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Triflavin, an Arg-Gly-Asp-containing Peptide, Inhibits Tumor Cell-induced Platelet Aggregation

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In this study, we examined the effect of triflavin, an Arg-Gly-Asp (RGD)-containing snake venom peptide, on human cervical carcinoma (HeLa) cell- and B16-F10 mouse melanoma cell-induced platelet aggregation (TCIPA) in heparinized platelet-rich plasma. TCIPA appears to play an important role in the development of certain experimental tumor metastases. Two ADP-scavenging agents, apyrase (10 U/ml) and creatine phosphate (CP) (5 m*M*)/creatine phosphokinase (CPK) (5 U/ml) completely inhibited B16-F10 TCIPA, but hirudin (5 U/ml) had no effect. In contrast, apyrase and CP/CPK did not inhibit HeLa TCIPA while hirudin completely inhibited it. Furthermore, HeLa cells initially induced platelet aggregation and then blood coagulation at a later stage. In addition, HeLa cells shortened, in a concentration-dependent manner, the recalcification time of normal as well as factor VIII- and IX-deficient human plasma, but did not affect the recalcification time of factor VII-deficient plasma. This suggests that HeLa TCIPA occurs via activation of the extrinsic pathway, probably owing to tumor cell expression of tissue factor-like activity. HeLa cell-induced thrombin generation was confirmed by detection of amidolytic activity towards a chromogenic substrate, S-2238 (H-D-Phe-Pip-Arg-p-NA). Triflavin and GRGDS inhibited, in a dose-dependent manner, TCIPA caused by either cell line. On a molar basis, triflavin was 10,000–30,000 times more potent than GRGDS in this regard. Moreover, monoclonal antibodies raised against glycoprotein (GP) IIb/IIIa complex (i.e., $7E_3$ and AP_2) and against GP Ib (i.e., AP_1) completely inhibited HeLa TCIPA. $7E_3$ and AP_2 inhibited B16-F10 TCIPA by up to 80% whereas AP_1 showed only 30% inhibition of B16-F10 TCIPA. In conclusion, the inhibitory effect of triflavin on HeLa and B16-F10 TCIPA may be mediated principally by the binding of triflavin to the fibrinogen receptor associated with GP IIb/IIIa complex on the platelet surface. However, GP Ib is also involved in HeLa TCIPA as thrombin formation is the key factor in triggering platelet aggregation caused by HeLa cells.

Key words: RGD-containing peptide — Triflavin — Fibrinogen receptor antagonist

Aggregation of host platelets by tumor cells may play an important role in the metastatic process.[1,2] This interaction is proposed to facilitate tumor cell attachment to endothelial cells and subendothelial matrix.[3,4] Ultrastructural studies have demonstrated tumor cell-platelet-endothelial cell interactions in the microvasculature following the intravenous injection of tumor cells.[3,5,6] In addition, platelets seem to secrete permeability factors[7,8] and tumor cell growth factors[9–11] which theoretically should assist in tumor cell penetration of the vessel wall and development of extravascular secondary tumor colonies. The role of platelets in tumor metastasis is also supported by the following observations: (1) injection of tumor cells with high metastatic potential causes a significant decrease in platelet counts *in vivo*[12,13]; (2) isolated tumor cells can cause activation (adhesion, aggregation, and release reaction) of platelets *in vitro*[12,13]; (3) aggregability of platelets in response to specific tumor cell types correlates with metastatic potential *in vivo*[13,14]; (4) drugs that interfere with platelet function or induce thrombocytopenia can also interfere with metastasis.[13,15]

Although the role of platelets in hematogenous metastasis of tumor cells is clear, details of the mechanisms of TCIPA[4] are still unclear. Several investigators have reported different mechanisms through which tumor cells activate platelets. For example, some tumor cells are procoagulant and generate thrombin, as shown by interference with their TCIPA by thrombin inhibitors such as hirudin.[2,16] Some tumor cells release cathepsin B or ADP as documented by inhibition of TCIPA, respectively, by cysteine proteinase inhibitors or ADP scavengers (such as apyrase).[17,18] Platelet membrane surface components may be involved in TCIPA, but their roles have not been elucidated. Although the mechanisms are still unclear,

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[4] Abbreviations: TCIPA, tumor cell-induced platelet aggregation; DMEM, Dulbecco's modified Eagle's medium; GRGDS, Gly-Arg-Gly-Asp-Ser; GRGES, Gly-Arg-Gly-Glu-Ser; FCS, fetal calf serum; GP, glycoprotein; PRP, platelet-rich plasma; PPP, platelet-poor plasma; PBS, phosphate-buffered saline; CP, creatine phosphate; CPK, creatine phosphokinase; CMF-HBSS, Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution.

platelet surface glycoproteins have been shown to play an important role in aggregation, adhesion and surface contact interaction.[19-21]

Recently, many trigramin-like antiplatelet peptides (or disintegrins) have been reported.[22-26] Trigramin, an RGD-containing peptide purified from snake venom, is a specific fibrinogen receptor antagonist.[27,28] Their physicochemical properties, amino acid sequences, and antithrombotic effect have been reported. These peptides all contain the RGD sequence, are rich in cysteine, and bind with high affinity to the surface of platelets. Triflavin, a trigramin-like peptide purified from *Trimeresurus flavoviridis* snake venom,[29,30] is three times more potent than trigramin in inhibiting platelet aggregation. Its primary structure of 70 amino acid residues includes 12 cysteines. It contains the RGD sequence at positions 49–51. Triflavin directly interferes with the interaction of fibrinogen with its receptor associated with the GP IIb/IIIa complex.[30-32]

In this study, we show that human cervical carcinoma (HeLa) cells as well as B16-F10 mouse melanoma cells induce aggregation in human heparinized PRP. However, HeLa cell-induced platelet aggregation occurred via thrombin formation associated with tissue factor-like activity, whereas B16-F10 mouse melanoma cells induced platelet aggregation via ADP release. Furthermore, we examined the effect of triflavin on HeLa cell- or B16-F10 mouse melanoma cell-induced platelet aggregation of human heparinized PRP, and compared its action with that of the synthetic peptide GRGDS and monoclonal antibodies against either GP IIb/IIIa complex or GP Ib.

