

$\alpha_v\beta_3$ Integrin Antagonist S247 Decreases Colon Cancer Metastasis and Angiogenesis and Improves Survival in Mice¹

Niels Reinmuth, Wenbiao Liu, Syed A. Ahmad, Fan Fan, Oliver Stoeltzing, Alexander A. Parikh, Corazon D. Bucana, Gary E. Gallick, Maureen A. Nickols, William F. Westlin, and Lee M. Ellis²

Departments of Cancer Biology [N. R., W. L., F. F., O. S., C. D. B., G. E. G., L. M. E.] and Surgical Oncology [S. A. A., A. A. P., L. M. E.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Oncology Pharmacology, Discovery Research, Pharmacia Corporation [M. A. N., W. F. W.], St. Louis, Missouri 63198

ABSTRACT

Members of the integrin family influence several aspects of tumor progression and metastasis, including cell survival, proliferation, and angiogenesis. Specific integrins such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are involved in regulating endothelial cell function, and thus angiogenesis. We evaluated the effect of the $\alpha_v\beta_3/\alpha_v\beta_5$ integrin antagonist S247 on the growth and angiogenesis of colon cancer liver metastases in an orthotopic murine model. Murine colon cancer cells were injected into the spleens of BALB/c mice to produce liver metastases. On day 7, miniature osmotic pumps were implanted into the subcutis to continuously infuse either saline or 70 mg/kg/day S247. All mice were sacrificed when control mice became moribund. Mice that received S247 developed significantly fewer liver metastases than did controls ($P < 0.05$). Using the same model, a subsequent survival study was performed. Mice were sacrificed when moribund as determined by an observer blinded to the treatment given. Treatment with S247 significantly prolonged overall survival ($P < 0.05$). Interestingly, primary tumors in the spleen were the cause of death in the S247-treated group as S247 appeared to have little effect on these tumors. Immunohistochemical staining demonstrated a significant reduction of vessels in liver metastases of S247-treated mice ($P < 0.001$), a significant increase in endothelial cell apoptosis ($P < 0.05$), and a significant decrease in pericyte coverage ($P < 0.0001$). To determine the role of S247 on angiogenesis, we examined the effect of S247 *in vitro* on human umbilical vein endothelial cells (HUVECs) and human vascular smooth muscle cells (hVSMCs). The addition of S247 to HUVECs and hVSMCs growing on vitronectin-coated flasks and in Matrigel significantly impaired cell growth and colony formation, respectively ($P < 0.05$). Furthermore, S247 completely inhibited the attachment of HUVECs and hVSMCs and increased apoptosis by six- to 9fold compared with controls. In *in vitro* invasion assays, S247-treated cells demonstrated decreased migration ($P < 0.05$). In conclusion, S247 demonstrated significant antimetastatic and antiangiogenic activity and impaired both endothelial and hVSMC/pericyte function *in vitro* and *in vivo*. The use of agents such as integrin antagonists that target multiple cell types involved in angiogenesis may be a more effective method of inhibiting angiogenesis than agents targeting only the endothelial cells.

INTRODUCTION

Integrins are heterodimeric transmembrane receptor complexes composed of noncovalently bound α and β chains that serve as receptors to the ECM.³ At present, 18 α and 8 β subunits are known,

forming 24 different $\alpha\beta$ heterodimers and thereby defining ligand specificities (reviewed in Refs. 1, 2). Integrins can mediate cellular adhesion to proteins of the ECM and to adjacent cells, and this ligand/receptor interaction can initiate intracellular signaling. Ligands for various integrins include fibronectin, collagen, and vitronectin, all components of the ECM. Certain integrins can also bind to soluble ligands such as fibrinogen or to other adhesion molecules on adjacent cells (2, 3).

Integrins appear to play a central role in mediating angiogenesis, which depends largely on EC interactions with the ECM and pericytes. Integrins appear to be involved in mediating EC adhesion to the ECM and, in turn, to contribute to EC migration, survival, and proliferation (1). Therefore, integrins may contribute not only to angiogenesis by initiating the development of new blood vessels but also to the survival of the newly formed vasculature.

