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Triflavin, an Arg-Gly-Asp-containing Antiplatelet Peptide Inhibits Cell-substratum Adhesion and Melanoma Cell-induced Lung Colonization

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Triflavin, an Arg-Gly-Asp (RGD) containing peptide purified from *Trimeresurus flavoviridis* snake venom, inhibits human platelet aggregation by blocking fibrinogen binding to fibrinogen receptors associated with glycoprotein IIb/IIIa complex. In this study, we show that triflavin (1–30 μg/mouse) inhibits B16-F10 melanoma cell-induced lung colonization in C57BL/6 mice in a dose-dependent manner. *In vitro*, triflavin dose-dependently inhibits adhesion of B16-F10 melanoma cells to extracellular matrices (ECMs; i.e., fibronectin, fibrinogen, vitronectin, and collagen type I). Triflavin is approximately 600–800 times more potent than GRGDS at inhibiting cell adhesion. In addition, triflavin dose-dependently inhibits B16-F10 cell-induced platelet aggregation. These results imply that the inhibitory effect of triflavin on the adhesion of tumor cells to ECMs (e.g., fibronectin, vitronectin and collagen type I) and/or tumor cell-induced platelet aggregation may be partially responsible for its antimetastatic activity in C57BL/6 mice.

Key words: RGD-containing peptide — Tumor cell metastasis — Extracellular matrix

Tumor cell metastasis is a complex process which may be divided into a number of distinct steps, several of which involve the traversal of extracellular matrix barriers.[1] In this process, tumor cell surface components may play an important role.[2,3] The initial and most important adhesion event in tumor cell metastasis is adhesion to the vascular endothelium.[4,5] Following adhesion of tumor cells to the endothelial cell surface, endothelial cell retraction is often observed,[6] resulting in the exposure of subendothelial basement membrane, which contains a variety of adhesive proteins including laminin, type IV and type V collagen, vitronectin and heparin sulfate proteoglycan. Adhesion of tumor cells to these basement membrane components is also an important step of the metastatic pathway. Recently, a family of cell surface adhesion receptors termed "cytoadhesins"[7] or "integrins"[8] has been described. These glycoproteins share similar heterodimeric structures consisting of α and β subunits noncovalently associated in a 1:1 ratio[9,10] and are primarily responsible for the attachment of cells to the extracellular matrix. Several reports show that the interaction of integrins with adhesion proteins is at least partially mediated by binding of integrin to the short hydrophilic amino acid sequence arginine-glycine-aspartic acid (RGD) within adhesion proteins.[11,12] We now know that RGD is present in a number of extracellular adhesion proteins including fibrinogen, vitronectin, type I collagen, thrombospondin, and von Willebrand factor (vWF). For example, a key cell-binding site of fibronectin has been identified as RGD; synthetic RGD peptides block the adhesion of cells to fibronectin in a competitive, reversible fashion.[11,13,14] Preincubation of tumor cells with the tetrapeptide RGDS causes a significant reduction in metastatic efficacy.[15] RGDS may thus affect the binding of tumor cells to one of the RGD-containing adhesion molecules. Furthermore, a heterodimeric adhesion receptor from human melanoma cells and endothelial cells mediates the attachment of these cells to immobilized vitronectin, vWF, and fibrinogen, and RGD-containing peptides block this adhesion.[10,16,17]

On the other hand, aggregation of host platelets by circulating tumor cells may play an important role in the process of metastasis by some types of tumor cells.[18] This interaction of host platelets with circulating tumor cells is proposed to facilitate tumor cell attachment to endothelial cells and subendothelial matrix.[19]

Recently, many trigramin-like antiplatelet peptides have been reported.[20–25] Trigramin, an RGD-containing peptide purified from venom of *Trimeresurus gramineus*, is a specific fibrinogen receptor antagonist with a high binding affinity (K_d, 10 n*M*) for platelet fibrinogen receptors.[26,27] The physicochemical properties, amino acid sequence, and antithrombotic effect of these trigramin-like peptides have been revealed.[20–25] These peptides all contain RGD, are rich in cysteine, and bind with high affinity to integrins on the surface of platelets and other cells. Triflavin is a trigramin-like antiplatelet peptide purified from *Trimeresurus flavoviridis* snake venom.[28,29] Its primary structure of 70 amino acid residues includes 12 cysteines.[30] It contains RGD in positions 49–51. Interestingly, the sequences of these trigramin-like peptides are highly conserved. Indeed, triflavin has 68%

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sequence identity with trigramin.[30] However, the IC_{50} of triflavin is 3-fold lower than that of trigramin in inhibiting platelet aggregation. Triflavin directly interferes with the interaction of fibrinogen with its specific receptor associated with the glycoprotein IIb/IIIa complex.