MATERIALS AND METHODS

Materials *T. flavoviridis* venom was purchased from LATOXAN, France and stored at −20°C. GRGDS was purchased from Peninsula Laboratories, USA. GRGES was synthesized by the Biochemical Institute, College of Medicine, National Taiwan University. Apyrase (grade III); heparin; hirudin (grade IV from leeches); CP and CPK (type I from rabbit muscle); neuraminidase (type X from *Clostridium perfringens*); phospholipase A_2 (from *Laticanda semifasciata*); collagenase (type VII from *Clostridium histolyticum*) and thrombin (from human plasma) were obtained from Sigma Chem. Co., USA. Aprotinin (Trasylol, from bovine lung) was obtained from Boehringer, Germany. Tissue thromboplastin reagent (Simplastin^R Excel standard) was purchased from Organon Teknika, Boxtel, NL. Coagulation factor-deficient human plasmas (deficient in factor VII, VIII, or IX) were obtained from Sigma Chem. Co. Monoclonal antibody 7E3 was kindly donated by Dr. Barry Coller, State University of New York, USA. Antibodies AP_1, AP_2 and AP_3 were the gift of Drs. P. Newman and T. Kunicki, Milwaukee Blood Center, USA. 7E3 and AP_2 were raised against GP IIb/IIIa complex,[33,34] AP_1 against GP Ib[34] and AP_3 against GP IIIa.[34] Cell culture reagents, including fetal calf serum, were from GIBCO.

Purification of triflavin Triflavin was purified from *T. flavoviridis* venom as previously described.[29] The procedure consisted of Fractogel TSK MW-50 gel filtration, CM-Sephadex C-50 column chromatography and gel filtration on Sephadex G-75 and G-50 column. The last step of purification was accomplished on a reverse-phase HPLC C18 column. The purified peptide migrates as a single band on SDS-PAGE, and its molecular mass was estimated to be 7,500 daltons. Its N-terminal sequence was shown to be Gly-Glu-Glu-Cys-Asp……, and it has the Arg-Gly-Asp sequence at residues 49–51 in the carboxy-terminal domain.[30]

Cell culture HeLa cells were obtained from the Institute of Bacteriology, College of Medicine, National Taiwan University. B16-F10 mouse melanoma cells were obtained from the Institute of Preventive Medicine, Taiwan. Both cell lines were grown in DMEM containing 10% FCS and 1% L-glutamine. Cells were passed and harvested for experiments before reaching confluence. Cells viability was determined by trypan blue exclusion and was usually greater than 90%. Cell-free supernatant was prepared from tumor cell suspension by centrifugation at 120*g* for 5 min.

Aggregation studies Human blood was anticoagulated with heparin (final concentration, 2.5 U/ml), and PRP was prepared by centrifugation at 120*g* for 10 min at room temperature. PPP was prepared from the remaining blood by additional centrifugation at 500*g* for 10 min. PRP was adjusted with PPP to contain about 3.0×10^8 platelets per ml. HeLa cells and B16-F10 mouse melanoma cells were detached with EDTA (1 m*M*)/trypsin (0.25%, w/v) and washed thoroughly with CMF-HBSS to remove residual FCS. Cells finally were resuspended in CMF-HBSS. Platelet aggregation of PRP was measured turbidimetrically by a Lumi-aggregometer (Chrono-log). PRP (400 μl) was pre-warmed at 37°C for 2 min in a silicone-treated glass cuvette. Triflavin, monoclonal antibody or peptide was added 1 min before addition of 30 μl of HeLa cells or B16-F10 mouse melanoma cell (1.0×10^6 cells/ml, final concentration). The reaction was allowed to proceed for at least 10 min and extent of aggregation was expressed as a percentage of the control (tumor cells only). The degree of aggregation was expressed as changes in light transmission (ΔT).

Measurement of amidolytic activity Four hundred μl of PRP or PPP (containing 0.38% sodium citrate) supplemented with 2 m*M* $CaCl_2$ was prewarmed at 37°C for 2 min, then triflavin (1 μg/ml) or normal saline was added 1 min before the addition of 50 μl of HeLa cells (5×10^6

cells/ml, final concentration). At various times (0, 2 and 6 min), this aggegation reaction was terminated by adding 50 μl of 50 m*M* EDTA in 50 m*M* sodium citrate (pH 7.5). In some experiments, addition of HBSS buffer in equal volume (instead of HeLa cell suspension) was also performed as the control. The reaction mixtures were centrifuged (1,000*g*) for 10 min at 4°C. The sediments obtained were washed twice with 150 m*M* Tris-HCl buffer (pH 8.4) containing aprotinin (2 μg/ml, final concentration). The sediments finally were resuspended in 500 μl of the same buffer and stored at 4°C before the amidolytic assay.

The chromogenic substrate, H-D-Phe-Pip-Arg-p-NA (S-2238, Kabi Diagnostica, Sweden), which is usually used as a thrombin substrate, was dissolved in distilled water at a concentration of 1 m*M*. The test sample (500 μl) or thrombin (0.01–1 U/ml) was incubated with chromogenic substrate solution (500 μl) for 20 min at 37°C. This reaction was terminated by adding 0.2 ml of 50% acetic acid and the mixture was centrifuged at 1,000*g* for 10 min. The *p*-nitroanilide formation due to hydrolysis of Arg-ester (S-2238) by thrombin was monitored at 405 nm using a spectrophotometer (U3200, Hitachi Ltd., Tokyo). The activity of test samples was expressed in thrombin units.

Measurement of procoagulant activity Procoagulant activity of the tumor cells was measured in terms of plasma recalcification time.[35] PPP was prepared from whole blood, collected from healthy human volunteers and mixed with 3.8% (w/v) sodium citrate (9:1, v/v). In brief, one hundred μl of fresh normal citrated PPP or human plasmas deficient in factors VII, VIII or IX was incubated with 100 μl of cell suspension with various numbers of tumor cells for 2 min at 37°C, and then 100 μl of prewarmed 25 m*M* $CaCl_2$ was added, and the plasma clotting time recorded. Tissue thromboplastin was used in this system as a positive control for activating the extrinsic coagulation pathway.

RESULTS

Platelet aggregation induced by tumor cells HeLa cells and B16-F10 mouse melanoma cells induced platelet aggregation of heparinized human PRP in a concentration-dependent manner (Fig. 1). Both HeLa cells and B16-F10 cells at concentrations of more than 5×10^5 cells/ml caused aggregation. The tracings of aggregation induced by HeLa cells (at $\geq5\times10^6$ cells/ml) were interrupted by delayed fibrin clot formation (Fig. 1a), which is macroscopic. In contrast, B16-F10 cells did not induce fibrin clot formation even at a concentration of 1×10^7 cells/ml. However, at 1×10^6 cells/ml HeLa cells induced only aggregation without concomitant coagulation of PRP 15 min after the addition of cells (Fig. 1a).

