PA activity remains to be determined in detail, but no such enzymatic enhancement was observed in the rat model as an increase in PIC. The PIC analysis used in our study might not be suitable for the rat, since the levels of PIC were under the detection limit in every sample (data not shown). Though it may be true that garlic has no effect on t-PA activity in rats, we cannot conclude this until a sensitive PIC assay is applied to rat plasma. Thus, at the moment, it remains unclear whether garlic functions as a t-PA enhancer in plasminogen activation in rats.

Since there was no difference in body weight or the intake of diet among the three groups (Tables 2 and 3), toxic, tasty, or unexpected effects of the garlic preparation can be excluded. In addition, microscopic observation of HUVECs showed no morphological changes in cells even under a high concentration of garlic (1 mg/ml) (data not shown), suggesting that garlic is a non-toxic, safe substance in cellular circumstances.

The *in situ* loop model employed in our study induced

thrombus formation by fibrin generation followed by platelet aggregation, and thus it might be suitable for observing the ordinary process of blood clot formation by platelet and coagulation systems, which is frequently caused by endothelial injury<sup>22)</sup> and blood flow disturbance. Though the platelet system was not analyzed, the coagulation factors were consumed in the control group, indicating that both extrinsic and intrinsic pathways were induced, as shown in the prolongation of PT and APTT (Fig. 5A, B). This activation of the coagulation system was confirmed by elevated thrombin activity (as TAT) and FDP (Fig. 5C, D). Since FDP reflects the degradation of fibrin, a significant amount of fibrin clot, though not observed directly in the loop, was presumably formed and subsequently degraded by the fibrinolytic system in the control rats. Under such conditions, the rats administered garlic exhibited significantly reduced TAT and a tendency to decrease FDP, suggesting that garlic considerably attenuated the formation of thrombus in the model. This might essentially explain how odorless garlic prevents pathological fibrin-thrombus formation in addition to platelet aggregation in humans.

The antithrombotic property also refers to relaxing of blood vessels (*i.e.* anti-hypertension), because enlargement of vessel interspace can help blood circulation in occlusion by blood clot. We also measured two vasomotor factors from HUVECs stimulated by garlic. One is the vasoconstriction protein endothelin-1 (ET-1) and the other vasorelaxing substance nitric oxide (NO) which can be measured as endothelial nitric oxide synthase (eNOS). In contrast to the results that garlic caused NO-dependent relaxation and inhibited ET-1-induced constriction in rats,<sup>23)</sup> no significant changes in ET-1 or eNOS expression were found in the presence of garlic (data not shown). However, S-allyl cysteine inhibited NO without changes in eNOS in HUVECs.<sup>24)</sup> Thus the effects seemed to be different in humans and rats, or else they depend on the component of garlic. Our preparation of odorless garlic did not directly regulate these factors in HUVECs. It remains to be analyzed whether garlic can reduce hypertension in animals such as the spontaneously hypertensive rat.

It has not yet been investigated which substances in the garlic preparation were essentially involved in antithrombotic function (such as the anticoagulation effect by decreasing thrombin in the rat and such as the hyperfibrinolytic effect by increasing t-PA activity on fibrin film) evidenced in this study. Though allicin, one of the major components in garlic, possesses several biological activities,<sup>25)</sup> it seems that allicin does not function in promoting t-PA activity, since the garlic preparation contains less allicin in the process of production due to inactivation of alliinase, thus being odorless. However, it was uncertain whether alliin in the diet was converted to allicin after it was taken into the rats. We did not examine other garlic products with and without odor. Thus, it is also uncertain whether the

antithrombotic activity is specific to our garlic preparation. Since garlic possesses a lot of physiological functions, the composition and the amounts of each responsible element in the various functions may differ among species and methods of manufacture. Thus it is natural that the physiological functions of garlic are not homogenous and depend on the amount of active components as well as their combination in a garlic preparation.

Therefore it has not yet been confirmed which component contributes to the antithrombotic activity in the rat model.

In summary, this odorless garlic powder did not stimulate t-PA or PAI-1 in HUVECs, but it promoted plasminogen activation by t-PA *in vitro*. In addition, the garlic strongly reduced coagulation activity, which would prevent blood-clot formation in the rat pathological thrombus model. These results may explain much evidence that garlic and its preparations are beneficial substances for blood and vasculature. Thus, our preparation of odorless garlic was found to be useful as a supplement the mechanism of which is basically characterized as antithrombotic function.

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