In this study, We describe the inhibitory effect of triflavin on the adhesion of B16-F10 mouse melanoma cells to ECMs[4] (e. g., fibronectin, fibrinogen, vitronectin, laminin, collagen type I and type IV) and B16-F10 cell-induced platelet aggregation. We also show that triflavin dose-dependently inhibits B16 cell-induced lung colonization in C57BL/6 mice. Therefore, we suggest that its antimetastatic activity may be related to its inhibitory effect on the adhesion of melanoma cells to these ECMs and B16 cell-induced platelet aggregation.

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 and YIGSR were synthesized by the Biochemical Institute, College of Medicine, National Taiwan University. Fibrinogen was purchased from Kabi, Sweden. Fibronectin (from bovine plasma), vitronectin (from human plasma), type IV collagen (from mouse sarcoma), type I collagen (from calf skin) and laminin (from basement membrane of mouse sarcoma) were obtained from Sigma Chem. Co., USA. Cell culture reagents including FCS were from GIBCO, USA. Triflavin was prepared according to the previously described method.[28] In brief, the procedure consisted of Fractogel TSK HW-50, CM-Sephadex C-50 column chromatography and gel filtrations on Sephadex G-75 and G-50 columns. The last step of purification was accomplished by a reverse-phase HPLC C18 column. The purified triflavin migrates as a single band and its molecular mass was estimated to be 7,500 daltons on SDS-PAGE (20% gel).

Cell culture The B16-F10 mouse melanoma cells were obtained from the Institute of Preventive Medicine, Taipei, Taiwan, and grown in DMEM containing 10% FCS and 1% L-glutamine. Cells were passaged and harvested for experiments before reaching confluence.

Adhesion assays B16-F10 melanoma cells were detached with EDTA (1 m*M*)/trypsin (0.25%, w/v) and washed thoroughly with DMEM to remove residual FCS. Cells were resuspended in DMEM at a concentration of 1×10^4 cells/ml. Plates (96-well; Costar, USA) were previously coated overnight at 4°C with 50 μl of fibronectin (30 μg/ml), fibrinogen (40 μg/ml), vitronectin (15 μg/ml), laminin (15 μg/ml), collagen type I (80 μg/ml) or type IV (80 μg/ml) in phosphate-buffered saline. A 300 μl aliquot of cells in the absence or presence of triflavin or GRGDS (1×10^4/ml of Hanks' balanced salt solution with glucose and 0.5% BSA) was placed into each well and incubated for 90 min at 37°C. Nonadherent cells were removed by aspiration and the cells were gently washed with PBS. The adherent cells were then fixed with 2% glutaraldehyde for 10 min and stained with 2% Giemsa for 20 min. Cells were viewed at 100× magnification using a Nikon inverted phase-contrast microscope. Cells were counted with a 1.0 mm^2 reticle in the eyepiece.

Metastasis assays An aliquot of 5×10^4 melanoma cells (B16-F10) with or without triflavin was injected slowly into the lateral tail vein of C57BL/6 mouse (age, 6–7 weeks). Fourteen days later, the mice were killed by cervical dislocation and their lungs were removed and fixed in 10% formaldehyde. The number of surface melanoma colonies was counted by eye or with a dissecting microscope (Nikon, UFX-II).

Aggregation study Human blood was anticoagulated with heparin (final concentration, 5 U/ml), and platelet-rich plasma (PRP) was prepared by centrifugation at 120*g* for 10 min at room temperature. Platelet-poor plasm (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. Platelet aggregation of PRP was measured turbidimetrically by using a Lumi-aggregometer (Chrono-log). PRP (400 μl) was pre-warmed at 37°C for 2 min in a silicone-treated glass cuvette. Triflavin was added 1 min before addition of 30 μl of B16-F10 cells (1.0×10^6 cells/ml, final concentration). The reaction was allowed to proceed for at least 10 min and the extent of aggregation was expressed as a percentage of the control (saline). The degree of aggregation was expressed as change of light transmission.

