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Enhancement of Fibrinolytic Activity of U937 Cells by Malformin A_1

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We have found that malformin A_1, a cyclopentapeptide metabolite of *Aspergillus niger*, enhanced 2.0- to 3.2-fold the ^{125}I-fibrin clot lysis when incubated at 1~10 μM with both U937 cells and blood plasma, both of which were essential to the malformin A_1 action. The effect was inhibited by ε-aminocaproic acid and anti-urokinase serum, but not by anti-tissue-type plasminogen activator IgG, showing that the enhancement was mediated by urokinase-catalyzed plasminogen activation. However, malformin A_1 affected neither cellular urokinase activity nor cell-free reactions involved in the fibrinolytic pathway. Malformin-treated, washed cell had an increased capacity to degrade fibrin in the presence of plasma. These results suggest that malformin A_1 enhances fibrinolytic activity by affecting cell-mediated response to initiate and/or propagate fibrinolytic activity.

The fibrinolytic system is implicated in several pathophysiological proteolytic processes in the blood vessel, including thrombolysis, hemostasis, aneurysm formation, neovascularization, restenosis, and atherosclerosis[1]. This system comprises an inactive proenzyme, plasminogen, which is converted to the active enzyme plasmin by two physiological plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Fibrinolysis is regulated by physiological inhibitors such as α_2-antiplasmin and plasminogen activator inhibitor-1 (PAI-1) at the level of plasmin and plasminogen activators[2]. The aberration of the regulatory system may lead to thrombotic states that are implicated in the pathogenesis of thromboembolic diseases such as myocardial infarction and stroke[2].

During the course of screening to identify agents that stimulate fibrinolytic activity, we have isolated malformin A_1, a disulfide form of *cyclo*(-D-Cys-D-Cys-L-Val-D-Leu-L-Ile-), from a culture of *Aspergillus niger* F7586. Malformin is reported to induce root curvatures and malformations in the plants[3,4], while its effect in mammalian system is unknown. In this paper, we show that malformin A_1 effectively enhances fibrinolytic activity of human monocytoid U937 cells.

Materials and Methods

Materials

The following proteins and chemicals were obtained from the commercial sources: fibrinogen, plasmin and S-2251 from Sigma, USA; goat anti-human tPA IgG from Biopool, Sweden; rabbit anti-human uPA serum and uPA from JCR Pharmaceuticals, Japan; Spectrozyme UK from American Diagnostica, USA; plasminogen from Enzyme Research Laboratories, USA; α_2-antiplasmin from Cosmo Bio, Japan; glutaryl-Gly-Arg-MCA from Peptides Institute, Japan. PAI-1 was purified according to the method of LAWRENCE *et al.*[5] from conditioned medium of human HT1080 cells and re-activated before use. Malformin A_1 was purified from a culture of *A. niger* F7586 that had been isolated from a soil sample collected in Okinawa, Japan. Plactin D was chemically synthesized in our laboratory[6]. Fibrinogen was radioiodinated by the iodine monochloride method[7] to a specific activity of 200~400 cpm/ng. Venous blood was drawn from healthy volunteers in 13 mM sodium citrate and centrifuged to obtain platelet-poor plasma. U937 cells were grown in RPMI-1640 medium supplemented with 10% of fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin as described[6].

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Determination of Fibrinolytic Activity of U937 Cells

^{125}I-Fibrin clot was prepared in 96-well microplate using ^{125}I-fibrinogen (~2000 cpm/μg) as described[8]. The ^{125}I-fibrin plates were washed twice with PBS (20 mM sodium phosphate and 150 mM NaCl, pH 7.4) containing 0.1% Tween 80 and once with PBS, then incubated at 37°C for 1 hour in PBS containing 5 mg/ml gelatin. After removing buffer, each well received 70 μl of PBS containing 2.5 mg/ml gelatin, U937 cells and/or plasma as described for each result. After incubation at 37°C for 1~4 hours, aliquot (35 μl) of the mixture was removed to determine radioactivity released from the ^{125}I-fibrin clot.

Cell-free Assays

All the following assays were performed at 37°C in TBS (50 mM Tris-HCl and 100 mM NaCl, pH 7.4) containing 0.01% Tween 80, and changes in absorbance at 405 nm were measured with an interval of 5~6 minutes using a model 450 microplate reader (Bio-Rad). Pro-uPA activation by plasmin was assayed by incubating 20 nM pro-uPA with 250 pM plasmin and 100 μM Spectrozyme UK. Plasminogen activation by pro-uPA was determined by incubating 50 nM plasminogen with 50 nM pro-uPA and 100 μM S-2251. Plasmin inhibition by α_2-antiplasmin was assayed by incubating 50 nM plasmin with 3~3,000 nM α_2-antiplasmin and 100 μM S-2251. Fibrin degradation by plasminogen and uPA was assayed by incubating ^{125}I-fibrin at 37°C for 3 hours with 20 nM plasminogen and 0.05 U/ml uPA in the absence or presence of 2~50% (v/v) plasma in PBS containing 2.5 mg/ml gelatin. Other determinations shown in Table 1 were carried out essentially as described previously[9~11].