To date, the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ appear to be the ones most closely associated with tumor angiogenesis (4, 5). Although only minimally expressed in quiescent blood vessels, $\alpha_v\beta_3$ is significantly up-regulated during angiogenesis *in vivo* (6). The interaction of $\alpha_v\beta_3$ and the ECM has been identified as a crucial event for EC survival in nascent vessels (7). In some cancer systems such as breast cancer, $\alpha_v\beta_3$ expression correlates with the aggressiveness of the disease (6). An alternative angiogenic mechanism seems to be provided by the integrin $\alpha_v\beta_5$, which may be involved in VEGF-mediated angiogenesis (8). Therefore, antibodies, antagonists, and small inhibitory peptides to these receptors have been developed as potential antiangiogenic strategies. For example, in one study, application of an $\alpha_v\beta_3$ monoclonal antibody suppressed angiogenesis of human melanoma fragments on chick chorioallantoic membranes and induced apoptosis of vascular ECs (7). In a mouse model using s.c. implanted RKO colon cancer cells, treatment with an $\alpha_v\beta_3$ antagonist resulted in decreased tumor growth and neovascularization as well as an increased tumor apoptotic index *in vivo*, as determined by immunohistochemical analysis (9). These studies imply that $\alpha_v\beta_3$ may be a potential target on ECs for specific antiangiogenic therapy.

A structure-activity-relationship-based rational medicinal chemistry effort led to the discovery of a small peptidomimetic antagonist of $\alpha_v\beta_3$ and $\alpha_v\beta_5$, S247 (10, 11). S247 is a potent antagonist of $\alpha_v\beta_3$ (IC₅₀ for inhibition of adhesion of transfected HEK 293 cells to vitronectin = 0.4 nM) and $\alpha_v\beta_5$ (IC₅₀ = 1.5 nM) *in vitro*. Oral administration of S247 to BALB/c mice implanted with s.c. CT26 murine colon carcinoma cells led to a significant, dose-dependent inhibition of primary tumor growth, as well as inhibition of the concomitant hypercalcemia observed in this model (10). Moreover, administration of S247 to mice in which MDA-MB-435 human breast carcinomas were orthotopically implanted in the mammary fat pads produced a dramatic reduction (>90%) in metastatic burden in the lungs (10, 11).

Inhibiting angiogenesis may be a rationale for preventing the growth of colon cancer hepatic metastases (12, 13). In the current

Received 9/19/02; accepted 3/3/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported, in part, by the Dr. Mildred Scheel Stiftung für Krebsforschung, Deutsche Krebshilfe (to N. R.), NIH Grant T-32 09599 (to S. A. A., A. A. P.), the Gillson Longenbaugh Foundation (to G. E. G., L. M. E.), and the RGK Foundation (to L. M. E.).

² To whom requests for reprints should be addressed, at Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 444, Houston, TX 77030-4009. Phone: (713) 792-6926; Fax: (713) 792-4689. E-mail: lellis@mail.mdanderson.org.

³ The abbreviations used are: ECM, extracellular matrix; EC, endothelial cell; HEK, human embryonic kidney; S247, (3S)-3-(3-bromo-5-chloro-2-hydroxyphenyl)-3-[[N-((5-(5-hydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)amino)pyridin-3-yl)carbonyl]glycyl]amino]-propanoic acid; VEGF, vascular endothelial growth factor; hVSMC, human vascular smooth muscle cell; HRP, horseradish peroxidase; IgG, immunoglobulin G; FAK, focal adhesion kinase; SMA, smooth muscle actin; PDGF, platelet-derived growth factor;

HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; OD, optical density; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; MTT, 3-[4,5-dimethyl-thiazol-2-yl]2,5-diphenyltetrazolium bromide.

study, we evaluated the effect of the integrin antagonist S247 on the growth and angiogenesis of colon cancer liver metastases in an orthotopic murine model. We also sought to determine the cellular processes involved in angiogenesis that were altered by the inhibition of integrin activity. Treatment with S247 significantly impaired the development of liver metastases and improved survival. This observation was associated with a decrease in blood vessel counts, an increase in apoptotic ECs, and a decrease in vessel coverage by perivascular cells (presumed to be pericytes). *In vitro*, S247 had similar effects on both ECs and hVSMCs. These observations not only demonstrate that S247 is a novel and effective antiangiogenic compound but also suggest that targeting $\alpha_v\beta_3$ may inhibit both ECs and perivascular cells. We propose that through the reduction of vessel coverage by perivascular cells, ECs might be more susceptible to apoptotic stimuli, thereby enhancing the antiangiogenic effect of therapy.