Cytotoxicity and ^{3}H-thymidine uptake assay To rule out the possibility that triflavin was cytotoxic to B16-F10 mouse melanoma cells, cell viability was evaluated before and 24, 48, and 72 h after addition of triflavin (20 μg/ml) to cell-coated plates. The cells were detached and stained with trypan blue. The number of dye-excluding cells was counted as described above.

Cell viability was also examined by measuring cellular thymidine uptake. Confluent cultures of cells maintained in a 24-well plates were incubated for 24, 48, or 72 h with 20 μg/ml of triflavin. At the final 8 h of the incubation, 5 μCi of [methyl-^{3}H]thymidine (specific activity, 25 Ci/mmol, Amersham, USA) was added to each well. At the end of the incubation, cells were detached and washed

[4] Abbreviations: ECM, extracellular matrix; GRGDS, Gly-Arg-Gly-Asp-Ser; GRGES, Gly-Arg-Gly-Glu-Ser; YIGSR, Tyr-Ile-Gly-Ser-Arg; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin.

three times with PBS. [Methyl-^{3}H] thymidine incorporation was quantified by liquid scintillation counting.

RESULTS

B16-F10 melanoma cells were seeded onto plates coated with ECMs. The cells adhered most efficiently to laminin, vitronectin, fibronectin and fibrinogen-coated plates (data not shown). BSA and gelatin, however, were poor substrates for adhesion even at concentrations of up to 1 mg/ml. Cells became attached less efficiently to collagen type IV and least efficiently to collagen type I-coated ones (both at 15 μg/ml). At higher concentration of type IV collagen (80 μg/ml), however, cells adhered equally well to laminin (15 μg/ml). Cell attachment to type I collagen (80 μg/ml) was only 68.4% as efficient as compared to laminin (15 μg/ml) (data not shown). The above observations suggest that the major component of interstitial connective tissue, collagen type I, is less effective for melanoma cell adhesion when compared with other ECMs (i.e., fibronectin, laminin, and collagen type IV). In subsequent experiments we used appropriate concentrations of these ECM components to investigate the inhibitory effect of triflavin on cell-substratum adhesion.

Effects of triflavin on the adhesion of melanoma cells to ECMs Inhibition of B16-F10 mouse melanoma cells attachment to fibronectin by varying concentrations of triflavin and GRGDS are shown in Figs. 1 and 2. After 90 min incubation at a cell concentration of 1×10^4 cells/ml more than 50% of the cells attached and spread well on fibronectin-coated wells (Fig. 1a). As expected, adhe-

