Triflavin, an Arg-Gly-Asp–Containing Peptide, Inhibits the Adhesion of Tumor Cells to Matrix Proteins *via* Binding to Multiple Integrin Receptors Expressed on Human Hepatoma Cells (44038)

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Abstract. **Integrins are a superfamily of cell surface glycoproteins that promote cellular adhesion. The interaction of integrins with extracellular matrices such as fibronectin and vitronectin has been shown to be mediated through an arginine-glycine-aspartic acid (RGD) sequence within adhesive proteins.**

Triflavin, a 7.5-kDa cysteine-rich polypeptide purified from *Trimeresurus flavoviridis* snake venom, belongs to a family of RGD-containing peptides, termed disintegrins. Disintegrins have been isolated from the venom of various vipers and have been shown to be potent inhibitors of platelet aggregation. In this study, we found that human hepatoma cell adhesion to immobilized matrix proteins (i.e, fibronectin, collagen, laminin, and vitronectin) was differentially affected by various anti-integrin monoclonal antibodies (mAbs) (i.e., $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$) as well as by the peptide GRGDS. Indirect flow cytometric analysis of hepatoma cells with anti-integrin mAbs demonstrated that $\alpha_6\beta_1$ was uniformly expressed at a high density, while $\alpha_3\beta_1$ and $\alpha_5\beta_1$ were moderately expressed and $\alpha_v\beta_3$ was expressed in small amounts on hepatoma cells, consistent with the results obtained from immunofluorescence microscopic analysis. When immobilized on plastic wells, triflavin promoted hepatoma cell attachment; this cell attachment was inhibited by either GRGDS or mAbs against integrins ($\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$). In addition, the binding of FITC-conjugated triflavin to hepatoma cells was inhibited by GRGDS, anti-$\alpha_3\beta_1$, anti-$\alpha_5\beta_1$, and anti-$\alpha_v\beta_3$ mAbs. Among these mAbs, anti-$\alpha_5\beta_1$ exerted the most pronounced inhibitory effect (>70%) on the binding of triflavin to hepatoma cells. Taken together, these results suggest that triflavin binds *via* its RGD sequence to multiple integrin receptors (i.e., $\alpha_5\beta_1$, $\alpha_3\beta_1$, and $\alpha_v\beta_3$) expressed on the surface of hepatoma cells, resulting in inhibition of hepatoma cell adhesion to extracellular matrices (i.e., fibronectin and vitronectin).

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Metastasis is a complicated phenomenon that involves tumor cell dissemination and the interaction of tumor cells with host cells (i.e., platelets, endothelial cells) and subendothelial matrices. Cellular interactions are mediated by cell-surface molecules expressed by tumor cells as well as host cells. These surface molecules also mediate cell-cell recognition, adhesion, and interaction with subendothelial matrix components (1). A family of cell surface adhesion receptors termed "integrins" has been described (2). The integrins comprise a superfamily of transmembrane receptors that participate in cell-cell

and cell-substrata interactions. Integrin receptors are membrane-spanning heterodimers consisting of noncovalently associated α and β subunits (3, 4). Many of the extracellular matrix receptors (e.g., the family of VLA antigens) are composed of a common β_1 subunit associated with one of several α chains (5). VLA-5 ($\alpha_5\beta_1$) and VLA-4 ($\alpha_4\beta_1$) interact with fibronectin, VLA-6 ($\alpha_6\beta_1$) binds to laminin, and VLA-1 ($\alpha_1\beta_1$) and VLA-2 ($\alpha_2\beta_1$) bind both collagen and laminin (6). The VLA-3 ($\alpha_3\beta_1$) structure is especially versatile, acting as a receptor for fibronectin, collagen, and laminin (7, 8). Two β_3 integrins on the platelet membrane, GPIIb/IIIa ($\alpha_{IIb}\beta_3$) and the vitronectin receptor ($\alpha_v\beta_3$), share the same β subunit. Both GPIIb/IIIa and the vitronectin receptor have been shown to function as promiscuous receptors for the adhesive proteins fibrinogen, vitronectin, fibronectin, von Willebrand factor, and thrombospondin which are involved in platelet-subendothelium and platelet-platelet interactions. The multiple ligand-binding capability of these receptors is due to their ability to recognize Arg-Gly-Asp (RGD) sequences (9, 10). However, there must be secondary sites apart from the specific binding site on ligands that determines the ligand specificity of integrins. $\alpha_5\beta_1$ Complex (VLA-5, the classical fibronectin receptor) recognizes RGD-containing sequence in the cell-binding domain of fibronectin but is generally fibronectin specific (11). There is evidence that tumor cells interact with RGD-containing proteins during invasion. Gehlsen *et al.* (12) showed that RGD peptide blocked tumor cell invasion of basement membranes. In addition, RGD and its polymers were reported to inhibit experimental metastasis (13, 14).