MATERIALS AND METHODS

S247. S247 is a highly potent and selective antagonist that inhibits binding of purified $\alpha_v\beta_3$ to vitronectin in a solid-phase receptor binding assay *in vitro* ($IC_{50} = 0.18$ nM; Ref. 14). In preliminary studies, human placenta-derived $\alpha_v\beta_3$ or human platelet-derived $\alpha_{IIb}\beta_3$ (15) were bound to 96-well microtiter plates and incubated with human plasma-derived vitronectin (14) conjugated to biotin (16) for detection purposes. Densitometric determination after coin-cubation of the labeled ligand with a competitor provides data that when analyzed using four-parameter fitting techniques (17) yields the IC_{50} . Comparison of the IC_{50} s for each receptor is a means to assess compound selectivity between the two known β_3 integrins, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ and to predict the effect of a compound in cell-based *in vitro* and *in vivo* efficacy models. S247 is greater than three orders of magnitude more selective for $\alpha_v\beta_3$ than for $\alpha_{IIb}\beta_3$, with an IC_{50} for purified $\alpha_{IIb}\beta_3$ binding to vitronectin of 244 nM.

Characterization of S247 in cell-based assays dependent on $\alpha_v\beta_3$ function indicated that this compound is effective at antagonizing physiological $\alpha_v\beta_3$. In human $\alpha_v\beta_3$ -transfected HEK 293 cell binding assays, S247 inhibited adhesion to vitronectin with an IC_{50} of 0.4 nM. Evaluation of the ability of S247 to antagonize $\alpha_v\beta_3$ in the HEK 293 cell transfectants demonstrated that this compound inhibited cell adhesion dependent on $\alpha_v\beta_3$ ($IC_{50} = 1.5$ nM). This is a desirable property in an angiogenesis inhibitor as $\alpha_v\beta_3$ plays a significant role in the formation of neovasculature and in tumor cell adhesion and migration. S247 and a biologically inactive analogue, S286, were obtained from Pharmacia Corporation (Oncology Pharmacology, Discovery Research, Pharmacia Corporation, St. Louis, MO).

Chemicals and Cell Cultures. Human vitronectin was purchased from Promega (Madison, WI). The following antibodies were used for immunohistochemical and Western blot analysis: monoclonal rat antimouse CD31 (PharMingen, San Diego, CA); HRP-conjugated goat antirat IgG and Texas Red-conjugated goat antirat IgG (Jackson Research Laboratories, West Grove, PA); HRP-conjugated rat antimouse IgG (Serotec, Harlan Bioproducts for Science, Inc., Indianapolis, IN); HRP-conjugated goat antimouse IgG (Jackson Research Laboratories); goat antimouse IgG (Jackson Research Laboratories); HRP-conjugated goat antimouse and HRP-conjugated goat antirabbit IgG (Bio-Rad Laboratories, Hercules, CA); mouse antihuman FAK (Becton Dickinson; for Western blotting); mouse antihuman FAK (Upstate Biotechnology, Lake Placid, NY; for immunoprecipitation); rabbit antihuman phosphorylated Akt and total Akt (Cell Signaling, Beverly, MA); and mouse antihuman α -SMA (Dako, Carpinteria, CA). Recombinant VEGF and PDGF were purchased from R&D Systems (Minneapolis, MN).

CT26 murine colon carcinoma cells, syngeneic to BALB/c mice, were obtained from the National Cancer Institute Tumor Bank (Bethesda, MD). HT29 human colon cancer cells and hVSMCs were purchased from the American Type Culture Collection (Manassas, VA). HUVECs were obtained from the American Type Culture Collection or Clonetics (San Diego, CA). Both colon cancer cell lines lack the integrin $\alpha_v\beta_3$ but express $\alpha_v\beta_5$ (12, 18, 19). Cells were maintained as previously described (20) and used in passage 2–6 after their receipt from the supplier. HT29-derived conditioned medium was harvested from 70% confluent cell cultures grown in 1% FBS-containing

medium for 48 h. For animal injection, cells were harvested from 50–60% confluent cultures and suspended in serum-free HBSS after determination of >90% viability by trypan blue dye exclusion.

Animals and Tumor Cell Inoculations. Eight-week-old male BALB/c mice (obtained from the National Cancer Institute's Animal Production Area, Frederick, MD) were acclimated for at least 1 week and caged in groups of 5. Each mouse was anesthetized by i.p. injection of 10 μ l of pentobarbital sodium/gram body weight. Single-cell suspensions (10,000 cells/50 μ l/mouse) were injected into the spleen as described previously (21). All animal studies were conducted according to a protocol approved by the Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center.