Effect of ADP-scavenging agents and hirudin on TCIPA Both cell types were used for all subsequent platelet aggregation studies at a concentration of 1×10^6 cells/ml. B16-F10 cells at this concentration induced near-maximal platelet aggregation (ΔT, 60%) (Fig. 1b). Pretreatment with the ADP-scavenging agents apyrase (10 U/ml, final concentration) or CP/CPK (5 m*M*/5 U/ml) in PRP did not inhibit HeLa TCIPA but completely inhibited B16-F10 TCIPA (Fig. 2b, c). This indicates that ADP is essential for platelet aggregation caused by B16-F10 cells but is not essential for that caused by HeLa cells. Incubation of PRP with hirudin (5 U/ml), a

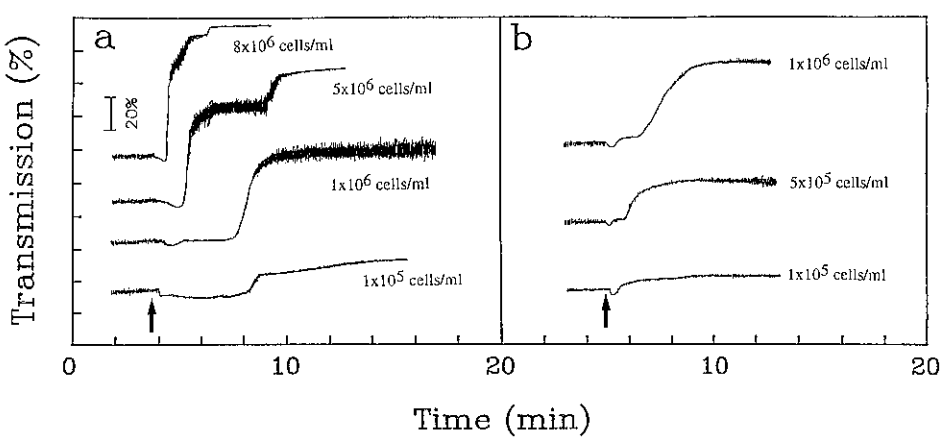

Fig. 1. Concentration-effect relationship of platelet aggregation induced by (a) HeLa cells (b) B16-F10 cells in human heparinized PRP. The arrow indicates the addition of tumor cells. Various numbers of tumor cells were added to heparinized PRP to trigger platelet aggregation, and the degree of aggregation was expressed as changes in light transmission (ΔT).

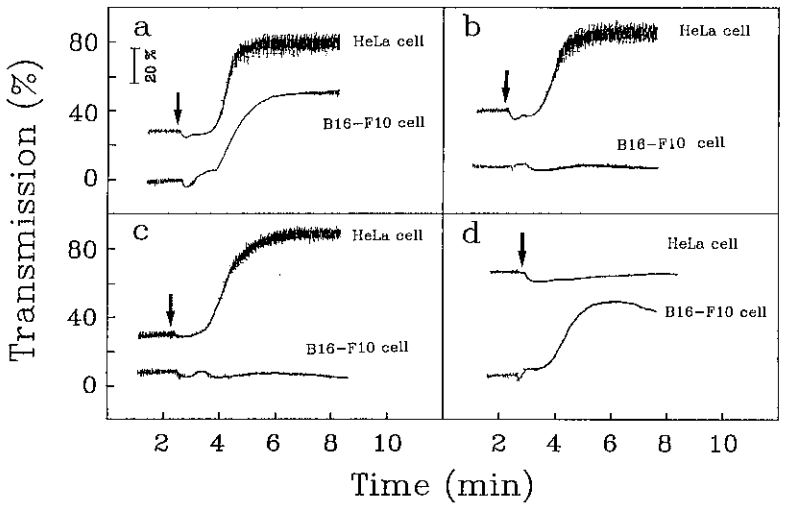

Fig. 2. Effect of apyrase, CP/CPK and hirudin on TCIPA. Heparinized human PRP (400 μl) was preincubated with (a) saline, control; (b) apyrase (10 U/ml); (c) CP (5 mM)/CPK (5 U/ml); (d) hirudin (5 U/ml) at 37°C for 2 min, then 30 μl of tumor cells was added (1×10^6 cells/ml, ↓) to induce platelet aggregation. Platelet aggregation was monitored turbidimetrically and expressed as changes in light transmission (ΔT). For details of the experimental procedure see "Materials and Methods."

thrombin inhibitor, completely inhibited the aggregation response caused by HeLa cells, but did not affect that caused by B16-F10 cells (Fig. 2d). Increasing the concentration of heparin in PRP from 2 U/ml to 4 U/ml markedly prolonged the lag time and resulted in loss of aggregation response in studies with HeLa cells, indicating that HeLa TCIPA is thrombin-dependent. To determine the source of thrombin and ADP generated in mixture of platelets and either HeLa cells or B16-F10 cells, we prepared cell-free supernatant and found that either type of supernatant added in equivalent volume (30 μl) to platelets failed to induce aggregation; this suggests that neither thrombin required for HeLa TCIPA nor ADP required for B16-F10 TCIPA was directly derived from the tumor cells. In addition, pretreatment of either cell type with either trypsin (1 mg/ml, final concentration), neuraminidase (1 U/ml, final concentration), collagenase (1 mg/ml, final concentration) or phospholipase A_2 (25 μg/ml, final concentration) was performed at 37°C for 30 min. We found that only phospholipase A_2 pretreatment almost completely blocked platelet aggregation caused by HeLa cells (data not shown), suggesting that phospholipid is involved in this reaction. On the other hand, preincubation of B16-F10 cells with trypsin (100 μg/ml) reduced TCIPA by 90% (data not shown). Furthermore, pretreatment of B16-F10 cells with apyrase (10 U/ml) at 37°C for 1 min followed by washing did not inhibit B16-F10 TCIPA (data not shown), suggesting that ADP involved in B16-F10 TCIPA is most likely secreted from platelets and that the secretion process may be related to the trypsin-sensitive membrane proteins on B16-F10 cells.

