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Rhodostomin, A Snake Venom Disintegrin, Inhibits Angiogenesis Elicited by Basic Fibroblast Growth Factor and Suppresses Tumor Growth by A Selective $\alpha_v\beta_3$ Blockade of Endothelial Cells

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ABSTRACT

Angiogenesis consists of the proliferation, migration, and differentiation of endothelial cells, although angiogenic factor and integrin-extracellular matrix interaction modulate this process. We report here that a snake venom-derived disintegrin, rhodostomin, inhibited distinct steps in angiogenesis elicited by basic fibroblast growth factor (bFGF), and also suppressed in vivo murine melanoma tumor growth. Rhodostomin dose-dependently inhibited bFGF-induced human umbilical vein endothelial cell (HUVEC) proliferation as examined by cell number count, metabolic activity, and BrdU incorporation assays with submicromolar IC_{50} values. However, it apparently did not affect the viability of murine B16F10 melanoma cells, even up to 50 μM. Rhodostomin also inhibited HUVEC migration and invasion evoked by bFGF, and tube formation of bFGF-treated HUVECs in Matrigel. Moreover, rhodostomin selectively inhibited bFGF-, but not vascular endothelial growth factor-associated angiogenesis in the chick chorioallantoic membrane model. Furthermore, rhodostomin blocked both bFGF- and B16F10-induced neovascularization in murine Matrigel plug model and suppressed the growth of subcutaneously inoculated B16F10 solid tumor, leading to a prolonged survival of the rhodostomin-treated C57BL/6 mice. The antiangiogenic effect of rhodostomin on bFGF-treated HUVECs is related to the integrin $\alpha_v\beta_3$ blockade, as evidenced by its selective inhibition on the binding of 7E3, a monoclonal antibody (mAb) raised against $\alpha_v\beta_3$, but not that of P1F6, an $\alpha_v\beta_5$ mAb toward both naive and bFGF-primed HUVECs. Moreover, 7E3 specifically blocked fluorescein isothiocyanate-conjugated rhodostomin binding to HUVEC, whereas P1F6 and anti-integrin α_2, α_3, α_4, or α_5 mAbs did not.

Angiogenesis, which consists of a complex process of the development of new capillaries from pre-existing vessels, plays a critical role in a variety of normal physiological events, such as the female menstrual cycle, bone remodeling, and wound healing. In contrast, many pathological conditions are also characterized by undesirable neovascularization, including diabetic retinopathy, tumor growth, metastasis, and various inflammation diseases (Folkman, 1995). Growth and progression of primary solid tumors is highly dependent on angiogenesis. An avascular tumor rarely grows to a size larger than 2 to 3 mm^3 and contains up to a few million cells. Once a tumor becomes vascularized, the expansion of tumor mass is rapid (Hanahan and Folkman, 1996). Tumor angiogenesis involves several processes, including endothelial proliferation, migration, invasion, and tube formation, that are regulated by cell adhesion receptors and specific angiogenic growth factors produced by tumor cells and the surrounding stroma (Folkman and Shing, 1992; Sastry and Horwitz, 1996). In preclinical models, agents that target the tumor vasculature (i.e., angiostatin and endostatin) have been shown to prevent or delay tumor growth and even to promote tumor regression or dormancy (O'Reilly et al., 1997; Klohs and Hamby, 1999).

Integrins are a family of heterodimeric transmembrane receptors that mediate cell-cell and cell-extracellular matrix interaction (Hynes, 1992). The function of integrin during angiogenesis has been studied extensively with $\alpha_v\beta_3$, which is one of several Arg-Gly-Asp (RGD)-dependent integrins (Byzova ey al., 1998). In vivo, expression of this integrin is strongly up-regulated in angiogenic endothelial cells, where it plays a critical role in angiogenesis induced by basic fibroblast growth factor (bFGF), tumor necrosis factor-α, or frag-

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ABBREVIATIONS: RGD, Arg-Gly-Asp; bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; VEGF, vascular endothelial growth factor; mAb, monoclonal antibody; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; BrdU, bromodeoxyuridine.

ments of human tumors in corneal or chick chorioallantoic membrane (CAM) model (Brooks et al., 1994a). It has been reported that bFGF exerts its effects on cultured endothelial cells through the modulation of integrin-dependent vascular cell adhesion events (Enenstein et al., 1992; Yeh et al., 1999). Friedlander et al. (1995) demonstrated that angiogenesis depends on cytokines and vascular cell integrins. $\alpha_v\beta_3$ is required when angiogenesis is stimulated with bFGF or tumor necrosis factor-α, whereas angiogenesis induced by vascular endothelial growth factor (VEGF), transforming growth factor-β, or phorbol ester depends on $\alpha_v\beta_5$. These facts imply that antiangiogenic agents may be selective for certain angiogenic responses, depending on the mechanism(s) involved in a given angiogenic disease or process.

Disintegrins are a family of low-molecular-weight, RGD-containing peptides that bind specifically to integrins $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ expressed on platelets and other cells including vascular endothelial cells and some tumor cells (Gould et al., 1990). In addition to their potent antiplatelet activity, studies of disintegrins have revealed a new use in the diagnosis of cardiovascular diseases and the design of therapeutic agents in arterial thrombosis, osteoporosis, and angiogenesis-related tumor growth and metastasis (Huang, 1998). It has been shown that disintegrin inhibited adhesion of tumor cells to extracellular matrices and in vivo experimental metastasis of B16 murine melanoma cells (Sheu et al., 1992; Kang et al., 2000). Recently, other investigators and we demonstrated that disintegrin inhibits either spontaneous or tumor associated angiogenesis (Yeh et al., 1998; Kang et al., 1999; Zhou et al., 1999). However, it remains to be elucidated whether disintegrin affects the distinct steps in growth factor-induced angiogenesis. In the present work, we report that rhodostomin, a snake venom-derived disintegrin, inhibited several angiogenic responses elicited by bFGF, including pro-