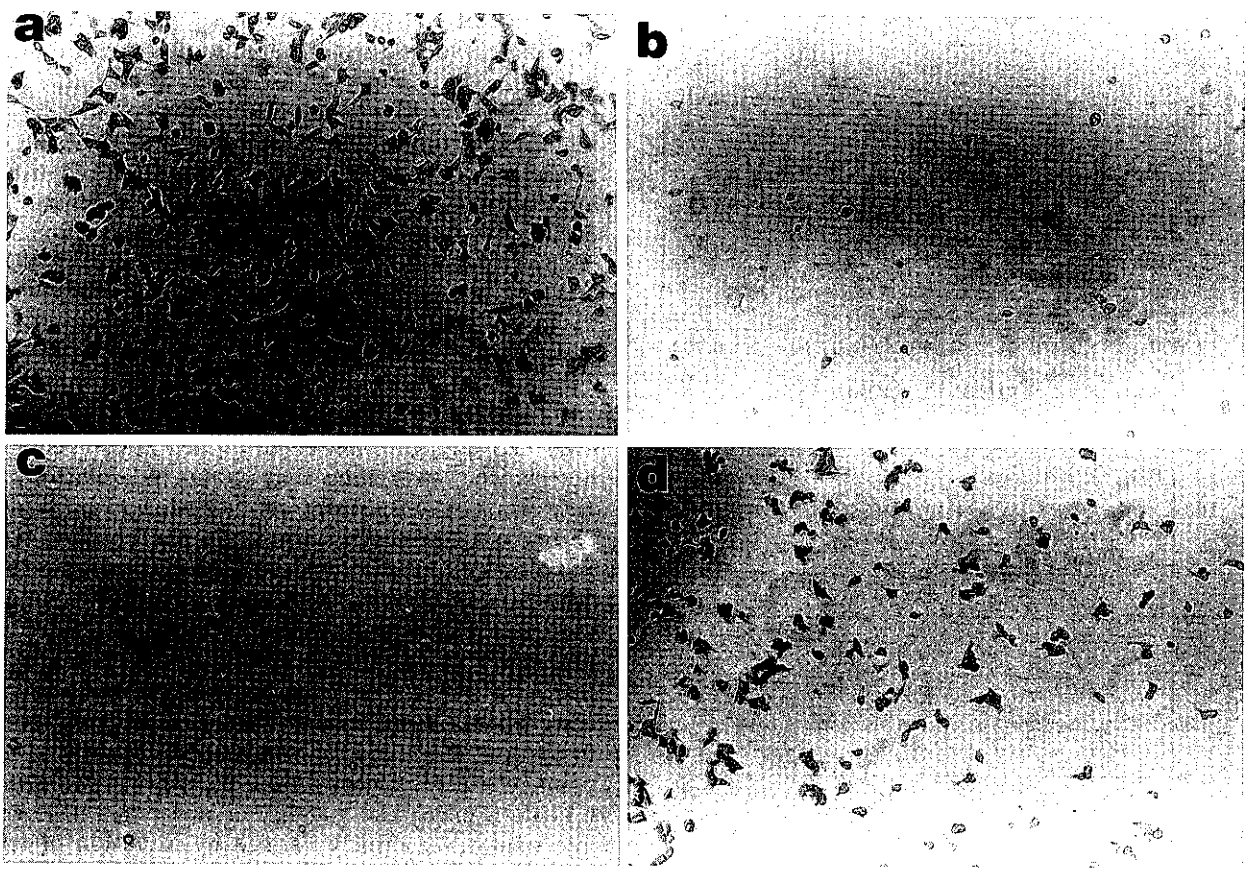

Fig. 1. Adhesion of B16-F10 mouse melanoma cells immobilized fibronectin in the presence of triflavin or GRGDS. (a) Control, no peptide, (b) triflavin (0.13 μM), (c) triflavin (0.2 μM), (d) GRGDS (41 μM). Plates (96-well) were treated overnight at 4°C with 50 μl of fibronectin (30 μg/ml) in PBS. Cells were harvested with EDTA (1 mM)/trypsin (0.25%, w/v) and washed three times in serum-free DMEM to remove residual FCS. A 300 μl aliquot of cells (1×10^4/ml) was added to each well. Cells were incubated for 90 min at 37°C. Nonadherent cells were removed by aspiration and the wells were washed with PBS. Adherent cells were fixed with 2% glutaraldehyde for 10 min and stained with 2% Giemsa for 20 min. Cells were viewed at 100× magnification, and counted with a 1.0-mm^2 reticle in the 10× eyepiece.

sion of melanoma cells to fibronectin was inhibited by triflavin in a dose-dependent manner (Figs. 1b, 1c and 2A). At 0.07 μM, triflavin inhibited cell attachment by 55%. At 0.2 μM, triflavin inhibited cell attachment by 88%. Addition of the GRGDS peptide (41–164 μM) also dose-dependently inhibited attachment of melanoma cells to fibronectin (Fig. 1d, Fig. 2B). At 164 μM, GRGDS showed 78% inhibition. On a molar basis, triflavin was about 800 times more potent than GRGDS at inhibiting cell attachment.

Fig. 2 shows that triflavin and GRGDS also inhibited melanoma cell attachment to fibrinogen in a dose-dependent manner (with IC_{50} ≤ 0.07 μM and ≤ 41 μM, respectively). At 0.07 μM, as shown in Fig. 2A, triflavin inhibited cell attachment to vitronectin about 40%. At 0.28 μM, triflavin almost completely inhibited cell attachment (96%) (Fig. 2A). Addition of GRGDS peptide (41–144 μM) also dose-dependently inhibited attachment of cells to vitronectin (Fig. 2B). On a molar basis, triflavin was about 500 times more potent than GRGDS at inhibiting cell adhesion to vitronectin.