Determination of Cellular uPA Activity

U937 cells (5×10^6 /ml in 100 μl of PBS containing 2.5 mg/ml gelatin) were incubated with the indicated concentrations of plasma at 37°C for 30 minutes in the absence or presence of malformin A_1. Subsequently, cells were washed twice with TBS and suspended with 100 μl of TBS containing 100 μM glutaryl-Gly-Arg-MCA. After incubation at 22°C for 1 hour, the mixture was centrifuged at 2,000×g for 1 minute to obtain supernatant (80 μl), which was then mixed with 920 μl of 3.3% acetic acid to quench the reaction. The fluorescence intensity of the solution was measured using excitation and emission wavelengths of 380 and 480 nm, respectively.

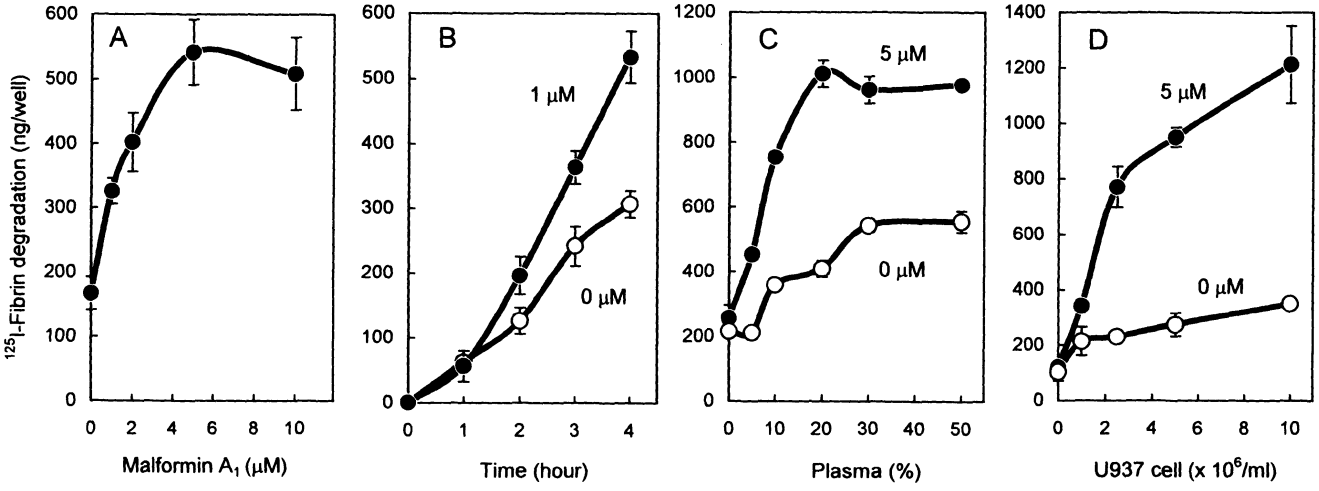
Results and Discussion

When U937 cells were incubated at 37°C for 3 hours on ^{125}I-fibrin plate in the presence of 20% (v/v) blood plasma, fibrin degradation was elevated 2.0- to 3.2- fold by malformin A_1 at a concentration of 1~10 μM (Fig. 1A). The enhancement was not seen by 1 hour, while it was apparent after 2 to 4 hours of incubation (Fig. 1B). When cells were incubated in the absence of plasma, fibrin degradation was not enhanced by malformin (Fig. 1C). The incubation with plasma at >5% was sufficient to obtain the malformin A_1 effect, and the activity remained constant at concentrations ranging from 20 to 50% (Fig. 1C). The malformin A_1 effect was also dependent on the presence of U937 cells. As shown in Fig. 1D, no increase in ^{125}I-fibrin degradation was observed in the absence of U937 cells, while enhancement was prominent at $>2.5\times10^6$ /ml.