For one experiment, mice were randomly assigned to the S247 treatment group or the control group (10 mice/group). On postinjection day 7, miniature osmotic pumps (Alzet Durect, Cupertino, CA) were implanted into the subcutis on the backs of the mice. The pumps contained either saline (control) or 70 mg/kg/day S247 dissolved in saline and delivered the substances at a rate of 25 μ l/h over 14 days. All mice were sacrificed when control mice became moribund (postinjection day 20). A second experiment was designed to additionally investigate the development and response of colon cancer metastases to S247 treatment. Mice underwent splenic injections with CT26 cells as described above, and osmotic pumps (delivering 70 mg/kg/day S247 or saline; 5 mice/group) were implanted on day 7. Additionally, a bolus injection of 10.75 mg/kg S247 or saline was given i.p. as a loading dose to accelerate the achievement of an effective serum concentration to potentially improve the efficacy of this therapy. The serum concentration of S247 was then maintained by the osmotic pump. Mice were sacrificed on day 7 (before treatment), day 14, or day 21 (after 7 or 14 days of treatment) after splenic injection.

Simultaneously, a survival study was performed [$n = 15$ mice (S247) and 17 mice (saline)]. Seven days after splenic injection of CT26 cells as described above, osmotic pumps delivering 70 mg/kg/day S247 or saline over 28 days were implanted s.c., and mice received an i.p. bolus injection of 10.75 mg/kg S247 or saline. Mice were checked twice daily and sacrificed when moribund as determined by an observer blinded to the therapy delivered. Remaining mice were sacrificed after 28 days of treatment.

Necropsy and Tissue Preparation. The mice were sacrificed by carbon dioxide asphyxiation and cervical dislocation. Spleens and livers were excised and weighed, and the numbers of surface liver metastases were counted. If no metastases were visible, the livers were cut in 1–2-mm slices to search for metastases within the parenchyma. Tumor sections were embedded in optimal cutting temperature compound (Sakura, Torrance, CA), frozen at -70°C , and later sectioned (8–10- μ m thick), mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA), air-dried for 30 min, and stored at -20°C .

Standard Immunohistochemistry. To study the overall tissue morphology, slides were stained with H&E (21). For assessment of angiogenesis, tumor vessels were stained for CD31 as previously described (21) and visualized with 3,3'-diaminobenzidine (Research Genetics, Huntsville, AL). Background counterstaining was performed with Gill's 3 hematoxylin (Sigma, St. Louis, MO) and mounted with Universal Mount (Research Genetics). For determination of pericyte coverage, double-staining for CD31 and α -SMA was performed using a previously described modification (22) of a procedure developed by Eberhard *et al.* (23).

Immunofluorescent Staining for CD31 and TUNEL. For immunofluorescent staining, frozen tissue was fixed in cold acetone and chloroform, washed with PBS, and incubated with primary and secondary antibodies as described previously (21, 22). The TUNEL assay was performed with a commercial kit (Promega) according to the manufacturer's protocol. Finally, tissue was counterstained and mounted with 4',6-diamidino-2-phenylindole fluorescence mounting medium (Vector Laboratories, Inc., Burlingame, CA). ECs were identified by red fluorescence, and DNA fragmentation was detected by localized green (tumor cell apoptosis) and yellow (EC apoptosis) fluorescence within the nuclei of apoptotic cells.

Quantification of Apoptotic Cells. Tumor and EC apoptosis was determined by localized green (tumor cell) or green with red, producing yellow (EC) fluorescence. Apoptotic tumor cells were counted in four random areas at $\times 100$ magnification. Quantification of apoptotic ECs was expressed as a mean of the ratio of apoptotic ECs to the total number of ECs in four different areas containing distinct TUNEL-positive cells at $\times 200$ magnification. Morphologically necrotic areas were excluded from all analyses.

Quantification of Vessel Density and Pericyte Coverage. Tumor vessels were counted at the tumor edge in high vessel density areas (hot spots) in four distinct fields (at 3, 6, 9, and 12 o'clock) at $\times 100$ magnification, a modification of the method described by Weidner (24). Necrotic areas were excluded.