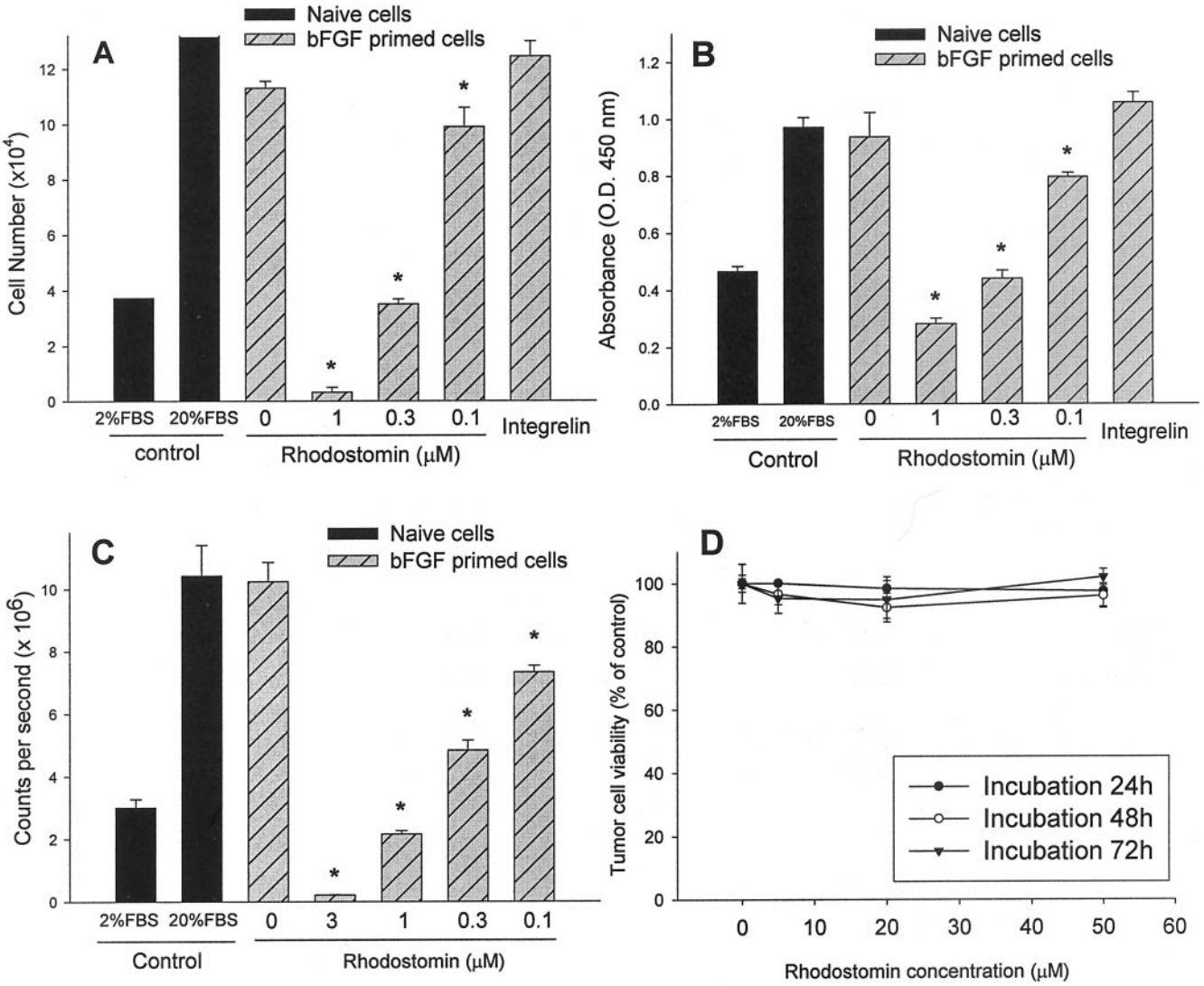

Fig. 1. Effect of rhodostomin on bFGF-induced HUVEC proliferation and on B16F10 melanoma cell viability. A–C, HUVECs (4×10^4/ml) were seeded on 24-well plates. After attachment, cells were treated with indicated concentration of test compounds for 48 h in the presence or absence (naive) of bFGF (30 ng/ml). Cell proliferation was measured by cell number counting (A), metabolic activity using WST-1 reagent (B), and BrdU incorporation (C) as described under *Experimental Procedures*. For control, HUVECs were grown in M199 containing 2% FBS or 20% FBS for 48 h and then assayed for cell proliferation. D, the inhibition of B16F10 melanoma cell viability was analyzed in the absence (control) or presence of various concentration of rhodostomin. After incubation for 24, 48, or 72 h, cells were stained with MTT and lysed. The absorbance was read by an enzyme-linked immunosorbent assay reader. Data are presented as mean ± S.E.M. ($n = 3$). *$P < 0.01$ versus bFGF-primed cells in the absence of rhodostomin (A, B, and C).

liferation, migration, invasion, and differentiation in human endothelial cells in vitro and in chick CAM model in vivo. On the other hand, the antitumor activity of rhodostomin was also evaluated by examining its effects on B16F10 melanoma tumor-induced angiogenesis using a murine Matrigel plug model and on the tumor mass of subcutaneously inoculated tumor cells and the survival rate in tumor-bearing C57BL/6 mice.

Experimental Procedures

Materials. Rhodostomin was purified from the snake venom of *Calloselasma rhodostoma* as described previously (Huang et al., 1990). Monoclonal antibodies (mAbs) 7E3, raised against integrin $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ (Coller, 1985), and anti-LIBS1, raised against ligand-induced binding sites of β_3 integrin (Frelinger et al., 1990), were kindly donated from Dr. B. Coller (The Mount Sinai Hospital, New York, NY) and Dr. M. H. Ginsberg (The Scripps Research Institute, La Jolla, CA), respectively. mAb P1F6 raised against integrin $\alpha_v\beta_5$, anti-α_2, α_3, α_4, and α_5 integrin mAbs were purchased from Chemicon (Temecula, CA). Eptifibatide (Integrelin), a cyclic heptapeptide based on a Lys-Gly-Asp sequence that specifically antagonizes integrin $\alpha_{IIb}\beta_3$ but not integrin $\alpha_v\beta_3$ (Scarborough et al., 1993), was kindly donated from COR Therapeutics (South San Francisco, CA). Human recombinant bFGF and all cultured reagents were purchased from Life Technologies (Gaithersburg, MD). Human recombinant $VEGF_{165}$ was from R & D Systems (Minneapolis, MN). Toluidine blue O, gelatin, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC) was from Molecular Probes (Eugene, OR).

Cell Culture. HUVECs obtained from umbilical cord veins were prepared by the method described previously (Jaffe et al., 1973). Isolated endothelial cells identified by positive immunofluorescent staining of human von Willebrand factor antigen (DAKO, Carpinteria, CA), were maintained in M199 containing 20% fetal bovine serum (FBS) and other supplements. HUVECs were used between the second and fourth passages. The B16F10 murine melanoma cells were obtained from the National Institute of Preventive Medicine, Department of Health, Executive Yuan (Taipei, Taiwan) and maintained in Dulbecco's modified Egale's medium containing 10% FBS.

Proliferation and Viability Assays. HUVECs (2×10^4 cells/well) were seeded to 24-well plates (Costar, Cambridge, MA) for attachment. After incubation with tested peptides for 30 min before the addition of bFGF (30 ng/ml) for 48 h, cells were used in three proliferation assays. For cell number counting, HUVECs were detached from the plate and counted by hemacytometer (Hausser Sci, Horsham, PA). Cell metabolic assays were measured using a cell proliferation reagent (WST-1; Roche Molecular Biochemicals, Mannheim, Germany) for the microtiter tetrazolium assay. After incubation with WST-1 labeling reagent, the number of living cells was determined by measuring the ratio of developed color absorbance at 450 and 610 nm. BrdU incorporation was assayed by the protocol of enzyme-linked immunosorbent assay chemiluminescence detection kit (Roche Molecular Biochemicals) and by a luminescence counter (TopCount; Packard Instruments, Meriden, CT). The counts per second correlate directly to the amount of DNA synthesis and hereby the number of proliferating cells.

For viability assay, cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h. After lysis in dimethyl sulfoxide (DMSO), cell lysates were measured for the developed color absorbance at 550 nm. Cytotoxic effect of rhodostomin on HUVEC was measured by trypan blue exclusion in cell suspension after 2 h incubation. Apoptosis determination was performed by propidium iodide (Sigma) labeling as examined by flow cytometry (Yeh et al., 1998).