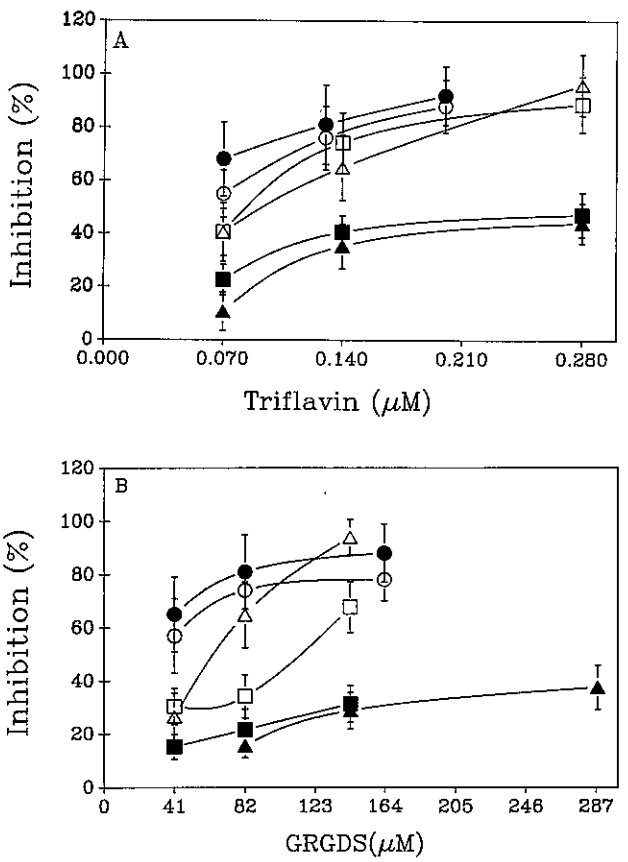

Fig. 2. Dose-response relation of triflavin (A) and GRGDS (B) action on B16-F10 cell adhesion to immobilized ECMs. Fibronectin (30 μg/ml, ○), fibrinogen (40 μg/ml, ●), vitronectin (15 μg/ml, △), laminin (15 μg/ml, ▲), collagen type I (80 μg/ml, □) and type IV (80 μg/ml, ■). The cell adhesion assay was carried out as described in the legend to Fig. 1. Inhibition of cell adhesion is shown as a percentage of the control. Data are presented as mean ± SE (n = 6–7).

Laminin is a large, major noncollagenous glycoprotein component of basement membrane.[31] As shown in Fig. 2, triflavin and GRGDS were less effective in suppressing cell attachment to laminin than to fibronectin, fibrinogen and vitronectin. At 0.28 μM, triflavin showed a maximal inhibitory effect of 44%. The sequence YIGSR has been implicated in mediating tumor cell adhesion to laminin.[32] However, YIGSR (1 mg/ml) inhibited cell adhesion to laminin with a maximal inhibition of 73% (data not shown). This may explain why triflavin did not effectively inhibit cell adhesion to laminin.

Collagen type IV is the most important component in basement membrane and collagen type I is the predominant type of collagen in interstitial connective tissue.[33] Therefore, we assessed the ability of triflavin to inhibit collagen type I and type IV-mediated cell attachment (Fig. 2A). Triflavin and GRGDS were more potent inhibitors of the collagen type I-mediated adhesion than of the type IV-mediated adhesion. At 0.28 μM, triflavin inhibited collagen type I-mediated cell adhesion about 89%, and collagen type IV-mediated cell adhesion about 50%. Triflavin was much more active than GRGDS at blocking attachment to either substrate. However, triflavin and GRGDS were approximately equally effective at inhibiting attachment to laminin and collagen type IV (maximal inhibition, 40–50%).

In contrast, in the above experiments, GRGES (150 μM) had no significant effect on cell attachment (data not shown), suggesting that RGD-containing peptides may specifically interrupt cell adhesion to immobilized fibronectin, fibrinogen, vitronectin and collagen type I. Futhermore, in addition to reducing the number of cells attached, both GRGDS and triflavin inhibited the spreading of melanoma cells on matrix-coated wells (Fig. 1).

Effect of triflavin on metastasis of B16-F10 melanoma cells To investigate further whether triflavin inhibits the formation of pulmonary melanoma metastasis, melanoma cells were coinjected intravenously with triflavin into C57BL/6 mice. The viability of melanoma cells before injection was routinely verified by exclusion of trypan blue (>90%). Fourteen days later, visible colonies in lung were found (Figs. 3 and 4). Coinjection of purified triflavin (1–30 μg per mouse) with B16-F10 cells (5×10^4) resulted in a marked reduction of lung metastatic colonies in C57BL/6 mice (Figs. 3 and 4). The inhibitory activity was dose-dependent and afforded $\geq$ 50% inhibition at 1–2 μg per mouse. In our assay system, the dose of