The percentage of pericyte-covered vessels was then determined by the method of Eberhard *et al.* [percentage of pericyte coverage = (number of tumor microvessels that demonstrate pericyte colocalization/total number of tumor microvessels) $\times 100$; Ref. 23]. Pericyte-covered vessels were counted in four distinct high-density areas (hot spots) near the tumor edge at $\times 200$ magnification. Large tumor vessels were excluded (22, 23). (Note: Pericytes were defined as a single layer of α -SMA-positive cells surrounding CD31-positive cells.)

Determination of Cell Growth *in Vitro*. All *in vitro* experiments were performed at least in triplicate. For *in vitro* assays, 96-well plates were precoated with vitronectin (2 μ g/ml) for 18 h at 4°C and then washed with PBS containing 2% BSA (Sigma).

To examine monolayer cell growth, HUVECs, hVSMCs, HT29 human colon cancer cells, and CT26 murine colon cancer cells were plated in vitronectin-coated wells in equal densities. After attachment, cells were incubated in 10% FBS-containing medium with or without 10 μ M S247 or S286. After 48 h, the cell number was assessed by performing an MTT assay (22) or by using alamarBlue (BioSource International, Camarillo, CA) as a redox indicator.

To determine three-dimensional growth in an ECM-like environment, HUVECs and hVSMCs were seeded into Matrigel (Collaborative Biomedical Products, Bedford, MA) that was prepared as indicated by the manufacturer. After attachment, cells were treated with 10% FBS-containing medium with or without S247 (10 μ M) and/or VEGF (10 ng/ml for HUVECs), or PDGF-BB (20 mg/ml for hVSMCs). After 8 days of treatment, the colonies with diameters $> 10 \mu$ m were counted.

Determination of S247's Effect on $\alpha_v\beta_3$ Signaling. To determine the effect of S247 on $\alpha_v\beta_3$ signaling, HUVECs were trypsinized and suspended in medium containing 10% FBS and 2 μ g/ml vitronectin with or without S247 or S286 (10 μ M). After 30 min, protein was harvested from cell lysates, and FAK was immunoprecipitated from 300 μ g of the protein. Finally, Western blotting was performed for phosphorylated tyrosine and FAK as described previously (25). In addition, FAK was immunoprecipitated from HUVEC monolayer cultures treated with 10 μ M S247 for 30 min. In a separate experiment, HUVECs growing on vitronectin-coated flasks were incubated with or without 10 ng/ml VEGF for 1 h in 10% FBS-containing medium. S247 or S286 (10 μ M) was then added for 30 min, and protein was harvested from cell lysates. Western blot analysis was done for total and phosphorylated forms of Akt.

Determination of S247's Effect on Cell Attachment. To determine the effect of S247 on cell attachment, HUVECs and hVSMCs were suspended in 10% FBS-containing medium and then seeded on vitronectin-coated 96-well plates with or without S247 or S286 (10 μ M). After 30 min, cells were washed, and attached cells were counted at $\times 200$ magnification. In addition, MTT solution in 10% FBS-containing medium was added, and the absorbance was read at 570 nm after 90 min as described above.

Determination of Apoptosis of S247-treated Cells. HUVECs, hVSMCs, and HT29 and CT26 colon cancer cells were plated on vitronectin-coated flasks and incubated for 48 h to a final confluence of 60% in 10% FBS-containing medium. S247 or S286 was added to a final concentration of 10 μ M. Floating and attached cells were harvested after 8 and 24 h (brief trypsinization), resuspended in minimal essential medium containing 10% FBS, stained with 50 μ g/ml propidium iodide (Sigma), and analyzed by flow cytometry.

Determination of Migration and Invasion of S247-treated HUVECs and hVSMCs *in Vitro*. Matrigel-coated invasion chambers (Becton Dickinson) were seeded with 40,000 HUVECs or hVSMCs in 10% FBS-containing medium. After 2 h, the medium was changed to 10% FBS-containing medium (chambers) or HT29-derived conditioned medium (wells), and S247 or S286 was added to a final concentration of 5 μ M. We used a lower concentration of S247 in this experiment to reduce the pronounced effect of S247 on cell detachment. After 20 h, the invasion assay was stopped, nonmigrated cells were removed, and migrated cells were fixed and stained using a HEMA-3 kit (Biochemical Sciences, Swedesboro, NJ). Migrated cells were counted in five distinct areas at $\times 100$ magnification (22).