Migration and Invasion Assays. HUVEC migration and invasion assays were performed using Transwell (8.0-μm pore size; Costar) as described previously with modification (Leavesley et al., 1993). Polycarbonate filters (Transwell inserts) were coated with 0.25% gelatin and the lower chamber was filled with 0.6 ml of M199 in the absence or presence of bFGF (30 ng/ml). HUVECs (5×10^4 cells/ml) were incubated with or without tested peptides and an aliquot (200 μl) of cells was plated to the upper chamber of Transwell. After a 16-h incubation, all nonmigrant cells were removed from the upper face of the Transwell membrane with a cotton swab, and the migrant cells were fixed and stained with 0.5% toluidine blue in 4% paraformaldehyde. Migration was quantified by counting the number of stained cells per 100x field with an inverted contrast phase microscope (Nikon, Japan) and photographed.

For cell invasion assays, the filters were coated with Matrigel, a basement membrane matrix extracted from Engelbreth-Holm-Swarm mouse sarcoma (Becton Dickinson). Matrigel was diluted to 4 mg/ml using serum-free M199 at 4°C and an aliquot (20 μl) of Matrigel (80 μg/well) was added to each filter insert and incubated for 30 min at 37°C to form an uniform three-dimensional gel.

Quantification of HUVEC Tube Formation. Matrigel tube formation assays were performed as described previously with minor modification (Grant et al., 1989). Matrigel was diluted to 1 mg/ml in the presence or absence of bFGF (30 ng/ml) and added to 24-well plates in a total volume of 200 μl in each well to form a gel layer. After incubation with or without tested peptides for 24 h, HUVECs (2×10^5 cells) were stained with toluidine blue and photographed under microscope. Total tube length from each randomly chosen field was quantified using image analysis software of Image-Pro Plus (version 3.0; Media Cybernetics, Inc., Baltimore, MD).

Chick CAM Angiogenesis Assay. Eggs of 10-day-old chick embryos were opened with a 1.0-cm square window that allowed direct access to underlying CAM by the method described previously (Masood et al., 1999). Filter paper disk (13 mm; Minipore, Bedford, MA) saturated with bFGF or VEGF (both in 200 ng/disk) in the presence of test compounds or an equal aliquot of buffer (final in 20 μl) was applied to the top of CAM. After a 48-h incubation, CAM tissues under disks were resected and photographed by digital camera (Nikon, Tokyo, Japan).

Flow Cytometry. Determination of the integrins expressed on both naive and angiogenic factors-primed HUVECs was performed by flow cytometric analyses. Briefly, growth factor (i.e., bFGF or VEGF, both in 30 ng/ml for a 48-h incubation)-primed or naive HUVECs were fixed with 1% paraformaldehyde and then labeled with primary anti-integrin mAbs at 4°C for 1 h. After incubation with FITC-conjugated goat anti-mouse IgG (CALTAG Lab., Burlingame, CA), cells were analyzed with FACS Calibur (Becton Dickinson, Mountain View, CA) using excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals from 10,000 gated cells, to exclude any debris, were collected to calculate mean fluorescence intensity of single cell. To evaluate the inhibitory activity of rhodostomin, cells were incubated with various concentration of rhodostomin for 30 min before the addition of primary antibody.

To assess the effect of anti-integrin mAb on rhodostomin binding to HUVECs, rhodostomin and BSA were first conjugated with FITC as described previously (Yeh et al., 1998). The concentration of FITC-conjugated proteins was determined by bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Fixed HUVECs were preincubated either with nonimmune IgG or anti-integrin mAbs for 1 h at 4°C. After incubation, cells were washed twice and labeled with FITC-conjugated rhodostomin (1.3 μM). Nonspecific binding was determined by incubating cells with FITC-conjugated BSA.

Matrigel Plug Angiogenesis Assay. Matrigel plug assays were performed as described previously with modification (Prewett et al., 1999). An aliquot (0.5 ml) of Matrigel supplemented with bFGF (500 ng) or B16F10 (3×10^6 cells) in the presence or absence of rhodostomin (50 μM) was injected subcutaneously into the dorsal region of C57BL/6 male mice (6–8 weeks of age, 21.96 ± 0.19 g of weight).

After 14 days, Matrigel plugs were resected, fixed in 3% formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin by a standard method. For immunohistochemical staining, samples that had been deparaffinized by xylene and rehydrated were incubated with 3% H_2O_2 for 10 min. After incubation with rabbit anti-mouse von Willebrand factor antibody (1:50 dilution; Serotec, Oxford, UK), sections were incubated with biotinylated goat anti-rabbit IgG (1:40; DAKO) for 30 min, followed by incubation with avidin-biotin-horseradish peroxidase (DAKO). The sections were further developed with 3′,3′-diaminobenzidine (0.5 mg/ml) and counterstained with hematoxylin. The normal serum instead of the primary antibody was used for negative controls.

Growth of Solid Tumor in Mice. C57BL/6 mice were subcutaneously injected with B16F10 melanoma cells in a volume of 0.3 ml (3×10^6/mouse) into the dorsal midline with or without tested peptides. Tumors grown on mice of tested groups were measured twice weekly with caliper and the tumor volumes were calculated by the formula with volume = (4/3) π (length/2)(width/2) [(length + width)/4]. Volume sizes were reported as mean volumes ± S.E.M. (n = 9–11). The mice were either sacrificed on day 17 after implantation, at which point the control mice began to die, or followed up to examine their survival rates until all control mice died.

Results

Effect of Rhodostomin on bFGF-Induced HUVEC Proliferation and B16F10 Viability. The assays for cell proliferation were performed by counting cells with a hemocytometer and by determining the cell metabolic activity with a tetrazolium salt (i.e., WST-1). Furthermore, the measurement of DNA synthesis by BrdU incorporation was used to be a proliferation marker. As shown in Fig. 1, endothelial cells incubated with bFGF (30 ng/ml) in 2% FBS for 48 h, as well as those grown in medium containing 20% FBS, showed a 2.5- to 3.5-fold increase in three proliferation assays compared with control cells (grown in 2% FBS). Rhodostomin but not eptifibatide (10 μM) inhibited bFGF-induced HUVECs proliferation, as examined by cell number counting (Fig. 1A), cell metabolic activity (Fig. 1B), and BrdU labeling index (Fig. 1C), in a dose-dependent manner, with IC_{50} values of 0.24, 0.36, and 0.27 μM, respectively. Moreover, at a higher concentrations (i.e., 1 μM), rhodostomin significantly inhibited HUVECs proliferation compared with those of resting cells (control of 2% FBS, $P < 0.01$ for Fig. 1, A and B; $P < 0.05$ for Fig. 1C). However, the viability of B16F10 melanoma tumor cells was not decreased significantly upon incubation with rhodostomin (5–50 μM) for 24 to 72 h as examined by MTT assay (Fig. 1D). Furthermore, rhodostomin, as well as accutin, dose-dependently induced apoptosis when coincubated with HUVECs as examined by flow cytometric cell cycle analysis (Yeh et al., 1998; data not shown), but rhodostomin did not impair cell vitality as measured by trypan blue exclusion if HUVECs were incubated with rhodostomin in suspension (>90% exclusion, data not shown). These results indicate that the antiproliferative activity of rhodostomin is specifically restricted to anchorage-dependent cells (e.g., HUVECs).