Effect of triflavin on thrombin formation induced by HeLa cells in PRP and PPP D-Phe-Pip-Arg-p-NA (S-2238) is widely used to measure thrombin activity. Fig. 3

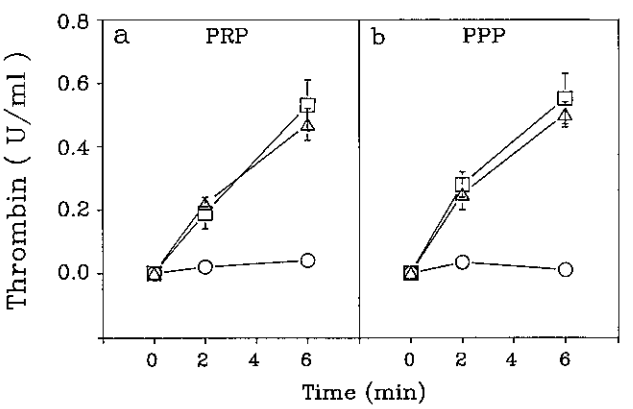

Fig. 3. Effect of triflavin on thrombin generation induced by HeLa cells. PRP or PPP (400 μl, containing 0.38% sodium citrate) supplemented with 2 mM $CaCl_2$ was prewarmed at 37°C for 2 min, then triflavin (1 μg/ml, △) or normal saline (□) was added 1 min before adding 50 μl of HeLa cells (5×10^6 cells/ml). At various incubation time periods, the reaction was stopped by adding 50 μl of 50 mM EDTA in 50 mM citrate solution. Samples for thrombin assay were prepared at various time periods by amidolytic assay and the quantity of thrombin generated in each incubation period was measured by using a chromogenic substrate (S-2238). In the control experiment (○), saline (instead of triflavin) was added to PRP or PPP 1 min before adding HBSS buffer (instead of HeLa cells). The ordinate represents the concentration of thrombin generated. Data are presented as mean±SE (n=3).

shows the thrombin activity generated after the addition of HeLa cells (5×10^6 cells/ml) in PRP or PPP. Thrombin generation induced by HeLa cells was not significantly affected by triflavin (1 μg/ml) in PRP (Fig. 3a). In the absence of platelets (PPP), HeLa cells also in-

duced thrombin formation to a similar extent to that in PRP (Fig. 3b). No significant thrombin generation was detected either in PRP or in PPP to which HBSS was added instead of HeLa cells (Fig. 3a, b). Triflavin thus did not interfere with thrombin generation induced by HeLa cells.

Effect of tumor cells on the recalcification time For the purpose of determining the basis of thrombin generation in HeLa TCIPA, we measured the effect of HeLa cells on plasma recalcification time. As shown in Table I, HeLa cells clearly shortened the recalcification time of normal human citrated PPP in a concentration-dependent manner. Furthermore, HeLa cells equally shortened the recalcification time of mormal, factor VIII- and factor IX-deficient plasmas. In contrast, these cells did not shorten the recalcification time of factor VII-deficient plasma. These data indicate that the procoagulant activity of HeLa cells was through the activation of factor VII in the extrinsic coagulation pathway, and that the cells thus probably have a tissue factor-like activity which is ultimately responsible for inducing platelet aggregation.

Effect of monoclonal antibodies on TCIPA When heparinized human PRP was preincubated with monoclonal antibody against GP IIb/IIIa complex, $7E_3$ (Fig. 4b), AP_2 (Fig. 4c) for 2 min at 37°C, all aggregation responses induced by B16-F10 cells were inhibited in a dose-dependent manner (Fig. 5a). These two monoclonal antibodies did not completely inhibit B16-F10 TCIPA, even at concentrations of up to 100 μg/ml. The maximal inhibitions of B16-F10 TCIPA by AP_2 and $7E_3$ were 83% and 80%, respectively. These findings imply that, in addition to platelet membrane GP IIb/IIIa complex, there are other platelet surface components involved in TCIPA. Therefore, we used monoclonal antibody against platelet membrane GP Ib (AP_1). However, AP_1 only slightly inhibited B16-F10 TCIPA (Fig. 4d and Fig. 5a); maximal inhibition was only about 30% even at 100

Table I. Recalcification Clotting Times of Normal and Coagulation Factor-deficient Plasmas in the Presence of Tumor Cells

	Plasma recalcification time (s)			
	Normal	Factor IX deficient	Factor VIII deficient	Factor VII deficient
HBSS buffer	365±41	>600	>600	310±23
Thromboplastin	13±2	20±4	11±4	302±17
HeLa cells				
5×10^5 cells/ml	82±15	91±9	87±13	325±26
1×10^6 cells/ml	50±11	53±8	61±6	316±22
5×10^6 cells/ml	27±5	22±6	25±5	308±28

Data are presented as means±SE (n=5–7).

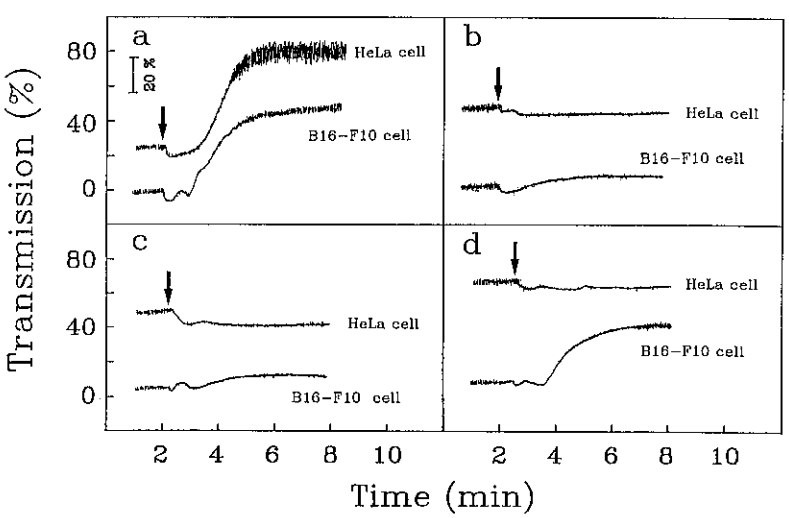

Fig. 4. Effect of anti-GP IIb/IIIa monoclonal antibody, $7E_3$, AP_2 and anti-GP Ib monoclonal antibody AP_1 on TCIPA. Heparinized human PRP (400 μl) was preincubated with (a) saline, control; (b) $7E_3$ (HeLa cells, 20 μg/ml; B16-F10 cells, 100 μg/ml); (c) AP_2 (HeLa cells, 30 μg/ml; B16-F10 cells, 100 μg/ml); (d) AP_1 (HeLa cells, 30 μg/ml; B16-F10 cells, 100 μg/ml) at 37°C for 2 min, followed by addition of tumor cells (1.0×10^6 cells/ml, ↓) to induce platelet aggregation.

μg/ml (Fig. 5a). As described above, HeLa cells induce platelet aggregation via a mechanism involving thrombin formation. Thrombin is known to stimulate platelets via binding to GP Ib on the platelet surface.[36] Platelet aggregation induced by HeLa cells was completely inhibited by preincubation with $7E_3$ (20 μg/ml), AP_2 and AP_1 (both at 30 μg/ml) (Figs. 4, 5b), but the monoclonal antibody against GP IIIa, AP_3 (50 μg/ml), showed no inhibitory effect on HeLa or B16-F10 TCIPA.