Statistical Analysis. Differences in Gaussian distributions were tested with the two-sided *t* test or Welch *t* test. Other analyses for statistical significance

were determined by the Mann-Whitney *t* test (InStat; GraphPad Software, San Diego, CA), unless otherwise specified. Significant outliers because of variations in tumor growth were excluded from additional analysis based on the Grubbs' test (GraphPad Software). Survival data were evaluated for statistical significance by the method of Kaplan and Meier using the log-rank test (Statistica 99, Kernel release 5.5A; StatSoft Inc., Tulsa, OK). The overall survival was calculated as the interval between implantation of tumor cells and death (or a moribund state as determined by a blinded observer).

RESULTS

Effect of S247 Therapy on Metastatic Potential of CT26 Colon Cancer Liver Metastases. No apparent toxicity from S247 or saline delivered via osmotic pump was observed. When control mice became moribund on day 20, the experiment was terminated. Necropsy confirmed that all control mice had liver metastases. Mice in the S247 treatment group developed significantly fewer liver metastases ($P < 0.01$; Fig. 1). This reduction in liver metastases occurred independent of a direct effect on primary tumor growth as the splenic tumor volume (primary tumor site) was not significantly different between the groups (mean \pm SE: controls, $835 \pm 198 \text{ mm}^3$; S247 group, $674 \pm 144 \text{ mm}^3$).

To investigate the effect of S247 in more detail, an experiment was implemented in which mice were sacrificed at various time intervals after tumor injection. No mice developed macroscopically visible liver metastases by day 7, before S247 or saline delivery began. All mice in the control group developed visible liver metastases within 14 days, but there were no visible liver metastases in the S247 treatment group after 14 days and significantly fewer visible liver metastases after 21 days ($P < 0.05$; Fig. 2).

Effect of S247 on Survival of Mice with Colon Cancer Liver Metastases. We also studied the effect of S247 on the survival of mice with liver metastases. After 28 days of treatment (day 36 of the experiment), the experiment was terminated because of the limited capacity of the osmotic pumps. At day 36, all 17 control mice had died, whereas 4 of 15 mice treated with S247 were still alive. Of these long-term survivors, 2 had no liver metastases, and the other 2 had one and three liver metastases each. Compared with saline, S247 treatment led to a significant improvement in overall survival ($P < 0.05$; Fig. 3). In contrast to the effect of S247 on the reduction of liver metastases, both control and S247-treated mice had large splenic tumors at the time the experiment was terminated (not significant; Fig. 3B). We believe that the mice in the S247 group died of their primary splenic

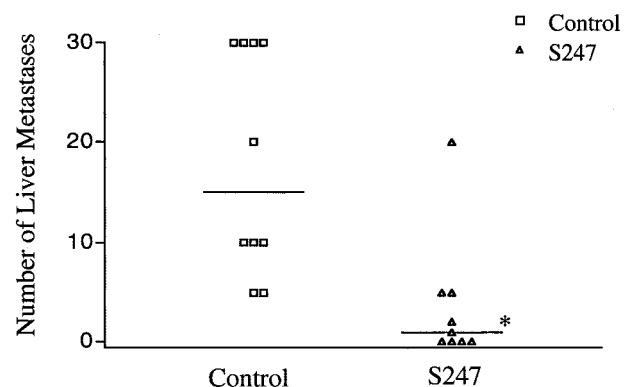


Fig. 1. Effect of therapy with the $\alpha_v\beta_3$ integrin antagonist S247 on the development of liver metastases. CT26 colon cancer cells were injected into the spleens of BALB/c mice. Starting on day 7, mice were treated with 70 mg/kg/day S247 or saline by continuous infusion via a s.c. implanted osmotic pump. When control animals became moribund, all mice were sacrificed, and liver metastases were harvested. Treatment with S247 significantly decreased the number of liver metastases ($*P < 0.01$; horizontal lines indicate median number of liver metastases).