Effects of Rhodostomin on bFGF-Induced HUVEC Migration and Invasion. Angiogenesis is highly dependent on endothelial cell motility and invasion. Therefore, we determined the effect of rhodostomin on HUVEC migration toward bFGF. Chemotactic migration was performed by Transwell containing insert filter coated with 0.25% gelatin. Adhesion of HUVECs toward gelatin was not affected by rhodostomin, even at a high dose of 2 μM (data not shown). As shown in Fig. 2, few control cells (without bFGF) were found in lower chamber, whereas a 3.4-fold increment of the migrated cells could be detected if bFGF was present as a chemotactic agent (Fig. 2A). After a 16-h incubation, rhodostomin (1 μM) showed a significant inhibition on HUVEC migration (77.6%, Fig. 2D), but 0.1 μM rhodostomin (Fig. 2C) and eptifibatide (10 μM, Fig. 2B) exerted little effect (5.1 and 2.1% inhibition, respectively). For the invasion assay, HUVECs were stimulated by bFGF and penetrated through a uniform layer of Matrigel, which consists of collagen type IV, heparan sulfate proteoglycan, entactin, and laminin. bFGF (30 ng/ml) induced a 5.5-fold increase in cell invasion through Matrigel compared with control (in the absence of

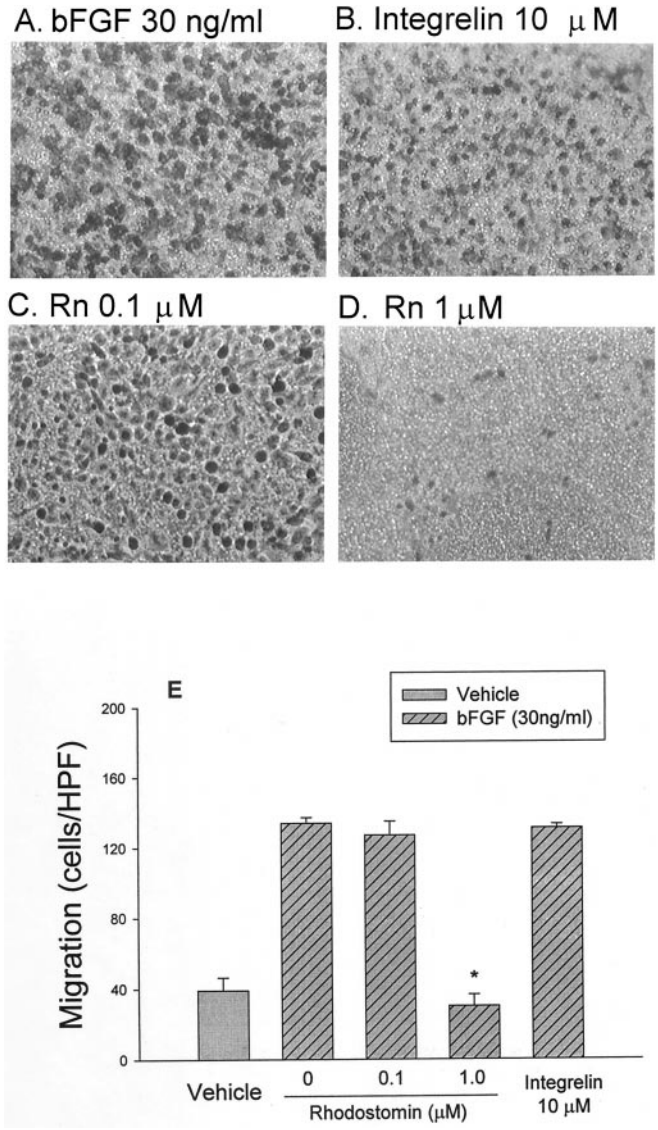

Fig. 2. Rhodostomin inhibited HUVEC migration toward bFGF. HUVECs (5×10^4/ml) were treated with indicated concentration of test compounds or PBS (vehicle) for 30 min, and then placed in the upper chamber of Transwell containing a gelatin-coated filter membrane. Chemotaxis was induced by adding bFGF (30 ng/ml) to the lower chamber for 16 h. After fixation and removal of nonmigrated cells, cells that migrated to the underside of filter membrane were photographed (A-D) and quantified (E) by phase-contrast light microscope under high power field (magnification, 100×). All experiments were conducted in triplicate and similar results were repeated at least four times. Data are presented as mean ± S.E.M. (n = 4–5). *$P < 0.01$ versus bFGF alone.

bFGF). Moreover, rhodostomin inhibited HUVEC invasion evoked by bFGF in a dose-dependent manner with an IC_{50} value of 0.37 μM (Fig. 3).

Effect of Rhodostomin on Angiogenesis in Vivo and in Vitro Elicited by bFGF. In vitro angiogenesis assays were carried out by using bFGF-treated HUVECs in a three-dimensional gel consisting of diluted Matrigel (1 mg/ml). As shown in Fig. 4, if HUVECs were plated on diluted Matrigel without the addition of exogenous growth factor, they showed only a few spontaneous tube formations, and most of them were still in a highly proliferating state with a cobblestone shape (Fig. 4A). However, when HUVECs were plated on the diluted Matrigel with addition of bFGF (30 ng/ml), cells displayed high motility, cell-cell communication, aligned, and formed an anastomosing capillary-like network within 18 h (Fig. 4B). Rhodostomin significantly decreased the total tube length in each of four randomly chosen fields in a dose-dependent manner, in contrast to the minimal effect of eptifibatide (10 μM, Fig. 4, C-F). Furthermore, in vivo chick CAM assays were performed under the induction of angiogenesis by both bFGF and VEGF (200 ng/embryo) for 48 h with or without the addition of rhodostomin (Fig. 5). As a control (PBS), formation of new blood vessels under disks on chick embryo CAMs could be observed (Fig. 5A). Either bFGF or VEGF induced a pronounced angiogenic response in this model (Fig. 5, B and E, respectively). Rhodostomin, at 2 μM, displayed a significant inhibition on bFGF-induced angiogenesis (Fig. 5C) compared with that of bFGF alone, but it slightly affected VEGF-associated angiogenesis (Fig. 5F). Similarly, the control peptide eptifibatide (2 μM) exerted little effect on bFGF-induced angiogenic response in the same model (Fig. 5D).

Elucidation of Targeted Receptors of Rhodostomin on Growth Factor-Activated Endothelial Cells. To identify which integrins expressed on HUVEC interact with rhodostomin, we first examined whether rhodostomin displaces the binding of anti-integrin mAb toward HUVEC by flow cytometry. As shown in Fig. 6A, the expression of integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$ on HUVECs in naive cells was probed by 7E3 or P1F6, respectively, and presented as the mean fluorescence

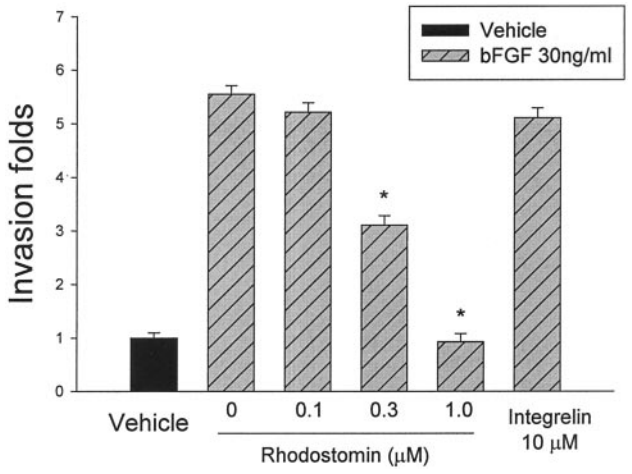

Fig. 3. Rhodostomin inhibited bFGF-induced HUVEC invasion through Matrigel. Invasion assay was performed using Transwell containing a Matrigel-coated filter membrane with or without addition of bFGF (30 ng/ml) in the lower chamber. HUVECs (5×10^4/ml) were placed in upper chamber in the presence or absence (vehicle) of test compounds. After a 16-h incubation, cells that invaded to the underside of filter membrane were quantified. All experiments were conducted in duplicate and repeated at least four times. Data are presented as mean ± S.E.M. ($n = 4$). *$P < 0.01$ versus bFGF alone.