Recently, many trigramin-like antiplatelet peptides (or disintegrins) have been reported (15–20). These peptides all contain RGD, are rich in cysteine, and bind with high affinity to integrins on the surface of platelets and other cells. Trigramin, an RGD-containing peptide purified from the venom of *Trimeresurus gramineus,* is a specific fibrinogen receptor antagonist with a high binding affinity (K_d, 20 n*M*) for the activated platelet fibrinogen receptor (21, 22). Triflavin is a trigramin-like antiplatelet peptide purified from *Trimeresurus flavoviridis* snake venom (23, 24) which is more potent than trigramin. Its primary structure consists of 70 amino acid residues including 12 cysteines with an RGD sequence at position 49–51 (25). Triflavin directly interferes with the interaction of fibrinogen with the glycoprotein IIb/IIIa complex (25, 26). We previously reported that triflavin inhibited B16-F10 murine melanoma cell-induced lung colonization in C57BL/6 mice in a dose-dependent manner (27). Furthermore, we also found that triflavin inhibited the adhesion of human hepatoma cells to extracellular matrices (i.e., fibronectin, fibrinogen, vitronectin, and collagen type I) in a dose-dependent manner (28). We suggested that triflavin exerts its antimetastatic effect mainly through blocking tumor cell adhesion to these extracellular matrices. In the present study, we further characterized the triflavin-binding integrins expressed on the surface of hepatoma cells. We show that the binding activity of triflavin is closely related to the RGD sequence within this antiplatelet peptide.

Materials and Methods

Materials. *T. flavoviridis* venom was purchased from Latoxan (Rosans, France) and stored at −20°C. Gly-Arg-Gly-Asp-Ser (GRGDS) was purchased from Peninsula Laboratories (Belmont, CA). GRGES was synthesized by the Biochemical Institute, College of Medicine, National Taiwan University. Fibronectin (from bovine plasma), vitronectin (from human plasma), collagen type I (from calf skin), and laminin (from the basement membrane of mouse sarcoma) were obtained from Sigma Chemical Co. (St. Louis, MO). The monoclonal antibodies (mAbs) used in this study, MCA794 (anti-$\alpha_3\beta_1$), MCA698 (anti-$\alpha_5\beta_1$), MCA699 (anti-$\alpha_6\beta_1$), and MCA757 (anti-$\alpha_v\beta_3$), were purchased from Serotec Ltd (Oxford, England). Control monoclonal antibody AP_1 raised against the platelet membrane glycoprotein Ib was kindly provided by Drs. P. Newman and T. Kuniki (Milwaukee Blood Center, Milwaukee, WI). FITC was purchased from Molecular Probe Inc. (Eugene, OR). Triflavin was prepared according to previously described methods (23). The purified triflavin migrated as a single band with a molecular mass of 7500 daltons on SDS-PAGE (20% gel).

Cell Culture. A human hepatoma cell line (J-5) was obtained from the Institute of Preventive Medicine, Taipei, Taiwan, and grown in Dulbecco's modified Eagles's medium (DMEM) containing 10% calf serum (FCS) and 1% L-glutamine. Cells were passaged and harvested for experiments before reaching confluence. Cell culture reagents including FCS were from Gibco (Grand Island, NY).

Adhesion Assays. Human hepatoma 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, Cambridge, MA) were previously coated overnight at 4°C with 50 μl of triflavin (50 μg/ml), vitronectin (15 μg/ml), fibronectin (30 μg/ml), laminin (15 μg/ml), or collagen type I (80 μg/ml) in phosphate-buffered saline (PBS). A 300-μl aliquot of cells (1×10^4 cells/ml in Hanks' balanced salt solution with glucose and 0.5% bovine serum albumin [BSA]) was added 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 counted with an image analyser (Pointek Computer Co., Ltd., Taipei, Taiwan), or with an 1.0 mm^2 reticle in the eyepiece using a Nikon inverted phase-contrast microscope and viewed at ×100 magnification. Competition assays were carried out by adding dilutions of inhibitory mAbs (5 μg/well) or various concentrations of GRGDS or GRGES to ligand-coated plates before conducting the adhesion assay exactly as described above.

Fluorescein-Conjugated Triflavin. One milligram of triflavin was dissolved in 0.2 ml of 0.1 *M* sodium bicarbonate. Twenty microliters of freshly prepared FITC solution (10 mg/ml in DMSO) was added to the protein solution for 1 hr at room temperature with continuous stirring. The reaction was stopped by adding 20 μl of freshly prepared 1.5 *M* hydroxylamine (pH 8.0–8.5) for another 30 min. Finally, the conjugated protein was separated from the residual labeling reagent and hydroxylamine on a Sephadex G-10 column (10 × 300 mm) equilibrated with PBS. The collected reaction was lyophilized, and protein concentration was estimated by the Lowry method (29). The concentration of FITC-conjugated triflavin was adjusted to 1 mg/ml.