The incubation of U937 cells with anti-uPA serum resulted in a decrease in fibrin degradation, while anti-tPA IgG did not inhibit the activity even at a concentration that cause >95% inhibition of tPA activity in a purified system. This observation demonstrated that uPA was a predominant plasminogen activator in U937 cells under these conditions. The malformin A_1-enhanced fibrinolytic activity was markedly inhibited by anti-uPA serum, but not by anti-tPA IgG (Fig. 2A). ε-Aminocaproic acid, which decreases fibrin degradation by inhibiting the binding of plasmin(ogen) to fibrin, also abolished the malformin effect (Fig. 2B). These results suggested that uPA-catalyzed plasminogen activation to plasmin was involved in the malformin A_1 enhancement of fibrinolytic activity. In cell-free systems, however, malformin A_1 did not affect the following reactions concerning fibrinolytic pathway: pro-uPA activation by plasmin, uPA activity, uPA inhibition by PAI-1, plasminogen activation by uPA, plasminogen activation by pro-uPA, plasmin activity, plasmin inhibition by α_2-antiplasmin and fibrin degradation by the plasminogen/uPA system in the absence and presence of varying concentrations of plasma (Table 1).

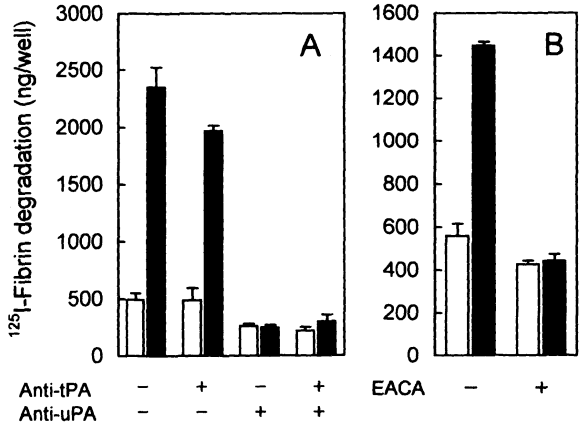
The above observations suggest that malformin A_1 specifically enhance U937 cell-mediated fibrinolytic process involving plasminogen activation by uPA. To address whether proteolytic enzyme(s) and/or other factor(s) secreted by cells account for the malformin A_1 effect, we incubated U937 cells in the absence or presence of malformin and/or plasma and obtained supernatant (conditioned medium) to determine fibrinolytic activity. As shown in Fig. 3A, fibrinolytic activity was low in any conditioned medium, and malformin A_1 effect was not seen. These results suggested that the presence of cells

Fig. 1. Enhancement of U937 cell-mediated ^{125}I-fibrin degradation by malformin A_1.



In the standard assay, ^{125}I-fibrin was incubated for 3 hours with U937 cells (5×10^6 /ml) and plasma (20%, v/v) in the absence or presence of the indicated concentrations of malformin A_1. Where indicated, the incubation conditions were varied. After incubation, the amount of ^{125}I-fibrin degradation products was determined. Each value represents the mean±SD from triplicate determinations.

Fig. 2. Effects of anti-plasminogen activator antibodies and ε-aminocaproic acid on malformin A_1-enhanced ^{125}I-fibrin degradation.



^{125}I-Fibrin was incubated with U937 cells (5×10^6 /ml), plasma (20%, v/v) and malformin A_1 (0 and 5 μM; open and filled bars, respectively) in the absence or presence of 10 μg/ml anti-tPA IgG and/or 110 μg/ml anti-uPA serum (A). In panel B, the reaction was performed in the absence or presence of 20 mM ε-aminocaproic acid (EACA). After incubation for 3 hours, the amount of ^{125}I-fibrin degradation products was determined. Each value represents the mean±SD from triplicate determinations.