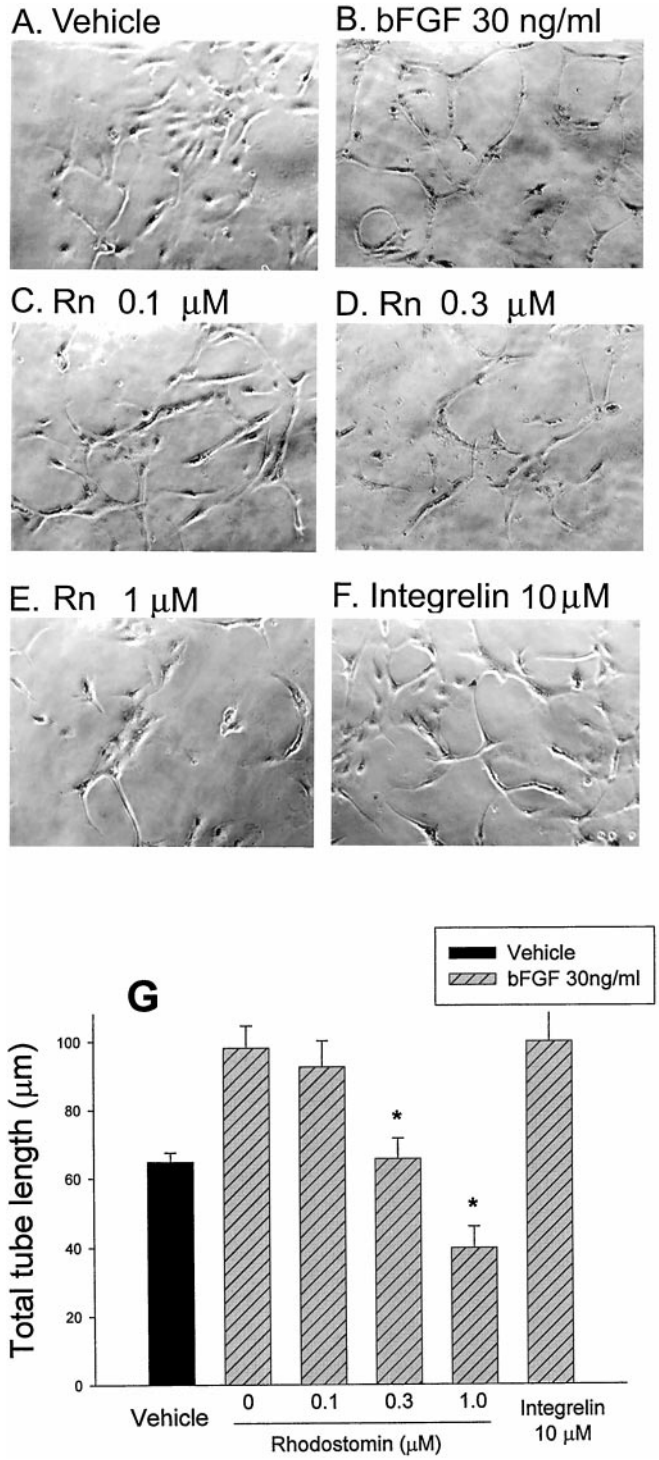

Fig. 4. Rhodostomin inhibited bFGF-induced HUVEC tube formation in diluted Matrigel. HUVECs (2×10^5/well) were treated with or without (vehicle, A and B) indicated concentration of test compounds, and then plated on diluted Matrigel (1 mg/ml) in the absence (A, no bFGF) or presence of bFGF (30 ng/ml, B–F) for 24 h. After washing and fixation, cells were observed under microscope at 40× magnification and photographed. This is a representative one of four similar experiments. G, total tube length from each randomly chosen field was quantified using image analysis system. Data are presented as mean ± S.E.M. ($n = 4$). *$P < 0.01$ versus bFGF alone.

intensity. Exposure to either bFGF or VEGF did not significantly affect the expression of $\alpha_v\beta_3$, but a 2-fold increment of $\alpha_v\beta_5$ expression was detected in VEGF-primed cells. Moreover, a conformational change of $\alpha_v\beta_3$ after bFGF or VEGF exposure was observed (Fig. 6A) as probed by an anti-LIBS1 mAb raised against ligand-induced binding sites of β_3 integrins (Frelinger et al., 1990). Rhodostomin inhibited 7E3 binding toward both naive and bFGF-primed HUVECs in a dose-dependent manner with IC_{50} values of 2.95 and 1.88 μM, respectively, but had little effect on P1F6 binding reaction under these conditions (Fig. 6B). Additionally, we conjugated rhodostomin with FITC and further performed the effects of anti-integrin mAbs on the interaction of FITC-conjugated rhodostomin with HUVEC. As shown in Fig. 7, incubation of HUVECs with 7E3 (20 μg/ml, Fig. 7A) significantly inhibited FITC-conjugated rhodostomin (1.3 μM) binding to HUVEC, whereas the incubation of HUVECs with P1F6 (20 μg/ml, Fig. 7B), anti-integrin α_2, α_3, α_4, or α_5 mAbs (all in 20 μg/ml, data not shown) showed little effect.

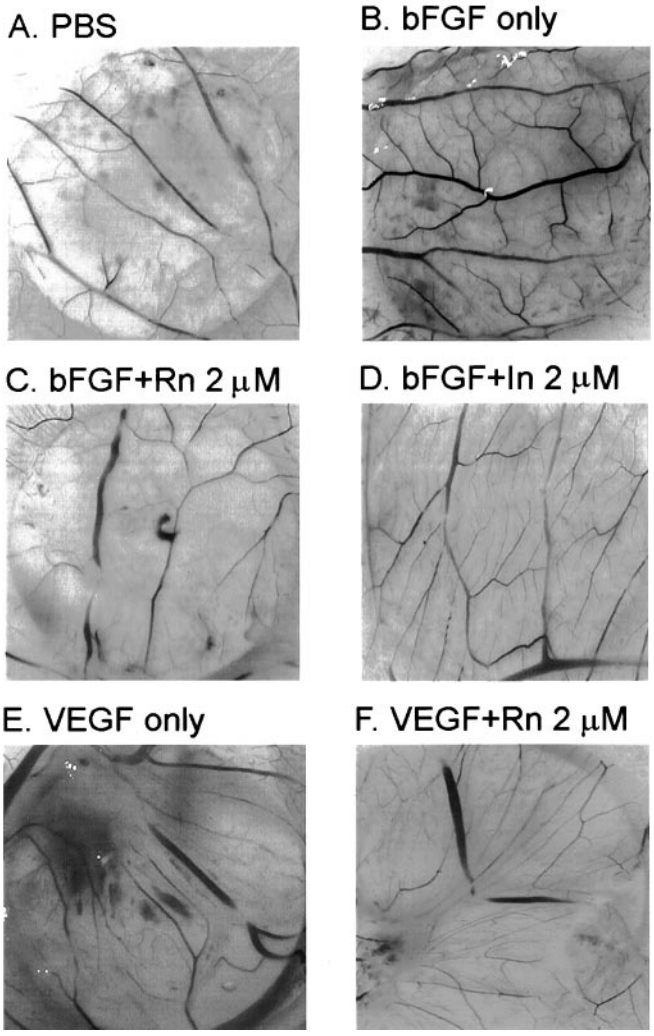

Fig. 5. Rhodostomin inhibited bFGF-induced angiogenesis in chick CAM assays. Filter disks soaked in buffer (A, vehicle), bFGF or VEGF alone (B and E, respectively, both in 200 ng/embryo), or growth factor plus indicated compounds in a total volume of 20 μl were applied on 10-day-old CAMs. After a 48-h incubation, each CAM under disk was resected, fixed and photographed. This experiment is representative of three similar experiments.