Effect of triflavin on TCIPA The binding of fibrinogen to its specific receptor is mainly through the RGD sequence within the fibrinogen molecule.[19] Triflavin, an RGD-containing antiplatelet peptide, was thus anticipated to inhibit TCIPA. Figs. 6 and 7 show that aggregation responses induced by B16-F10 cells or HeLa cells were inhibited in a dose-dependent fashion by triflavin (0.1–1.2 μg/ml; 13–160 n*M*) or GRGDS (0.1–1.0 mg/ml; 0.2–2.0 m*M*). GRGES (4 m*M*), however, had no significant effect (Fig. 6d). Pretreatment of platelets with triflavin (0.4 μg/ml; 53 n*M*) or GRGDS (0.6 mg/ml; 1.2 m*M*) completely inhibited HeLa TCIPA (Figs. 6b, c and 7). Maximal inhibition of B16-F10 TCIPA was about 80% by triflavin or GRGDS (Fig. 7). On a molar basis, triflavin (IC_{50}, 80 n*M*) is 10,000-fold more potent than GRGDS (IC_{50}, 0.81 m*M*) on B16-F10 TCIPA and about 30,000-fold more potent than GRGDS (IC_{50}, 8 n*M* vs. 0.31 m*M*) on HeLa TCIPA.

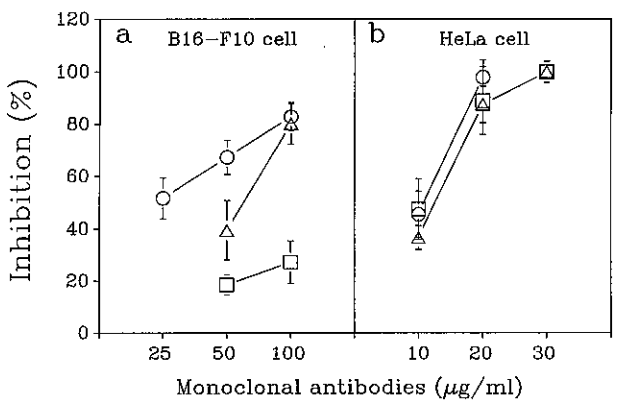

Fig. 5. Effect of various concentrations of anti-GP IIb/IIIa complex monoclonal antibody $7E_3$ (○), AP_2 (△) and anti-GP Ib monoclonal antibody AP_1 (□) on platelet aggregation induced by (a) B16-F10 cells, (b) HeLa cells. The data are presented as mean±SE (n=4). Experimental proceduces: see the legend to Fig. 2.

DISCUSSION

It is well known that some tumor cells induce platelet aggregation *in vitro* and that various mechanisms are involved in this phenomenon.[16] These aggregation reactions may be involved in hematogenous metastatic processes.[16] Gasic *et al.*[12] and Kohga *et al.*[37] have reported that inhibition of platelet aggregation induced by tumor cells and suppression of pulmonary metastasis induced by intravenous injection of tumor cells are closely related. Moreover, Kinjo[38] observed by electron microscopy that most of the AH130 cell emboli in lung were closely associated with platelet clumps. Furthermore, platelet aggregation inhibitors such as aspirin[12] or dipyridamole[39] have been used to prevent experimental blood-borne metastasis. In this study, HeLa cells produced a delayed TCIPA following a lag phase which, in turn, was dependent upon the concentration of tumor cells added. HeLa cells also induced clot formation at final cell concentrations over 5×10^6 cells/ml (Fig. 1a). This effect

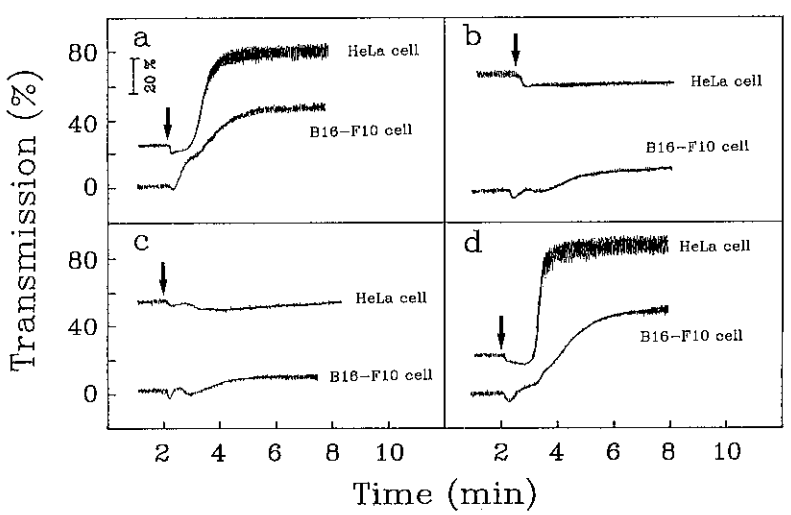

Fig. 6. Effect of triflavin, GRGDS and GRGES on TCIPA. Heparinized human PRP (400 μl) was preincubated with (a) saline, control; (b) triflavin (HeLa cells, 0.4 μg/ml, 0.052 μ*M*; B16-F10 cells, 1.0 μg/ml; 0.13 μ*M*); (c) GRGDS (HeLa cells, 0.6 mg/ml, 1.2 m*M*; B16-F10 cells, 1.0 mg/ml, 2 m*M*); (d) GRGES (4 m*M*) at 37°C for 2 min, followed by addition of tumor cells (1.0×10^6 cell/ml, ↓) to induce platelet aggregation.

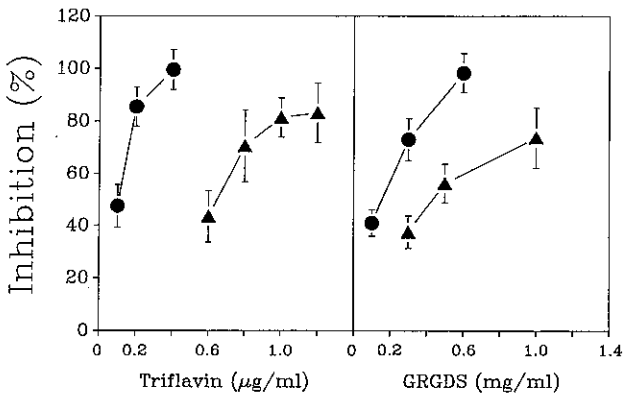

Fig. 7. Effect of various concentrations of triflavin and GRGDS on HeLa cell (●), and B16-F10 cell (▲) TCIPA. The data are presented as mean±SE (n=4). For experimental procedure, see the legend to Fig. 6.

always followed the initial phase of aggregation, since platelet aggregation requires less thrombin than does fibrin formation. In contrast, B16-F10 cells at final concentrations greater than 5×10^5 cells/ml caused platelet aggregation that was not followed by clot formation (Fig. 1b). HeLa cells were generally a more potent inducer of platelet aggregation in optimally heparinized PRP (2.5 U/ml), although heparin at higher concentration (5 U/ml) completely inhibited HeLa TCIPA.