Flow Cytometric Analysis. Human hepatoma cells were detached, washed thoroughly with PBS containing 1% BSA and 2% goat serum (Gibco, Gaithersburg, MD), and preincubated in the same buffer for 30 min at 4°C. Next, 2×10^5 cells/ml aliquots were treated individually with respective mAb specific for VLA (α_3, α_5, α_6) or $\alpha_v\beta_3$ in the same buffer (1:200 dilution of stock) for 45 min at 4°C. Cells were washed (three times) with buffer solution and then incubated with FITC-conjugated goat anti-mouse IgG (at a 1:100 dilution; Boehringer Mannheim Corp., Indianapolis, IN) for 45 min at 4°C. The cells were washed (three times) as above, and resuspended to 1 ml/tube in buffer solution. In other experiments, cells were directly treated with various concentrations of FITC-triflavin for 60 min at 4°C, then washed (three times) with buffer solution as described above, and finally resuspended to 1 ml/tube for assay. Fluorescein-labeled cells were assayed with a Coulter EPICS dual-laser cytometer (Coulter Electronics Inc., Hialeah, FL). FITC signals were detected and digitized in log base 10 configuration and the data was collected on an EPICS computer system. Data were collected in 256-channel resolution and 10,000 cells were counted per experimental group. Fluorescence intensity was directly proportional to the fluorescein label bound on the tumor cell surface. The relative fluorescence intensity was calculated by subtracting the mean control intensity (i.e., FITC-triflavin in the presence of 5 m*M* EDTA, or the group with addition of FITC-conjugated goat anti-mouse IgG only) from the mean experimental fluorescence intensity. All experiments were repeated at least four times to ensure reproducibility.

Immunofluorescence. Human hepatoma cells (1×10^5) were placed on four-chamber glass coverslips (Nunc, Inc., Naperville, IL) and allowed to grow for 2 days. Coverslips with plated cells (50%–65% confluent) were washed (three times) with PBS to remove serum proteins and fixed in 0.1% paraformaldehyde for 8 min. Coverslips were washed (two times) in PBS and blocked with 10% goat serum for 25 min. Cells were then treated with primary specific antibodies against VLA ($\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$) or $\alpha_v\beta_3$ (5 μg/chamber) for 1 hr at room temperature. The free primary antibody was removed by rinsing (three times) with PBS, and nonspecific binding was blocked with 10% goat serum. Finally, FITC-conjugated goat anti-IgG (1:100) was applied at 37°C for 1 hr. Negative controls were incubated with FITC-conjugated goat anti-mouse IgG only, or stained with equal amounts of irrelevant mAb AP_1 against platelet glycoprotein I_b, a non-integrin mAb, followed by addition of FITC-conjugated goat anti-mouse IgG. Representative fields of cells were photographed through an Olympus microscope equipped with epiluminescence optics (VANOX-S, Olympus).

Statistical Analysis. The experimental results are expressed as the mean ± SEM and accompanied by the number of observations. Data was assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, each group was then compared by the Newman-Keuls method. A *P* value less than 0.05 was considered significant.

Results

Integrin Expression on Hepatoma Cells. To determine whether integrin molecules are expressed on the surface of human hepatoma cells, an indirect immunofluorescence measurement using flow cytometric analysis was performed utilizing mAbs specific for VLA-3 ($\alpha_3\beta_1$), VLA-5 ($\alpha_5\beta_1$), VLA-6 ($\alpha_6\beta_1$), and VnR ($\alpha_v\beta_3$) as the primary ligands. As shown in Figure 1, hepatoma cells expressed various integrins of which VLA-6 ($\alpha_6\beta_1$) was expressed at a relatively high level, VLA-5 ($\alpha_5\beta_1$) and VLA-3 ($\alpha_3\beta_1$) were expressed at moderate levels and $\alpha_v\beta_3$ was present at the lowest level.

Localization of Integrins on Hepatoma Cells. To compare the distribution of integrins on the cell surface, an indirect immunofluorescence study was performed. Hepatoma cells were intensively stained by anti-$\alpha_6\beta_1$ and anti-$\alpha_3\beta_1$ mAbs (Fig. 2, a and b). The staining pattern with anti-$\alpha_5\beta_1$ was similar to the anti-

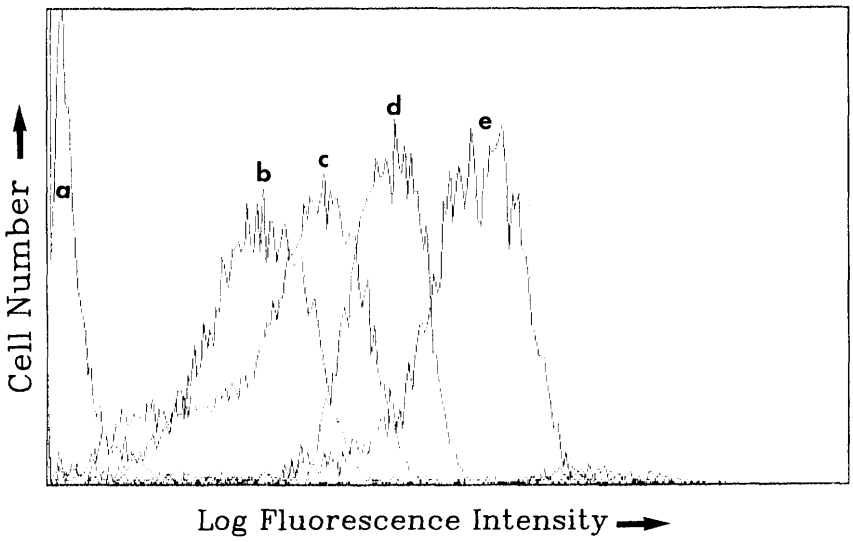

Figure 1. Expression of cell surface integrins on human hepatoma cells demonstrated by flow cytometric analysis. Hepatoma cells were harvested, washed, and stained with anti-integrin mAbs and FITC-conjugated goat anti-mouse IgG. For detailed procedures see Materials and Methods. The solid lines represent the fluorescence profile of the hepatoma cells after reaction with PBS (a), anti-$\alpha_v\beta_3$ (b), anti-$\alpha_3\beta_1$ (c), anti-$\alpha_5\beta_1$ (d), or anti-$\alpha_6\beta_1$ (e) mAbs before addition of FITC conjugate. The results represent a representative experiment from four similar experiments.