Effect of Rhodostomin on bFGF- and Tumor-Associated Angiogenesis in Matrigel Plug Assays. Matrigel serves as a vehicle for the slow release of angiogenic factor because it exists as a liquid at 4°C but forms a gel in vivo. The gel formed after subcutaneous injection of Matrigel alone was readily distinguished from surrounding tissue and produced little local reaction or angiogenic response (Passaniti et al., 1992). In the presence of bFGF (500 ng/mouse; Fig. 8A) or B16F10 murine melanoma cells (3×10^6/mouse; Fig. 8C). The Matrigel plug induced intense vascularization in vivo after 14 days after implantation as examined by immunohistological staining for von Willebrand factor, an endothelial cell-specific antigen. Rhodostomin (50 μM) significantly inhibited both bFGF (Fig. 8B) and B16F10 cell-induced angiogenesis (Fig. 7D) in the murine experimental model.

Effect of Rhodostomin on Subcutaneous B16F10 Tumor Growth and Survival Rate of Tumor-Bearing Mice. To examine the antitumor activity of disintegrin, we

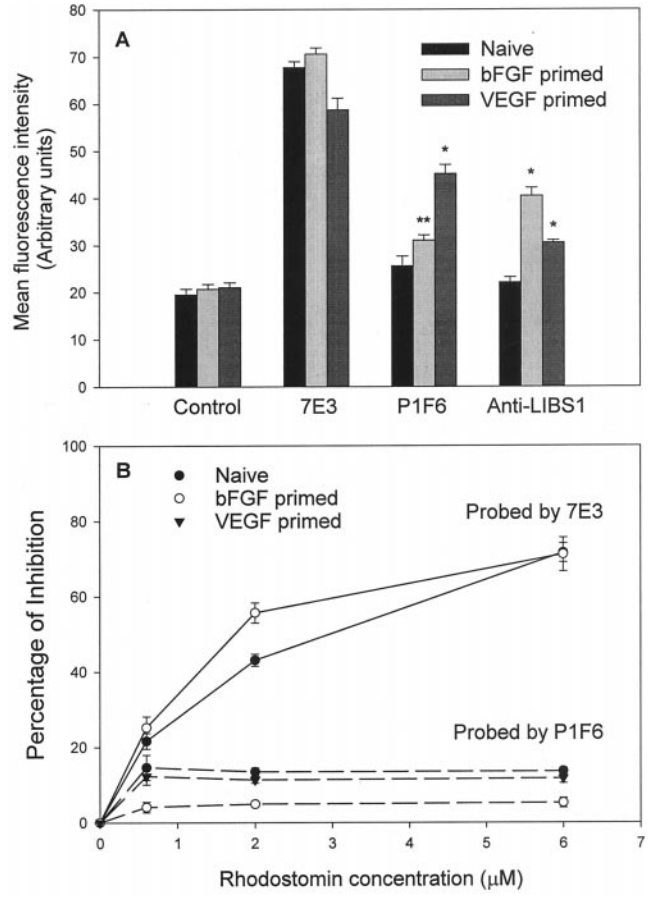

Fig. 6. A, effect of growth factors on HUVEC integrin expression and their binding affinity. HUVECs exposed to the standard medium (naive), bFGF or VEGF (both in 30 ng/ml, 48 h) were incubated with primary probe 7E3, P1F6 (both in 20 μg/ml), or anti-LIBS1 mAb (0.17 μM), and analyzed by flow cytometry. Nonspecific binding was performed by incubating HUVECs with nonimmune IgG (control, 1:50 dilution). Results are represented as mean fluorescence intensity ± S.E.M. ($n = 4$). *$P < 0.01$, **$P < 0.05$ versus naive. B, rhodostomin inhibited 7E3 but not P1F6 binding to bFGF-primed HUVECs. Naive or growth factor-primed HUVECs were pretreated with indicated concentration of rhodostomin and then probed with 7E3 or P1F6. After incubation with IgG-FITC, the mean fluorescence intensity of cells was measured by flow cytometry. Results are presented as percentage inhibition of mean fluorescence intensity ± S.E.M. ($n = 4$) compared with the respective vehicle (in the absence of rhodostomin).

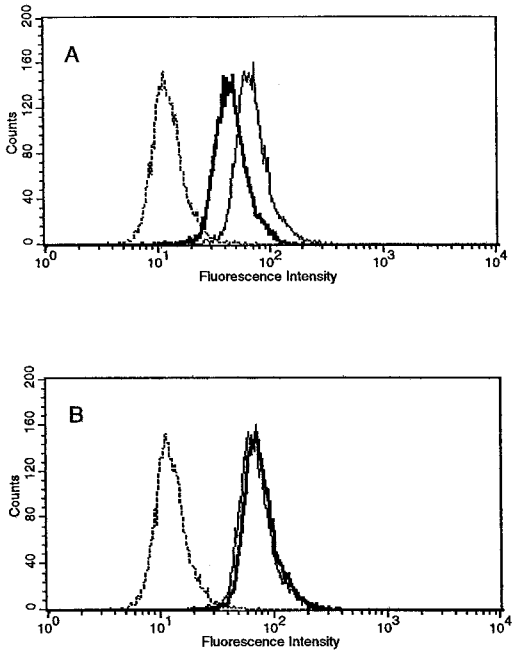

Fig. 7. Effect of 7E3 and P1F6 on FITC-conjugated rhodostomin interaction with HUVEC. HUVECs pretreated with 7E3 (A), P1F6 (B) (both in 20 μg/ml, heavy lines), or nonimmune IgG (1:50 dilution, A and B, thin lines) were incubated with FITC-conjugated rhodostomin (1.3 μM) and analyzed by flow cytometry. Nonspecific binding was carried out by incubating cells with FITC-conjugated BSA (A and B, dashed lines). The tracing of PBS (control HUVECs) and that of nonimmune IgG pretreated HUVECs was almost identical. Similar results were obtained in at least three separate experiments.

examined the effect of rhodostomin on B16F10 tumor model in C57BL/6 mice. As shown in Fig. 9A, the B16F10 tumor-bearing mice exhibited an aggressive tumor growth kinetic, as seen in the control group (PBS treatment). After coadministration of B16F10 cells with rhodostomin (5 and 50 μM), both tumor growth rate and tumor volumes were significantly reduced compared with those coinjected with B16F10 cells with PBS or eptifibatide (50 μM). Figure 9B showed the tumor-bearing mice receiving rhodostomin or eptifibatide in situ on day 17. It was necessary to sacrifice the animals receiving coinjection of B16F10 cells with PBS or eptifibatide (50 μM) because the tumors were large (i.e., 4806.56 $\pm$ 614.58 and 4530.7 $\pm$ 719.56 mm^3, respectively). In contrast, cells coinjected with rhodostomin (5 and 50 μM) grew significantly smaller tumors in mice (i.e., 2908.42 $\pm$ 445.39 and 485.29 $\pm$ 134.83 mm^3, respectively), compared with those arising from the control group ($P < 0.05$ and $P < 0.01$, respectively). Moreover, the survival rates of B16F10 tumor-bearing animals receiving rhodostomin (5 and 50 μM) were also obviously increased over those mice receiving vehicle (PBS) or eptifibatide (50 μM; Fig. 10). Thus, rhodostomin seems to be effective in treatment of mice suffering the solid tumor progression of B16F10 melanoma, not only by reducing the growth rate of tumor, but also by reducing the consequent mortality.