We used selective inhibitors to investigate the mechanisms of B16-F10 cells and HeLa TCIPA and also examined the inhibitory effects of the antiplatelet peptide triflavin and synthetic peptide GRGDS. Our studies show that B16-F10 TCIPA is ADP-dependent and that thrombin is probably not involved. B16-F10 TCIPA was abolished by treatment of tumor cells with trypsin. B16-F10 cells thus probably activate platelets by a direct interaction between platelets and a trypsin-sensitive protein domain on the surface of B16-F10 cells. However, this needs confirmation.

Furthermore, our studies show that HeLa TCIPA is a thrombin-dependent reaction (Fig. 2). As reported previously, some TCIPAs occur via generation of thrombin by activation of the blood-coagulation system.[35, 37, 40-42] For example, NCG human neuroblastoma cells,[41] human carcinoma lines Colo 205 and Colo 397[42] depend upon thrombin generated via the expression of tissue factor-like activity. During the lag period of platelet aggregation the accumulation of sufficient thrombin is required to trigger aggregation. This is consistent with the observation that thrombin inhibitors prolonged the lag period in dose-dependent manner, but did not reduce the maximal response of aggregation once platelets began to aggregate.[35, 43] The shortening of recalcification time by HeLa cells disclosed the procoagulant activity of HeLa cells (Table I). In general, at least three different types of tumor cells procoagulants have been described: (a) a factor VII-dependent tissue factor-like substance[36, 41, 42]; (b) proteinases activating factor X to Xa[44]; and (c) a phospholipid surface for prothrombinase complex formation.[45] Our studies of HeLa cells using coagulation factor-deficient plasma revealed procoagulant activity dependent upon the presence of factor VII but not factor VIII or IX. In this regard, tissue thromboplastin behaves very much like HeLa cells. This result suggests that HeLa TCIPA is mediated by thrombin generated through the tissue factor-like activity expressed on the tumor cell surface.[41] We found that HeLa TCIPA was inhibited by phospholipase A_2 pretreatment. This may imply that phospholipids are required for the expression of tissue factor-like activity. We have also demonstrated that the amount of thrombin generated in PPP following addition of HeLa cells was almost equal to that in PRP, indicating that thrombin generation by HeLa cells was independent of the presence of platelets.

We studied the effects of monoclonal antibodies, the anti-GP Ib antibody AP_1 and the anti-GP IIb/IIIa complex antibodies AP_2 and $7E_3$, to clarify the involvement of platelet surface glycoproteins in TCIPA.[46, 47] It is still unknown how platelet membrane glycoproteins are involved in the TCIPA. We found that both AP_1 and $7E_3$ or AP_2 completely inhibited HeLa TCIPA. These results are also consistent with the observation by Kitagawa *et al.*[21] that colon adenocarcinoma (M7609) TCIPA through thrombin generation is completely inhibited by anti-GP Ib (TM60) and anti-GP IIb/IIIa complex (TM83) antibodies. Nevertheless, $7E_3$ and AP_2 did not completely inhibit B16-F10 TCIPA (residual aggregation, 20%) and AP_1 showed less inhibition. The combination of two types of antibodies (i.e., AP_1 and AP_2) was shown to inhibit B16-F10 TCIPA almost completely. Therefore, both GP Ib and GP IIb/IIIa complex may be involved in the thrombin-dependent (HeLa cells) and thrombin-independent (B16-F10 cells) interaction between platelets and tumor cells. As described above, HeLa TCIPA occurs via a mechanism involving thrombin generation, so that its mechanism is similar to thrombin-induced platelet aggregation, whereas GP Ib is the receptor for thrombin and the GP IIb/IIIa complex is the binding site for fibrinogen after platelet activation by thrombin.[36] Therefore, it is reasonable that monoclonal antibodies against either GP Ib and GP IIb/IIIa complex are capable of inhibiting HeLa TCIPA completely. However, the mechanism by which ADP is released in the interaction between platelets and tumor cells is not clear. We found that trypsin-sensitive protein(s) on the B16-F10 cell surface may play a crucial role in mediating TCIPA. The

component on the platelet surface which interacts with the trypsin-sensitive protein is unknown.

It is well established that the platelet receptor recognition site on human fibrinogen involves the RGD sequence.[48] RGD is also present in two other proteins which mediate platelet-adhesive reactions, i.e., fibronectin and von Willebrand factor (vWF).[49,50] Therefore, peptides containing the RGD sequence can partially or fully inhibit fibrinogen binding to its specific receptor associated with GP IIb/IIIa complex. As expected, both triflavin and GRGDS completely inhibited HeLa TCIPA. However, neither triflavin nor GRGDS completely inhibited B16-F10 TCIPA (residual aggregation, 20%) (Figs. 6 and 7). $7E_3$ and AP_2 also showed similar results with B16-F10 TCIPA. When comparing the IC_{50} values on a molar basis, triflavin is 10,000–30,000-fold more potent than GRGDS in inhibiting TCIPA. In contrast, no significant inhibition of platelet aggregation was observed with the control peptide GRGES. These results suggest similar inhibitory mechanisms on TCIPA between triflavin and anti-GP IIb/IIIa monoclonal antibody, through the blockade of fibrinogen binding to its receptor on platelet surface membrane. Triflavin did not interfere with thrombin generation induced by HeLa cells in PRP or PPP (Fig. 3). In addition, triflavin did not affect the shortening of clotting time in normal PPP caused by HeLa cells. Triflavin thus acts directly on platelets rather than on plasma coagulation factors.

In our previous studies, we showed that triflavin inhibited B16-F10 cell lung colonization in C57BL/6 mice in a dose-dependent manner.[51] Because activation of platelets is required for lung colonization by B16-F10 cells,[13] inhibition of B16-F10 TCIPA by triflavin may be responsible for the inhibition of tumor metastasis by this peptide.