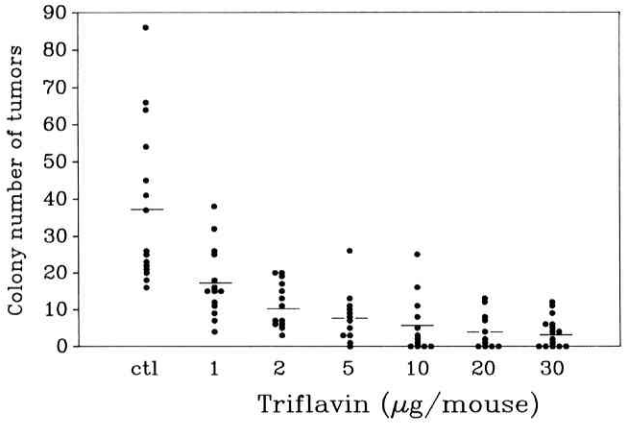

Fig. 3. Dose-response relation of triflavin (1–30 μg/mouse) action on lung colonization formation caused by B16-F10 melanoma cells. For details, see "Materials and Methods." The line (—) represents the average number of tumor colonies in lung. Data are presented as mean±SE (n=11–15).

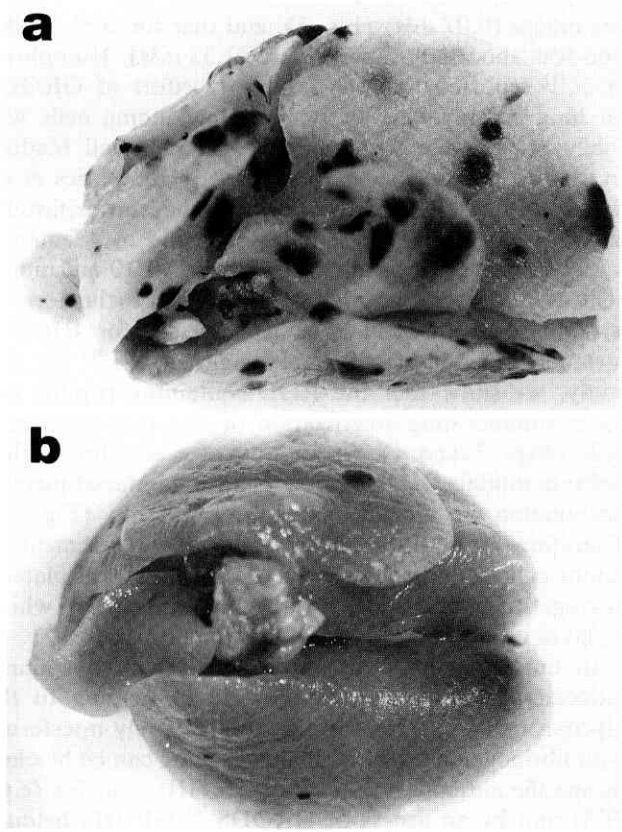

Fig. 4. Effect of triflavin on lung colonization induced by i.v. injection of B16-F10 mouse melanoma cells. (a) control, no peptide. (b) coinjection of triflavin (20 μg/ml) with melanoma cells. For details, see "Materials and Methods."

triflavin required for complete inhibition of colonization was estimated to be 20–30 μg per mouse ($\geq$90%). Humphries *et al.* showed that coinjection of GRGDS (3 mg/mouse) with 7×10^4 B16-F10 cells also resulted in a marked reduction of metastatic colonies in the lung.[15] In contrast, no significant decrease of lung tumor colony formation was observed with GRGES (3 mg/mouse) (data not shown). The effective concentration of triflavin was 0.06–0.2 μM (1–30 μg/mouse) assuming a 2-ml blood volume per mouse, consistent with that of triflavin for inhibiting cell adhesion.

Effect of triflavin on B16-F10 melanoma cell-induced platelet aggregation Pretreatment of platelets with triflavin (1.2 μg/ml; 0.16 μM) inhibited the aggregation reaction induced by B16-F10 cells (Fig. 5). GRGDS (2 mM) also had a similar inhibitory effect while GRGES (4 mM) had no significant effect (data not shown). B16-F10 cell-induced platelet aggregation was inhibited by triflavin (0.6–1.2 μg/ml; 0.08–0.16 μM) in a dose-dependent manner.

Effect of triflavin on proliferation of B16-F10 cells Incubation of B16 cells with triflavin (20 μg/ml) even for 72 h did not affect B16 cells proliferation as assessed by [methyl-^{3}H]thymidine uptake and trypan blue exclusion (data not shown). Moreover, a normal growth efficiency was observed after prolonged incubation of triflavin with melanoma cells. This suggests that triflavin is nontoxic to B16-F10 cells, and its inhibitory effect on attachment of B16 cells to ECMs can not be explained by simple cytotoxicity.