Discussion

During angiogenesis, a number of integrins expressed on the surface of activated endothelial cells regulate critical adhesive interactions. Each of these adhesive interactions may regulate distinct biological events, such as migration, proliferation, and differentiation. Eliceiri and Cheresh (1999) convincingly suggest a functional role of integrin $\alpha_v\beta_3$ in vascular development and tumor angiogenesis. Blockade of $\alpha_v\beta_3$ function by antibodies or RGD peptides disrupts blood vessel formation in various animal models. These $\alpha_v\beta_3$ antagonists perturb the growth and/or maturation of blood vessels without detectable alteration on the pre-existing blood vessels. Moreover, inhibition of blood vessels by α_v integrin antagonists not only blocks tumor-associated angiogenesis but also causes the regression of established tumor (Brooks et al., 1994b). These studies have created a drive to develop antagonists of α_v integrins as therapeutic agents for cancer (Mitjans et al., 2000).

The role of integrin $\alpha_v\beta_3$ in mediating crucial angiogenic

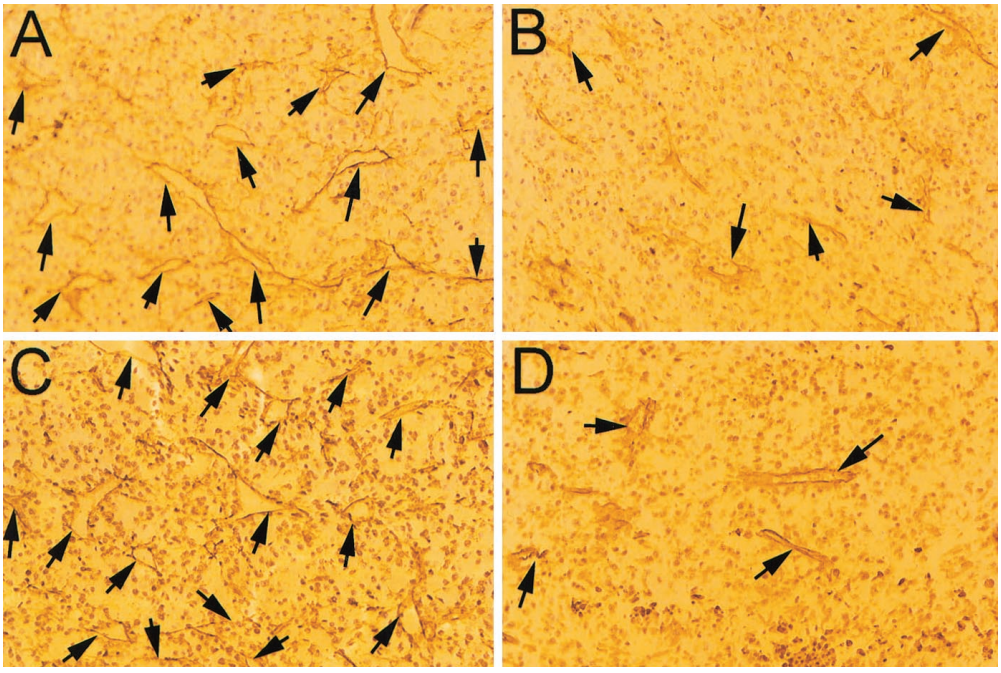

Fig. 8. Effect of rhodostomin on bFGF or B16F10 tumor melanoma cell-induced angiogenesis in Matrigel plug murine model. An aliquot of Matrigel (0.5 ml) supplemented with bFGF (500 ng, A and B) or B16F10 melanoma cells (3×10^6, C and D) in the absence (A and C) or presence of rhodostomin (50 μM, B and D) was subcutaneously injected into dorsal region of B57CL/6 mice. Analyses of the vasculature of respective Matrigel from the mice 14 days after implantation were performed by immunohistochemical staining for von Willebrand factor antigen that were indicated by arrowheads in figure. Magnification, 200×.

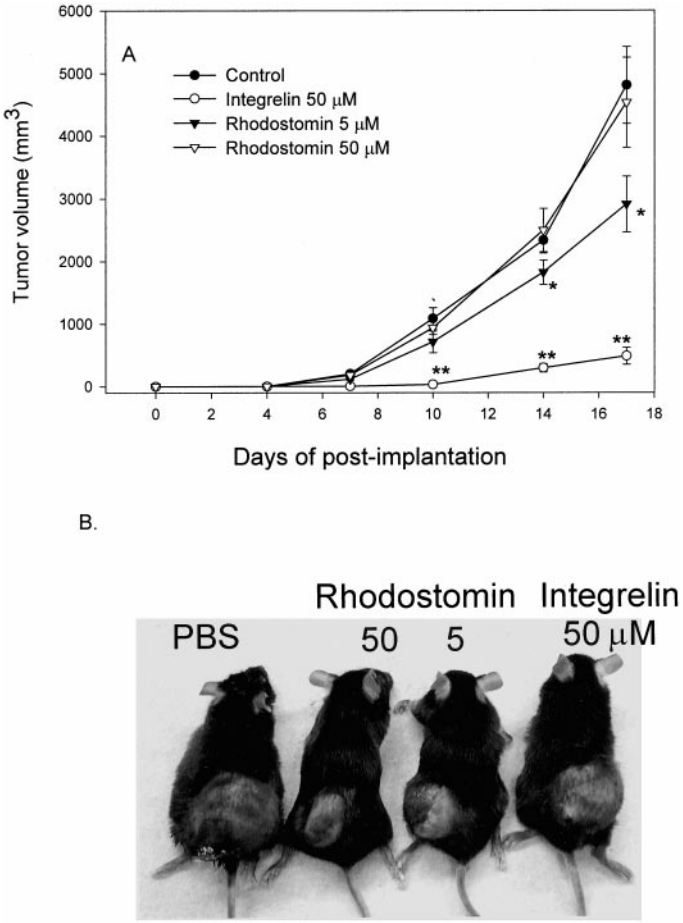

Fig. 9. Effect of rhodostomin on subcutaneous B16F10 tumor growth. A, B16F10 melanoma cells (3×10^6/mouse) were subcutaneously injected in dorsal region of B57CL/6 mice, in the absence (control, ●) or presence of rhodostomin [5 (▼) or 50 (○) μM] or eptifibatide (50 μM, ▽) in a final volume of 0.3 ml. Tumor volumes were monitored by periodic caliper measurements and presented as mean ± S.E.M., $n = 9$–11. *$P < 0.05$; **$P < 0.01$ compared with control group at the same day. B, tumor-bearing mice were shown in situ 17 days after coinjection of B16F10 cells with or without indicated compounds. This is a representative of one of three similar experiments.