This study shows that triflavin, an RGD-containing peptide, inhibits platelet aggregation caused by two types of tumor cells with different mechanisms. Triflavin may block the common pathway of platelet aggregation, i.e., the binding of fibrinogen to GP IIb/IIIa complex of the activated platelets, resulting in inhibition of TCIPA. If TCIPA plays an important role in promoting the metastatic process *in vivo*, triflavin might be considered as an adjuvant agent in prevention of tumor cell metastases.

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REFERENCES

1) Karpatkin, S. and Pearlstein, E. Role of platelets in tumor cell metastasis. *Ann. Int. Med.*, **95**, 636–641 (1981).
2) Gasic, G. J. Role of plasma, platelets and endothelial cells in tumor metastasis. *Cancer Metastasis Rev.*, **3**, 99–116 (1984).
3) Crissman, J. D., Hatfield, J. S., Harkins, C., Sloane, B. F. and Monn, K. V. Arrest and extravasation of B16 amelanotic melanoma in murine lungs. *Lab. Invest.*, **53**, 470–478 (1985).
4) Menter, D. G., Hatfield, J. S., Harkins, C., Sloane, B. F., Taylor, J. D., Crissman, J. D. and Honn, K. V. Tumor cell-platelet interaction *in vitro* and their relationship to *in vivo* arrest of hematogenously circulating tumor cells. *Clin. Exp. Metastasis*, **5**, 65–78 (1987).
5) Jones, D. S., Wallare, A. C. and Frazer, Z. E. Sequence of events in experimental metastasis of Walker-256 tumor: light, immunofluorescent and electron microscopic observations. *J. Natl. Cancer Inst.*, **46**, 493–504 (1971).
6) Chew, E. C. and Wallare, A. C. Demonstration of fibrin in early stages of experimental metastasis. *Cancer Res.*, **36**, 1904–1909 (1976).
7) Packham, M. A., Nishizawa, E. E. and Mustard, J. F. Response of platelets to tissue injury. *Biochem. Pharm.*, **17** (Suppl.), 171–184 (1968).
8) Nachman, R. U., Weksler, B. and Ferris, B. Characterization of human platelet vascular permeability-enhancing activity. *J. Clin. Invest.*, **51**, 549–556 (1972).
9) Eastment, C. T. and Sirbasku, D. A. Platelet derived growth factor for a hormone responsive rat mammary tumor cell line. *J. Cell Physiol.*, **97**, 17–28 (1978).
10) Hara, Y., Steiner, M. and Baldini, H. G. Platelet as a source of growth-promoting factor(s) for tumor cells. *Cancer Res.*, **40**, 1212–1216 (1980).
11) Kepner, N. and Lipton, A. A mitogenic factor for transformed fibroblasts from human platelets. *Cancer Res.*, **41**, 430–432 (1981).
12) Gasic, G. J., Casic, T. B., Galanti, N., Johnson, T. and Murphey, S. Platelet tumor cell interactions in mice. The role of platelet in the spread of malignant diseases. *Int. J. Cancer*, **11**, 704–718 (1973).
13) Mehta, P. Potential role of platelets in the pathogenesis of tumor metastasis. *Blood*, **63**, 55–63 (1984).
14) Pearlstein, E., Salk, P. L., Yogeeswaran, G. and Karpatkin, S. Correlation between spontaneous metastatic potential, platelet-aggregating activity of cell surface extracts, and cell surface sialylation in 10 metastatic-variant derivatives of a rat renal sarcoma cell line. *Proc. Natl. Acad. Sci. USA*, **77**, 4336–4339 (1980).
15) Honn, K. V., Busse, W. D. and Sloane, B. F. Prostacyclin and thromboxanes. Implications for their role in tumor cell