$\alpha_3\beta_1$ pattern (data not shown). In general, both $\alpha_6\beta_1$ and $\alpha_3\beta_1$ were diffusely distributed over the cell surface. The staining intensity of an mAb against the $\alpha_v\beta_3$ (Vn) receptor was less than the anti-$\alpha_6\beta_1$ and $\alpha_3\beta_1$ mAbs (Fig. 2c). In contrast, fluorescence was not observed when the secondary antibody (FITC-conjugated goat anti-mouse IgG) was added without the prior addition of anti-integrin mAbs (Fig. 2d), or when the irrelevant mAb AP_1 was used (data not shown).

Differential Effect of Anti-Integrin mAbs on Hepatoma Cell Adhesion to Matrix Proteins. To characterize further the adhesive properties of hepatoma cells and to investigate which integrins might mediate cell adhesion to various ligands, we used four anti-integrin mAbs to examine the interaction of hepatoma cells with ECMs. Anti-$\alpha_3\beta_1$ mAb inhibited cell adhesion to Fn (by 42%), but did not inhibit cell adhesion to collagen type I (Col I), laminin (Lm), or vitronectin (Vn). Anti-$\alpha_5\beta_1$ mAb inhibited cell adhesion to Fn by 87% but did not inhibit adhesion to either collagen type I (Col I), laminin (Lm), or vitronectin (Vn) (Fig. 3), consistent with the specificity of $\alpha_5\beta_1$ for Fn. Cell adhesion to Lm was specifically inhibited by anti-$\alpha_6\beta_1$ (75% inhibition), and cell attachment to Vn was specifically inhibited by anti-$\alpha_v\beta_3$ mAb (71% inhibition) (Fig. 3D). To determine whether cell adhesion was RGD dependent, RGD-containing peptides were tested. GRGDS (60 μM) inhibited cell attachment to Fn, Col I, Lm, and Vn by 47%, 27%, 13%, and 33%, respectively. In contrast, the control peptide GRGES (2 m*M*) had no significant effect on cell adhesion (Fig. 3). Because $\alpha_5\beta_1$ appears to be the major integrin responsible for Fn binding, the GRGDS peptide probably inhibited cell adhesion by blocking $\alpha_5\beta_1$ in these experiments. GRGDS (60 μM) produced a maximum of 40%–55% inhibition, whereas anti-$\alpha_3\beta_1$ mAb resulted in 35%–45% inhibition. A combination of GRGDS and anti-$\alpha_3\beta_1$ mAb resulted in additive in-

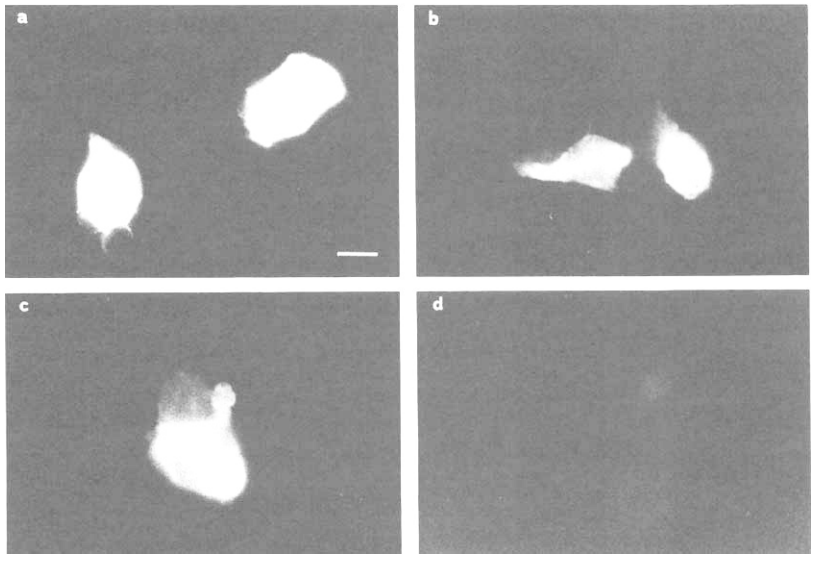

Figure 2. Immunofluorescence staining of integrins expressed on human hepatoma cells. Hepatoma cells were stained with anti-$\alpha_6\beta_1$ (a), anti-$\alpha_3\beta_1$ (b), or anti-$\alpha_v\beta_3$ (c) mAbs, or PBS (d) before addition of FITC-conjugated goat anti-mouse IgG. All photographs are in the same field. Bar, 10 μm.