processes has led to the conclusion that this integrin facilitates the survival of stimulated endothelial cells (Eliceiri and Cheresh, 1999). We showed here that rhodostomin inhibited bFGF-induced human endothelial cell proliferation at submicromolar concentrations as examined using various methods, including determination of total cell number, metabolic activity, and BrdU labeling index (Fig. 1, A-C). Even up to 50 μM, however, rhodostomin did not decrease B16F10 melanoma cell viability as determined by MTT assay (Fig. 1D). Several lines of evidence indicate that endothelial cell apoptosis plays a critical regulatory role in angiogenesis (Dimmeler and Zeiher, 2000). Moreover, primary cultured endothelial cells are anchorage-dependent. The selective anti-proliferating effect of rhodostomin on primary cultured endothelial cells is related to induce apoptosis as evidenced by flow cytometric cell cycle analysis, but it did not cause any cytotoxic effect as revealed by trypan blue exclusion. This is consistent with the previous observations that RGD peptides and disintegrin (i.e., accutin) induce an apoptotic effect toward HUVECs (Brooks et al., 1994b; Yeh et al., 1998), but not toward melanoma tumor cells (Mitjans et al., 2000; Allman et al., 2000). A similar result was also reported that $\alpha_v\beta_3$ integrin antagonist (i.e., echistatin, a disintegrin) induced detachment-activated apoptosis (Brassard et al., 1999).

A close interaction exists among cell motility, integrin receptors, and soluble angiogenic factors during each step of the angiogenic processes. Upon angiogenesis, integrin $\alpha_v\beta_3$ and the receptor for bFGF cooperate to promote signaling events, thereby facilitating the induction and/or maintenance of the angiogenic phenotype (Plopper et al., 1995). In the present study, we demonstrated that rhodostomin exhibited dose-dependent inhibitory effects on HUVEC migration and invasion evoked by bFGF (Figs. 2 and 3). Cytokines have been reported to trigger signaling pathways leading to modulate expression or/and ligand affinity of integrin $\alpha_v\beta_3$ on endothelial cells (Byzova et al., 1998; Yeh et al., 1999). Recent studies also showed that integrin $\alpha_v\beta_3$ could exist in different activation states (Byzova and Plow, 1998). Unlike the previous studies demonstrating that bFGF increases the expression of integrin $\alpha_v\beta_3$ in cultured endothelial cells (Enenstein et al., 1992; Klein et al., 1993), our data in this work indicate that exposure to bFGF promotes the ligand affinity of $\alpha_v\beta_3$ toward anti-LIBS1 mAb without increasing its expression on HUVECs as examined by flow cytometry (Fig. 6A). In addition, we observed that a decrease of IC_{50} value of rhodostomin on 7E3 binding to bFGF-primed cells compared with that of naive cells (1.88 versus 2.95 μM, respectively), suggesting that bFGF pretreatment may increase the binding affinity of integrin $\alpha_v\beta_3$ (Fig. 6B).

Several models have been used to study the role of cell adhesion molecules in angiogenesis, including Matrigel tube formation model, chick CAM assay, and murine Matrigel plug model (Stromblad and Cheresh, 1996). Rhodostomin is an effective inhibitor of bFGF-associated angiogenesis in vitro (Fig. 4) and in vivo (Fig. 5, A-D; Fig. 8, A and B) as examined by multiple models as described above. However, rhodostomin did not interfere with VEGF-associated forma-

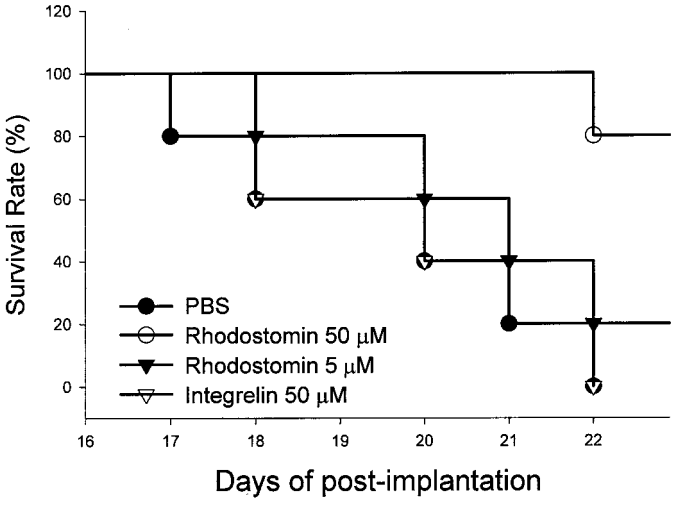

Fig. 10. Effect of rhodostomin on survival rate of B16F10 tumor-bearing mice. Mice (5 mice per group) were subcutaneously injected with 3×10^6 B16F10 melanoma tumor cells, in the absence (PBS, ●) or presence of rhodostomin [5 (▼) or 50 (○) μM] or eptifibatide (50 μM, ▽). The survival rates of tumor-bearing mice were calculated daily and the experiment was terminated when all the mice of control group died (at day 22).

tion of blood vessels in CAM model (Fig. 5, E and F). Regarding the binding site of rhodostomin toward HUVEC, our data demonstrate that rhodostomin dose-dependently inhibited binding of 7E3 (i.e., an anti-$\alpha_v\beta_3$ mAb) but not P1F6 (i.e., an anti-$\alpha_v\beta_5$ mAb) toward both naive and bFGF-primed HUVECs (Fig. 6B). We also showed that 7E3 but not P1F6 significantly inhibited FITC-rhodostomin binding to HUVEC (Fig. 7), confirming our observation that rhodostomin selectively inhibits 7E3 binding to HUVEC. Therefore, the action of mechanism of rhodostomin in suppressing bFGF-elicited angiogenesis is caused by the specific blockade of $\alpha_v\beta_3$ ligation rather than $\alpha_v\beta_5$ ligation of the activated endothelial cells. In addition to modulation of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins by bFGF and VEGF, respectively, as examined by flow cytometry (Fig. 6A), these results are consistent with the previous report that two cytokine-dependent pathways of angiogenesis have been proposed based on their dependence on distinct vascular cell α_v integrins (Friedlander et al., 1995). Furthermore, rhodostomin markedly inhibited tumor angiogenesis in murine Matrigel plug models elicited by B16F10 melanoma tumor cells (Fig. 8, C-D). The antitumor angiogenesis effects of rhodostomin may play a critical role in inhibiting growth rate of solid B16F10 melanoma tumor (Fig. 9) and in prolonging survival kinetics of tumor-bearing animals (Fig. 10). Similar results were also reported with salmosin and contortrostatin, two snake venom-derived disintegrins (Kang et al., 1999; Zhou et al., 2000).

In conclusion, many pharmacological and toxicological studies indicate that antagonists of $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ are well tolerated in different animal species, suggesting that these antagonists may be potentially effective and safe for therapeutic purpose. Most recently, the clinical potential of α_v-integrin antagonist (i.e., a humanized anti-$\alpha_v\beta_3$ mAb LM609 named Vitaxin) is currently being evaluated in phase II for late-stage cancer patients (Griffioen and Molema, 2000). Rhodostomin, a snake venom disintegrin, is an active inhibitor of bFGF- and tumor-induced angiogenesis responses, as evidenced by inhibition of HUVEC proliferation, migration, and differentiation, and in vivo angiogenesis elicited by bFGF and B16F10 melanoma cells, thereby inhibiting tumor growth and reducing mortality in mice. Taken together, disintegrin family, the naturally occurring RGD-containing small peptides, may be used as lead compounds for developing the potential therapeutic agents for angiogenesis-related diseases, including cancer.

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