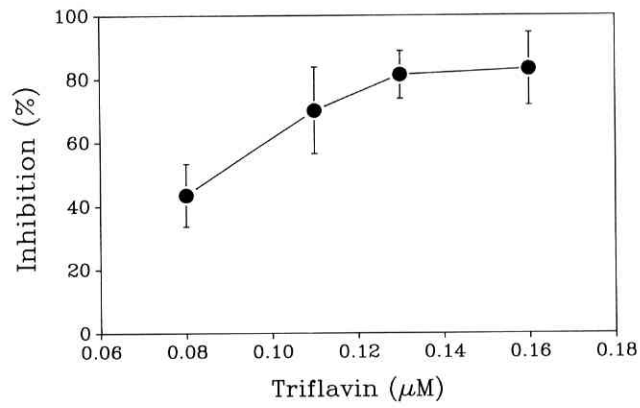

Fig. 5. Effect of triflavin on B16-F10 cell-induced platelet aggregation. Heparinized human platelet-rich plasma (PRP) (400 μl) was preincubated with various concentrations of triflavin at 37°C for 2 min, followed by addition of B16-F10 cells (1.0×10^6 cell/ml) for inducing platelet aggregation. The data are presented as mean±SE (n=4).

DISCUSSION

Triflavin, a potent platelet aggregation inhibitor purified from the venom of *T. flavoviridis* inhibits aggregation of human platelets by acting as a fibrinogen receptor antagonist. Its primary structure consists of 70 amino acid residues including 12 cysteines as well as the sequence Arg-Gly-Asp near the C-terminus (positions 49–51). This tripeptide sequence has been demonstrated to mediate interaction of triflavin with fibrinogen receptor associated with glycoprotein IIb-IIIa complex.[30] Many studies have demonstrated that the tripeptide RGD, present in many adhesive proteins, is the cell recognition site.[12,34] These RGD-containing peptides inhibit tumor cell adhesion to fibronectin, vitronectin, laminin, collagen and other adhesive proteins *in vitro* as well as tumor cell metastasis in many experimental models.[14,15,35–40] Extracellular adhesion-promoting proteins are thus likely to interact with adhesion receptors of tumor cells via the RGD sequence. Dedhar *et al.* have demonstrated that MG-63 human osteosarcoma cell expresses a receptor complex for collagen type I which recognizes the Arg-Gly-Asp sequence.[35] In addition, Kieffer *et al.* have recently reported that M21 human melanoma cells express a vitronectin receptor which mediates cellular adhesion to fibrinogen, vWF and vitronectin via an RGD-recognition site.[41]

In this paper, we report that triflavin, an RGD-containing peptide inhibits adhesion of B16-F10 cells to ECMs (e.g., fibronectin, fibrinogen, vitronectin, laminin, and collagen type I and IV). At 0.28 μM, triflavin almost completely inhibits the adhesion of tumor cells to fibronectin, vitronectin, and collagen type I, and to a lesser extent it inhibits adhesion to immobilized laminin and collagen type IV. Residual cell attachment may results from an RGD-independent process. For example, domain IIIb in the A chain of laminin contains an RGD sequence,[42] and the RGD-containing site of laminin is active in promoting cell adhesion and migration. Since RGD-containing peptides block adhesion to laminin and partially block migration on laminin, it is likely that this site of the intact molecule is biologically active. It has been established that laminin contains at least 4 active regions for cell adhesion; these include YIGSR, which is active in promoting adhesion and migration and in inhibiting tumor colonization of lungs in mice.[43] This may explain why triflavin only partially inhibits tumor cell attachment to immobilized laminin. In this study, synthetic peptide YIGSR (1 mg/ml) inhibits cell attachment to laminin about 73%, but can not inhibit cell attachment completely even at concentration up to 2 mg/ml. Although interference with cell attachment to either endothelium or exposed subendothelial basement membrane is the most likely mode of action of triflavin, the process of hematogenous metastasis is complex and other mechanisms may be involved. In this study, we compare the degree of cell attachment on immobilized ECMs. Triflavin and GRGDS significantly inhibit the adhesion of B16-F10 cells at concentrations of 0.07 μM and 41 μM, respectively. However, triflavin is at least 600–800 times more active than GRGDS. Triflavin has 12 half-cysteine residues at positions which afford six disulfide bonds in the native molecule.[30] The disulfide bonds may thus be very important in maintaining optimal configuration for expression of the biological activity. In contrast, GRGDS has a linear configuration, and is less active. We believe that these differences reflect the different affinity of the peptides for the integrin receptor. Meanwhile, synthetic peptide GRGES had no significant effect on tumor cell attachment to ECMs. This finding suggests that the conformation of the Arg-Gly-Asp sequence is important for receptor recognition.