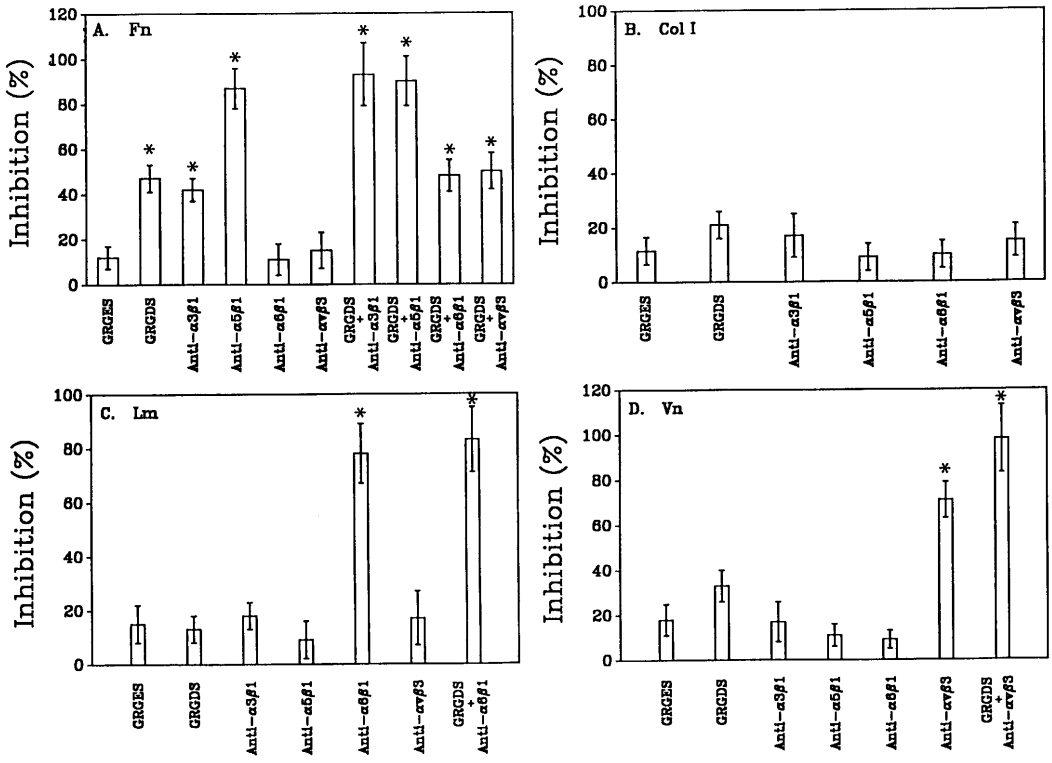

Figure 3. Inhibition of hepatoma cell adhesion to matrix proteins by various mAbs and GRGDS. Adhesion of cells to microplastic wells coated with (A) fibronectin (Fn, 30 μg/ml), (B) collagen type I (Col I, 80 μg/ml), (C) laminin (Lm, 15 μg/ml), or (D) vitronectin (Vn, 15 μg/ml) was examined in the presence of inhibitors. The following mAbs (5 μg/well) were tested for inhibition of cell adhesion: anti-$\alpha_3\beta_1$, anti-$\alpha_5\beta_1$, anti-$\alpha_6\beta_1$, and anti-$\alpha_v\beta_3$. The peptides GRGDS and GRGES were present at 60 μ*M* and 2 m*M*, respectively. Inhibition of cell adhesion is shown as a percentage of the control adhesion (in the absence of inhibitors). Data are presented as mean ± SEM (n = 4–5). *$P < 0.001$, a significant difference compared with the GRGES group.

hibition (93%) of cell adhesion to Fn (Fig. 3A). Furthermore, when GRGDS was added together with anti-$\alpha_5\beta_1$ mAb to Fn (Fig. 3A) or with anti-$\alpha_6\beta_1$ to Lm (Fig. 3C), it did not further increase the inhibitory effect of either mAb, indicating that anti-$\alpha_5\beta_1$ and anti-$\alpha_6\beta_1$ had likely reached their maximal inhibitory effects. These results suggest that the RGD sequence within Fn may mediate cell attachment to $\alpha_3\beta_1$ or $\alpha_5\beta_1$. However, GRGDS did not affect cell adhesion to laminin (Fig. 3C). A combination of GRGDS (60 μ*M*) and anti-$\alpha_v\beta_3$ mAb inhibited cell attachment to Vn by 98% in an additive manner (Fig. 3D).

Effect of GRGDS Peptide on FITC-Conjugated Triflavin Binding to Human Hepatoma Cells. The Arg-Gly-Asp (RGD) sequence is the domain responsible for cell recognition in a variety of adhesive proteins including fibronectin, fibrinogen, vitronectin, and collagen type I (11). This tripeptide appears to play an essential role in the interaction of these adhesive proteins with their individual receptors. We previously showed that triflavin may bind to RGD recognition sites on the cell surface resulting in the inhibition of tumor cell attachment to ECMs (28). Further evidence for this hypothesis is shown in Figure 4. FITC-triflavin directly bound to hepatoma cells in a concentration-dependent manner, whereas the presence of GRGDS (0.41 and 0.82 m*M*) inhibited triflavin binding to hepatoma cells (24% and 54%, respectively), indicating that the RGD sequence of triflavin plays an essential role in mediating triflavin binding to these cells. In the presence of 5 m*M* EDTA, FITC-triflavin binding to hepatoma cells was inhibited by 90%, indicating that triflavin binding activity is divalent cation dependent (data not shown).