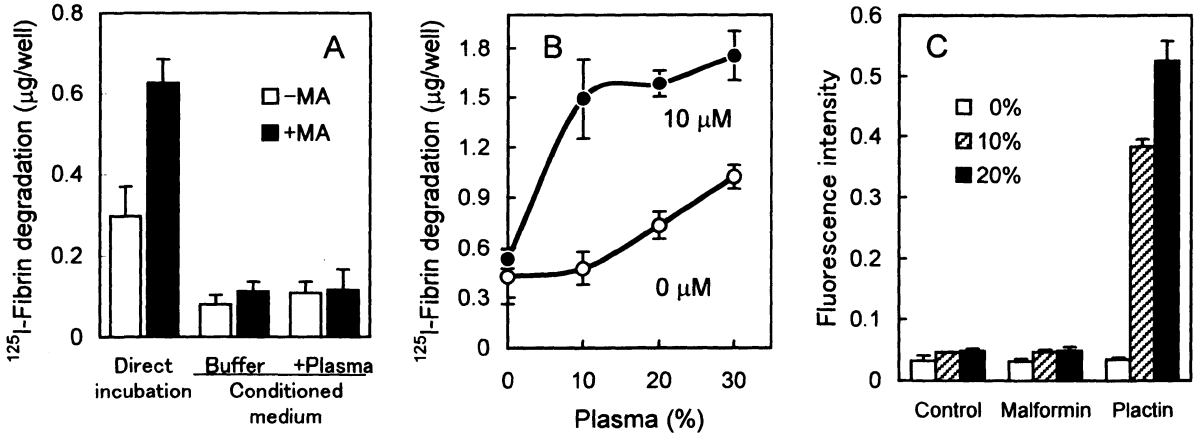
is required to promote both effective fibrinolysis and malformin action. When malformin A_1-treated, washed cells were incubated with ^{125}I-fibrin, a significant increase in fibrin degradation was observed as compared with the incubation with buffer-treated cells (Fig. 3B). In this assay, the malformin A_1 effect was observed even when plasma was not involved during the treatment of cells with the agent, while the presence of plasma in the second incubation was essential to the enhancement of fibrin degradation (Fig. 3B). From these results, it was suggested that the primary event in the malformin A_1 action occurred on the cell. However, the malformin A_1 effect was not sensitive to cycloheximide (data not shown), suggesting that this cell-surface event did not require *de novo* protein synthesis. The most likely event was the increase in cellular uPA activity, but the malformin A_1 treatment of U937 cells resulted in no change in uPA activity either in the presence or absence of plasma (Fig. 3C). This observation was in a striking contrast to the results obtained with plactin D [*cyclo*(-D-Val-L-Leu-D-Leu-L-Phe-D-Arg-)], another cyclic pentapeptide that causes a plasma-dependent increase in uPA activity of U937 cells[6]. Taken together, these results suggest that malformin A_1 enhances fibrin degradation by affecting unidentified cell-mediated response to initiate or propagate fibrinolytic activity.

Recently, several low molecular weight compounds have

Table 1. Effects of malformin A_1 on cell-free reactions on the fibrinolytic system.

Reaction	Activity in the presence of 2~30 μM malformin A_1 (% of control)
Pro-uPA activation by plasmin	98~103
uPA (amidolysis)	96~102
uPA inhibition by PAI-1	84~117
Plasminogen activation by uPA	100~109
Plasminogen activation by pro-uPA	100~103
Plasmin (amidolysis)	93~102
Plasmin inhibition by α_2-antiplasmin	95~107
Fibrin degradation by plasminogen and uPA	
in the absence of plasma	87~103
in the presence of plasma	78~108

Fig. 3. Fibrinolytic activity of U937 cell supernatant (conditioned medium) and malformin A_1-treated U937 cells.



(A) Fibrinolytic activity of conditioned medium. U937 cells (1×10^7/ml) were pretreated at 37°C for 3 hours with buffer or plasma (40%, v/v) in the absence or presence of 10 μM malformin A_1. After centrifugation, supernatant (conditioned medium) was removed and incubated with for 3 hours at a plasma concentration of 20% (v/v) to determine fibrinolytic activity. As controls, ^{125}I-fibrin was also incubated with U937 cells (5×10^6/ml) and plasma (20%, v/v) in the absence or presence of 5 μM malformin A_1 (direct incubation). (B) Fibrinolytic activity of malformin A_1-treated U937 cells. U937 cells (5×10^6/ml) were preincubated in PBS containing 2.5 mg/ml gelatin at 37°C for 1 hour in the absence or presence of malformin A_1 (10 μM). Subsequently, cells were washed and incubated with ^{125}I-fibrin in the presence of the indicated concentrations of plasma for 3 hours to determine fibrinolytic activity. (C) uPA activity of malformin A_1- and plactin D-treated U937 cells. U937 cells were preincubated with the indicated concentrations of plasma at 37°C for 30 minutes in the absence or presence of either malformin A_1 (5 μM) or plactin D (50 μM). Subsequently, cells were washed and incubated with glutaryl-Gly-Arg-MCA at 22°C for 1 hour to determine uPA activity. Similar results were obtained when the preincubation time was varied from 0 to 3 hours. Each value represents the mean±SD from triplicate determinations.

been found to enhance the fibrinolytic system. Some of these are candidates for the development of anti-thrombotic or thrombolytic agents. The actions of such agents are (i) induction of a change in plasminogen conformation resulting in a increased susceptibility to activation[9,11], (ii) enhancement of plasminogen binding to cells and fibrin[8], (iii) inhibition of plasminogen activator inhibitors[10,12], (iv) induction of plasminogen activator synthesis[10,13,14], and (v) increase in uPA activity possibly by enhancement of the conversion of inactive pro-uPA to active uPA[6]. Malformin A_1 affects none of these reactions, and its effect appears to be unique among known agents that enhance fibrinolytic activity.

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