Humphries *et al.*[15] have reported that GRGDS inhibits pulmonary metastatic spread of B16-F10 cells, but that millimolar concentrations are required for full inhibition. In our experiments, the dose of triflavin required for substantial inhibition of colonization was about 1 μg per mouse (0.07 μM) (Fig. 3), and that for 90% inhibition was about 20 μg per mouse (1.33 μM). Humphries *et al.*[15] reported that the inhibitory effect of GRGDS on lung colonization by B16-F10 melanoma cells was solely attributable to inhibition of tumor cell binding to fibronectin. In a subsequent paper, Humphries *et al.* suggested platelet involvement in the antimetastatic effects of GRGDS peptide.[44] According to Menter *et al.*,[45] platelet aggregation caused by B16-F10 melanoma cells is required for lung colonization. Furthemore, it was observed that ADP is also needed for B16-F10 melanoma cell-induced platelet aggregation.[46] In this study, we show that the RGD-containing peptide triflavin inhibits lung colonization of B16-F10 melanoma cells (Figs. 3 and 4). In addition, we also found that triflavin inhibits B16-F10 melanoma cell-induced platelet aggregation in human platelet-rich plasma (Fig. 5). Therefore, in addition to disrupting the attachment of tumor cells to immobilized ECMs, inhibition of platelet aggregation may be one of the mechanisms by which triflavin inhibits experimental lung metastasis.

In our previous study,[28] triflavin inhibited agonist-induced platelet aggregation by direct binding to the glycoprotein IIb/IIIa complex, competitively interfering with fibrinogen binding. Triflavin binding can be blocked by specific antibodies against GP IIb/IIIa complex (e.g., 7E3) and by an excess of GRGDS.[30] GP IIIa belongs to the $\beta 3$ integrin subfamily. Knudsen *et al.*[47] have demonstrated that a heterodimeric glycoprotein complex with a β subunit biochemically and immunologically similar to platelet IIIa plays a predominant role in the

adhesion of human melanoma cells to fibronectin, fibrinogen and vitronectin. In addition, Cheresh *et al.*[16,48,49] have reported that an RGD-dependent glycoprotein complex with functional and structural properties similar to those of the vitronectin receptor is expressed by melanoma cells. In addition, Grossi *et al.*[50] suggested that carcinoma cells, including human cervical carcinoma (MS751) and human colon carcinoma (clone A), express a plasma membrane receptor (i.e., immunological related GP IIb/IIIa) which is immunologically and functionally related to the platelet aggregation receptor complex (i.e., GP IIb/IIIa complex), and this IR GP IIb/IIIa is a multifunctional receptor which mediates tumor cell adhesion to a variety of biological substrata. Therefore, we speculated that inhibitory effect of triflavin on fibrinogen binding to platelets and its inhibitory effcet on melanoma cell adhesion to ECMs should show similar features. This may explain why triflavin inhibited cell-substratum adhesion, at least in part, by interfering with the specific receptor on B16-F10 melanoma cells. Therefore, triflavin exerts its inhibitory effect on tumor cell adhesion by binding to tumor cell integrin. The identity of the integrin on B16 melanoma cells to which triflavin binds is not known. Preliminary data from our laboratory indicate that ^{125}I-triflavin may directly bind to B16-F10 melanoma cells, but further study for characterization of the binding sites or binding affinity (K_d) of ^{125}I-triflavin on the tumor cell surface is needed.

This study reinforces the notion that triflavin inhibits pulmonary tumor metastasis by interfering with cellular adhesive processes, but the mechanism remains to be determined. Some likely explanations include (1) the disruption of cell attachment to extracellular-promoting proteins by binding to integrin receptor on B16-F10 melanoma cells through an RGD-dependent mechanism or (2) interference with tumor cell migration during invasion. Additionally, inhibition of tumor cell-induced platelet aggregation by triflavin may also play an important role. The usefulness of triflavin in inhibiting cell adhesion and migration could theoretically provide a reasonable basis for therapy or prevention of metastasis of cancer cells after surgical removal of a primary tumor.

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