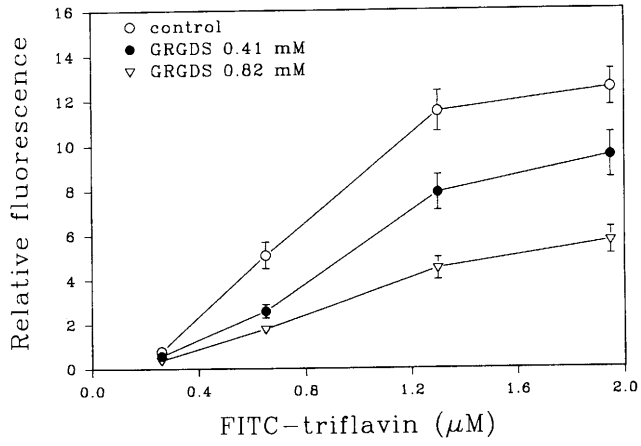

Figure 4. Flow cytometric analysis of FITC-conjugated triflavin binding to hepatoma cells. Cells were harvested, washed, and stained with FITC-triflavin (see Materials and Methods). The binding of FITC-triflavin to hepatoma cells in the absence (○) or presence of GRGDS (0.41 m*M* [●]; 0.82 m*M* [▽]) is shown. Data are presented as mean ± SEM (n = 4–5).

Differential Effect of Anti-Integrin mAbs on Hepatoma Cell Adhesion to Immobilized Triflavin. To determine further which integrins on the surface of the hepatoma cells mediate triflavin binding, cell adhesion inhibition studies were carried out. As shown in Figure 5, anti-$\alpha_3\beta_1$, anti-$\alpha_5\beta_1$, and anti-$\alpha_v\beta_3$ mAbs inhibited hepatoma cell adhesion to immobilized triflavin by 37%, 71%, and 42%, respectively. Moreover, the inhibitory activity of these mAbs did not increase at higher concentrations (10 μg/well) (data not shown). Thus, $\alpha_5\beta_1$ appeared to bind to the immobilized triflavin more efficiently than did $\alpha_3\beta_1$ or $\alpha_v\beta_3$. However, anti-$\alpha_6\beta_1$ mAb did not inhibit the adhesion of the hepatoma cells to triflavin (Fig. 5), whereas this mAb blocked the adhesion of hepatoma cells to immobilized Lm by 80% (Fig. 3C). On the other hand, GRGDS (0.5 m*M*) inhibited cell adhesion to immobilized triflavin by about 30% (data not shown). A combination of GRGDS (0.5 m*M*) with either anti-$\alpha_3\beta_1$, anti-$\alpha_5\beta_1$, or anti-$\alpha_v\beta_3$ inhibited cell adhesion to triflavin by 68%, 98%, and 71%, respectively, more pronounced inhibitions than produced by the individual mAbs (Fig. 5). These results suggest that triflavin binds to hepatoma cells through $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$. These results are consistent with the results shown in Figure 4 and our previous results (28) showing that human hepatoma cells bound to triflavin mainly *via* an RGD-dependent mechanism.

Effect of Anti-Integrin mAbs on FITC-Triflavin Binding to Hepatoma Cells. Figure 5 shows that anti-integrin mAbs inhibited hepatoma cell adhesion to immobilized triflavin. As shown in Figure 6, the relative fluorescence of FITC-triflavin binding to hepatoma cells in the absence of anti-integrin mAbs, taken as the control, was 11 ± 3. Among the anti-integrin mAbs tested, anti-$\alpha_5\beta_1$ inhibited FITC-triflavin binding to hepatoma cells by 95%, while the same concentration of anti-$\alpha_6\beta_1$ mAb did not significantly affect FITC-triflavin binding. Furthermore, mAbs against $\alpha_3\beta_1$ and $\alpha_v\beta_3$ produced about 59% and 72% inhibition of triflavin binding, respectively (Fig. 6B). These results correspond to the results shown in Figure 5.

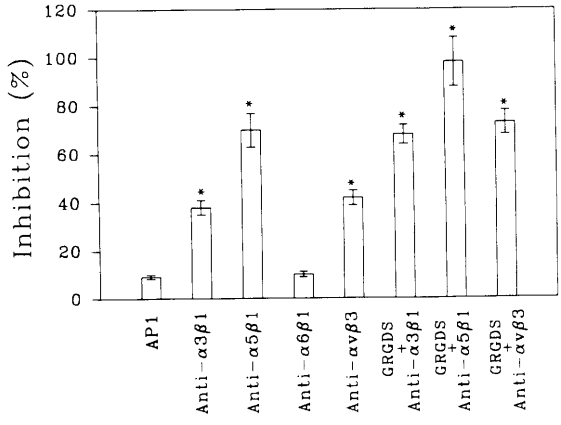

Figure 5. Inhibition of human hepatoma cell adhesion to immobilized triflavin. Adhesion of cells to microplastic wells coated with triflavin (50 μg/ml) was examined in the presence of inhibitors. The following mAbs (5 μg/well) were tested for inhibition of cell adhesion: anti-$\alpha_3\beta_1$, anti-$\alpha_5\beta_1$, anti-$\alpha_6\beta_1$, and anti-$\alpha_v\beta_3$. GRGDS was present at 0.5 m*M*. Inhibition of cell adhesion is shown as a percentage of control adhesion (in the absence of inhibitors). Data are presented as mean ± SEM (n = 4–5). *P < 0.001, a significant difference compared with the control monoclonal antibody AP_1.