metastasis. *Biochem. Pharmacol.*, **32**, 1–11 (1983).
16) Jamieson, G. A., Bastide, E. and Ordinas, A. Interaction of platelets and tumor cells. *In* "Platelets in Biology and Pathology," Vol. III, ed. E. MacIntyre and J. Gordon (1987). Elsevier, Amsterdam.
17) Sloane, B., Dunn, J. and Honn, K. Lysosomal cathepsin B: correlation with metastatic potential. *Science*, **212**, 1151–1153 (1981).
18) Bastida, E., Ordinas, A., Giardina, S. and Jamieson, G. Differentiation of platelet-aggregating effects of human tumor cell lines based on inhibitor studies with apyrase, hirudin, and phospholipase. *Cancer Res.*, **42**, 4348–4352 (1982).
19) Plow, E., Ginsberg, M. H. and Marguerie, G. A. Expression and function of adhesive protein on the platelet surface. *In* "Biochemistry of Platelets," ed. D. R. Philips and M. S. Schuman, pp. 225–256 (1986). Plenum Press, New York.
20) Ugen, K. E., Mahalingam, M., Klein, P. A. and Kao, K. J. Inhibition of tumor cell-induced platelet aggregation and experimental tumor metastasis by the synthetic Gly-Arg-Gly-Asp-Ser peptide. *J. Natl. Cancer Inst.*, **80**, 1461–1466 (1988).
21) Kitagawa, H., Yamamoto, N., Yamamoto, K., Tanoue, K., Kosaki, G. and Yamazaki, H. Involvement of platelet membrane glycoprotein Ib and glycoprotein IIb/IIIa complex in thrombin-dependent and -independent platelet aggregations induced by tumor cells. *Cancer Res.*, **49**, 537–541 (1989).
22) Gan, Z. R.. Gould, R. J., Jacobs, J. W., Friedman, P. A. and Polokoff, M. A. Echistatin, a potent platelet aggregation inhibitor from the venom of the viper, *Echis carinatus*. *J. Biol. Chem.*, **263**, 19827–19832 (1988).
23) Chao, B. H., Jakubowaski, J. A., Savage, B., Chow, E. P., Marzec, L. M., Harkeri, L. A. and Maraganore, J. M. *Agkistrodon piscivorus piscivorus* platelet aggregation inhibitor: a potent inhibitor of platelet activation. *Proc. Natl. Acad. Sci. USA*, **86**, 8050–8054 (1989).
24) Shebuski, R. J., Ramjit, D. Bencen, G. H. and Polokoff, M. A. Characterization and platelet inhibitory activity of Bitistatin, a potent arginine-glycine-aspartic acid-containing peptide from the venom of the viper *Bitis arietans*. *J. Biol. Chem.*, **264**, 21550–21556 (1989).
25) Huang, T. F., Wang, W. J., Teng, C. M. and Ouyang, C. Mechanism of action of the antiplatelet peptide, antin, from *Bitis arietans* venom. *Biochim. Biophys. Acta*, **1074**, 144–150 (1991).
26) Huang, T. F., Liu, C. Z., Ouyang, C. and Teng, C. M. Halysin, an Arg-Gly-Asp containing snake venom peptide, inhibits platelet aggregation by acting as fibrinogen receptor antagonist. *Biochem. Pharmacol.*, **42**, 1209–1219 (1991).
27) Huang, T. F., Holt, J. C., Lukasiewicz, H. and Niewiarowski, S. Trigramin, a low molecular weight peptide inhibiting fibrinogen interaction with platelet receptors expressed on glycoprotein IIb-IIIa complex. *J. Biol. Chem.*, **262**, 16157–16163 (1987).
28) Huang, T. F., Holt, J. C., Kirby, E. P. and Niewiarowski, S. Trigramin: primary structure and its inhibition of von Willebrand factor binding to glycoprotein IIb/IIIa complex on human platelet. *Biochemistry*, **28**, 661–666 (1989).
29) Huang, T. F., Sheu, J. R. and Teng, C. M. A potent antiplatelet peptide, triflavin from *Trimeresurus flavoviridis* snake venom. *Biochem. J.*, **277**, 351–357 (1991).
30) Huang, T. F., Sheu, J. R., Teng, C. M., Chen, S. W. and Liu, C. S. Triflavin, an antiplatelet Arg-Gly-Asp-containing peptide, is a specific antagonist of platelet membrane glycoprotein IIb/IIIa. *J. Biochem.*, **109**, 328–334 (1991).
31) Huang, T. F., Sheu, J. R. and Teng, C. M. Mechanism of action of a potent antiplatelet peptide, triflavin from *Trimeresurus flavoviridis* snake venom. *Thromb. Haemostasis*, **66**, 489–493 (1991).
32) Sheu, J. R., Teng, C. M. and Huang, T. F. Triflavin, an RGD-containing antiplatelet peptide, binds to GPIIIa of ADP-stimulated platelets. *Biochem. Biophys. Res. Commun.*, **189**, 1236–1242 (1992).
33) Coller, B. S. A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotin IIb/IIIa complex. *J. Clin. Invest.*, **76**, 101–108 (1985).
34) Kunicki, T. J. Organization of glycoproteins within the platelet plasma membrane. *In* "Platelet Membrane Glycoproteins," ed. J. N. George, A. T. Nurden and D. R. Phillips, pp. 87–103 (1985). Plenum Press, New York.
35) Pearlstein, E., Ambrogio, C., Gasic, G. and Karpatkin, S. Inhibition of the platelet aggregating activity of two human adenocarcinomas of the colon and an anaplastic murine tumor with a specific thrombin inhibitor dansyl-arginine N-(3-ethyl-1-5-pentanediyl) amide. *Cancer Res.*, **41**, 4535–4539 (1981).
36) Clemetson, K. J. Glycoproteins of the platelet plasma membrane. *In* "Platelet Membrane Glycoproteins," ed. J. N. George, A. T. Nurden and D. R. Philips, pp. 51–85 (1985). Plenum Press, New York.
37) Kohga, S., Kinjo, M., Tanaka, K., Ogawa, H., Ishihara, M. and Tanaka, N. Effect of 5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2,-1]pyridine hydrochloride (Ticlopidine), a platelet aggregation inhibitor, on blood-borne metastasis. *Cancer Res.*, **41**, 4710–4714 (1981).
38) Kinjo, M. Lodgement and extravasation of tumor cells in blood-borne metastasis; an electromicroscope study. *Br. J. Cancer*, **38**, 293–301 (1978).
39) Gastpar, H. Platelet-cancer cell interaction in metastasis formation: a possible therapeutic approach to metastasis prophylaxis. *J. Med.*, **8**, 103–114 (1977).
40) Tohgo, A., Tanaka, N. G. and Ogawa, H. Platelet aggregating activities of metastasizing tumor cells. II. Platelet aggregation as resulting from thrombin generation by tumor cells. *Invasion Metastasis*, **5**, 96–105 (1985).
41) Esumi, N., Todo, S. and Imashu, K. U. Platelet aggregating activity mediated by thrombin generation in the NCG

human neuroblastoma cell line. *Cancer Res.*, **47**, 2129–2135 (1987).
42) Scarlett, J. D., Thurlow, P. J., Connellan, J. M. and Louis, C. J. Plasma-dependent and -independent mechanisms of platelet aggregation induced by human tumor cell lines. *Thromb. Res.*, **46**, 715–726 (1987).
43) Tohgo, A. Tanaka, N. and Ogawa, H. Platelet aggregating activities of metastasizing tumor cells. II. Variety of the aggregation mechanisms. *Invasion Metastasis*, **4**, 134–145 (1984).
44) Gordon, S. G. and Cross, B. A. A factor X-activating cysteine protease from malignant tissue. *J. Clin. Invest.*, **67**, 1665–1671 (1981).
45) Van Dewater, L., Tracy, P. B., Aronson, D., Mann, K. G. and Dvorak, H. F. Tumor cells generation of thrombin via functional prothrombinase assembly. *Cancer Res.*, **45**, 5521–5525 (1985).
46) Bastida, E., Almirall, L. and Ordinas, A. Tumor cell-induced platelet aggregation is a glycoprotein-dependent and lipoxygenase-associated process. *Int. J. Cancer*, **39**, 760–763 (1987).
47) Grossi, I. M., Fitzgerald, L. A., Kendall, A., Taylor, J. D., Sloane, B. F. and Honn, K. V. Inhibition of human tumor cell-induced platelet aggregation by antibodies to platelet glycoprotein Ib and IIb/IIIa. *Proc. Soc. Exp. Biol. Med.*, **186**, 378–383 (1987).
48) Gartner, T. K. and Bennett, J. S. The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets. *J. Biol. Chem.*, **260**, 11891–11911 (1985).
49) Titani, K., Takio, K., Ericsson, L. H., Wade, R. D., Ashida, K., Walsh, S. A., Chopek, M. W., Sadler, J. E. and Fujikawa, K. Amino acid sequence of human von Willebrand factor. *Biochemistry*, **25**, 3171–3184 (1986).
50) Pierschbacher, M. D. and Ruoslahti, E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature*, **309**, 30–33 (1984).
51) Sheu, J. R., Lin, C. H., Chung, J. L., Teng, C. M. and Huang, T. F. Triflavin, an Arg-Gly-Asp-containing antiplatelet peptide inhibits cell-substratum adhesion and melanoma cell-induced colonization. *Jpn. J. Cancer Res.*, **83**, 885–893 (1992).