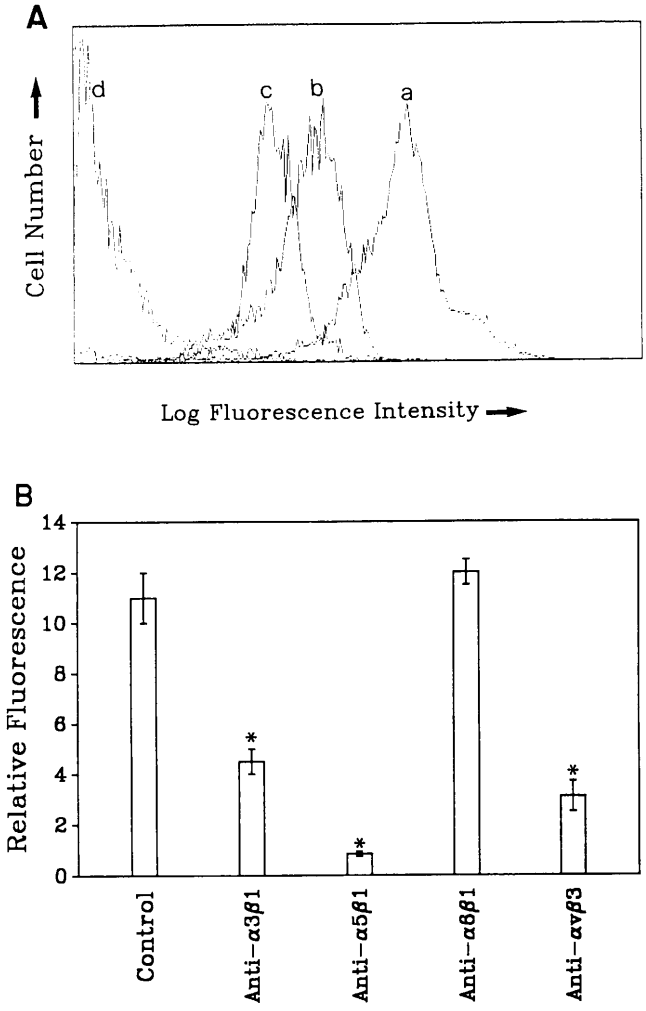

Figure 6. Flow cytometric analysis of FITC-conjugated triflavin binding to hepatoma cells in the absence or presence of anti-integrin mAbs. (A) The solid lines represents the fluorescence profile of FITC-triflavin (1.3 μ*M*) in the absence of mAb (a) or presence of anti-$\alpha_3\beta_1$ (b), anti-$\alpha_v\beta_3$ (c), and anti-$\alpha_5\beta_1$ (d) mAbs. The profile is a representative example of four similar experiments. (B) The relative fluorescence of FITC-triflavin (1.3 μ*M*) binding to cells in the absence or presence of anti-integrin mAbs. Data are presented as mean ± SEM (n = 4–5). *P < 0.001, a significant difference compared with the control group.

Discussion

Many studies have demonstrated that the tripeptide RGD acts as a cell recognition site in many integrins. RGD also appears to be present in the active

binding site in triflavin and other disintegrins (25, 30). Extracellular adhesion-promoting proteins thus likely interact with adhesion receptors on tumor cells *via* their RGD sequences. Dedhar *et al.* have demonstrated that MG-63 human osteosarcoma cells express a receptor complex for collagen type I which recognizes the RGD sequence (31). In addition, Kieffer *et al.* (32) have reported that M21 human melanoma cells express a vitronectin receptor which mediates cellular adhesion to fibrinogen, von Willebrand factor, and vitronectin *via* an RGD-recognition site. In a previous study (28), we showed that triflavin inhibited adhesion of human hepatoma cells to ECMs (e.g., fibronectin, fibronogen, vitronectin, and collagen type I). Furthermore, we also found that triflavin inhibited lung colonization of B16-F10 cells in C57 BL/6 mice (27), possibly by interfering with cellular adhesion processes. All these results suggest that the RGD sequence within the triflavin molecule is important for receptor recognition. Triflavin inhibits platelet aggregation by binding directly to the glycoprotein IIb/IIIa complex and thus competitively interfering with fibrinogen binding to activated platelets. The GPIIb/IIIa complex ($\alpha_{IIb}\beta_3$) is a member of structurally related glycoproteins in mammalian cells belonging to the integrin family (18).

More recently, cultured human melanocytes and melanoma cells have been shown to express a variety of integrins including several members of the β_1 subfamily (i.e., $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$). However, the expression of the β_3 subunit was exclusively restricted to tumorigenic cells (33). In this study, we detected the expression of various integrins on human hepatoma cells by flow cytometric analysis utilizing anti-integrin mAbs. $\alpha_6\beta_1$ was expressed at a relatively high level, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ were expressed at moderate levels, and the Vn receptor (VnR, $\alpha_v\beta_3$) was found at low level. Similar results were found after immunofluorescence staining and microscopic examination of cells (Fig. 2). In this study, anti-$\alpha_5\beta_1$, anti-$\alpha_6\beta_1$, and anti-$\alpha_v\beta_3$ were found to inhibit specifically hepatoma cell adhesion to immobilized Fn (Fig. 3A), Lm (Fig. 3C), and Vn (Fig. 3D), respectively. VnR ($\alpha_v\beta_3$) has been reported to function as a promiscuous receptor for the RGD-containing adhesive proteins fibrinogen, vitronectin, fibronectin, thrombospondin, laminin, and von Willebrand factor (34). In this study, however, anti-VnR mAb did not significantly affect hepatoma cell adhesion to Fn and Lm (Fig. 3, A and C). On the other hand, an anti-$\alpha_3\beta_1$ mAb inhibited the adhesion of hepatoma cells to immobilized Fn but not to Lm and Col I. Although previous studies clearly established the multiple binding function of $\alpha_3\beta_1$ as a receptor for Fn, Lm, and Col (7, 8, 34, 35), the interaction of $\alpha_3\beta_1$ with Col has also been disputed (36–38). Although the evidence for $\alpha_3\beta_1$ binding to various ligands is variable, $\alpha_3\beta_1$ does appear to interact with Fn (Fig. 3A). The role of $\alpha_3\beta_1$ in the attachment of many adherent cell lines (such as human hepatoma cells) to matrix ligands is not obvious, because other integrins, with overlapping function, may play a more dominant role. For example, an anti-$\alpha_5\beta_1$ mAb was more effective than an anti-$\alpha_3\beta_1$ in inhibiting hepatoma cell adhesion to Fn (fig. 3A).

To evaluate the role of RGD in mediating the binding of matrix proteins to integrins, a combination of the GRGDS peptide and anti-integrin mAbs was employed to inhibit adhesion of hepatoma cells to matrix proteins. GRGDS as well as an anti-$\alpha_3\beta_1$ mAb exhibited cell adhesion to Fn (Fig. 3A). The combination of GRGDS and an anti-$\alpha_3\beta_1$ mAb exhibited an additive inhibition (Fig. 3). Similarly, GRGDS and an anti-Vn receptor mAb ($\alpha_v\beta_3$) also inhibited cell adhesion to Vn in a additive manner (Fig. 3). Therefore, the integrins $\alpha_3\beta_1$, $\alpha_5\beta_1$, and the Vn receptor ($\alpha_v\beta_3$) expressed on human hepatoma cells exhibited RGD-dependent adhesion. This is consistent with the notion that the $\alpha_3\beta_1$ (39), $\alpha_5\beta_1$, and VnR (9) integrins recognize RGD sites within their respective ligands. However, we cannot rule out the possibility that $\alpha_5\beta_1$ and VnR might recognize an additional "non-RGD" site in Fn and Vn, respectively, or that GRGDS might also inhibit cell adhesion by blocking integrins other than the Fn or Vn receptor in an RGD-dependent manner.

Since triflavin is known to bind the RGD-dependent integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$) on platelets (25, 26), it is likely that triflavin can bind to RGD-dependent integrins expressed on hepatoma cells. The present results show that anti-$\alpha_5\beta_1$, anti-$\alpha_3\beta_1$, and anti-VnR but not anti-$\alpha_6\beta_1$ mAbs inhibited the adhesion of hepatoma cells to immobilized triflavin (Fig. 5), consistent with the results obtained from the FITC-triflavin binding study (Fig. 6). A combination of anti-$\alpha_3\beta_1$, anti-$\alpha_5\beta_1$, or anti-VnR mAbs with GRGDS, produced additive inhibition (Fig. 5). Furthermore, GRGDS dose-dependently inhibited FITC-triflavin binding to cells (Fig. 4). Taken together, these results suggest that triflavin binds to fibronectin receptors (i.e., $\alpha_5\beta_1$ and $\alpha_3\beta_1$) as well as the vitronectin receptor (i.e., $\alpha_v\beta_3$) *via* an RGD-dependent mechanism. Furthermore, Scarborough *et al.* (40) found that the relative activities of various disintegrins in blocking the adhesion protein binding functions of $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ were related to the amino acid sequences adjacent to their RGD sites (40).

In conclusion, our study indicates that human hepatoma cells express a high level of $\alpha_6\beta_1$, moderate levels of $\alpha_5\beta_1$ and $\alpha_3\beta_1$, and low amounts of the Vn receptor. Triflavin, *via* its RGD sequence, binds to multiple integrin receptors (i.e., $\alpha_5\beta_1$, $\alpha_3\beta_1$, and $\alpha_v\beta_3$) expressed on the surface of human hepatoma cells, thereby blocking hepatoma cells adhesion to extracellular matrices (i.e., fibronectin and vitronectin).

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