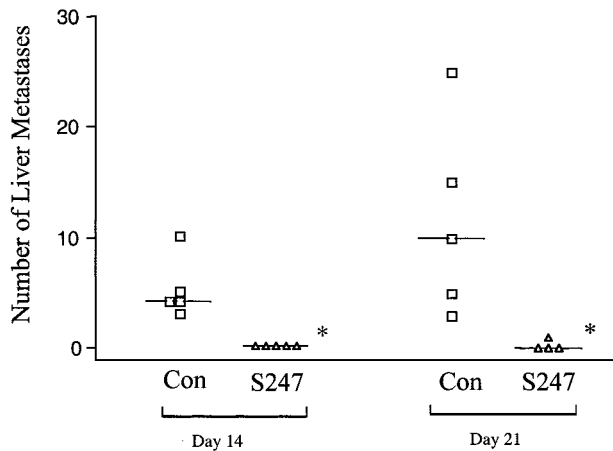


Fig. 2. Effect of therapy with S247 on the development of liver metastases after 14 and 21 days of tumor growth. CT26 colon cancer cells were injected into the spleens of BALB/c mice. On day 7, osmotic pumps containing 70 mg/kg/day S247 or saline were implanted for continuous therapy. Additionally, a bolus of 10.75 mg/kg S247 or saline was given i.p. Mice were sacrificed on day 7 (before implantation of the osmotic pumps), on day 14, or on day 21, and liver metastases were counted (*horizontal lines* indicate the median number of liver metastases). S247 led to significantly fewer liver metastases at 14 and 21 days (* $P < 0.05$).

bed tumors and gastric compression because there was minimal tumor burden in the liver. In contrast, animals from the control group appeared to have died of their hepatic tumor burdens, given the large amount of tumors found in the livers at necropsy.

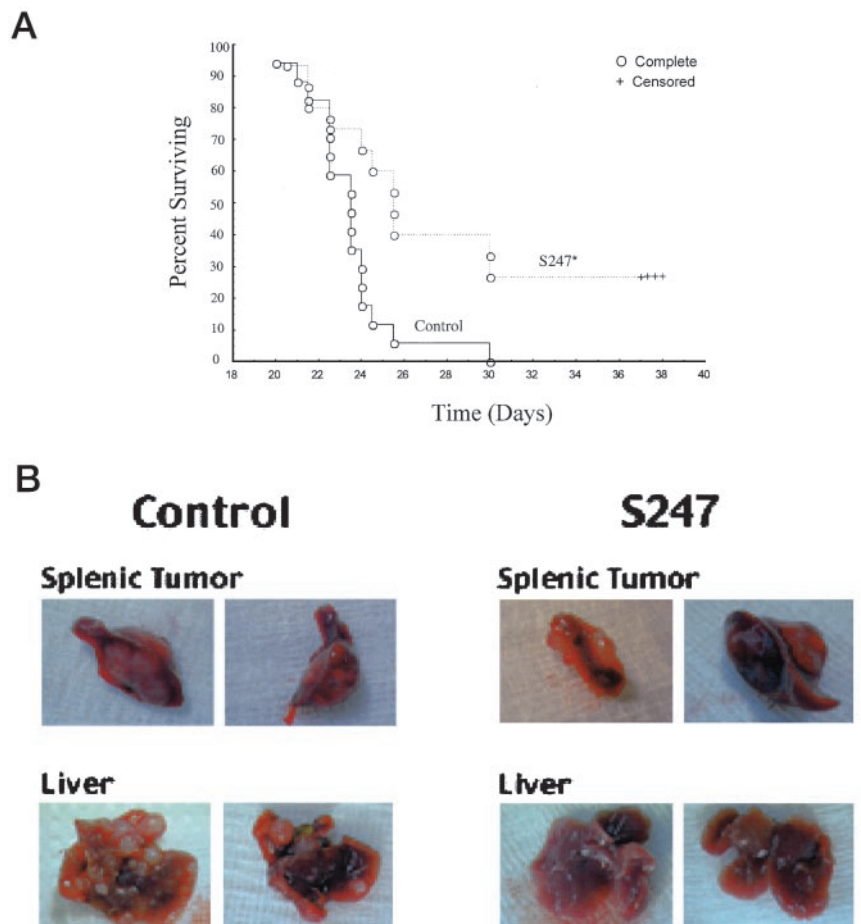
Effect of S247 on Tumor Cell and EC Apoptosis in Colon Cancer Liver Metastases. The impact of S247 on tumor cell and EC apoptosis was assessed by immunohistochemical analysis of liver

metastases from mice treated for 14 days with S247 or saline (Fig. 4). On H&E-stained sections, the metastases from S247-treated mice demonstrated vast necrotic regions that were nearly absent in controls (Fig. 4). Immunofluorescent TUNEL staining with and without concurrent staining for CD31 was performed to quantify tumor cell and EC apoptosis. In the control group, tumor cell and EC apoptosis was minimal, whereas S247-treated metastases demonstrated significantly more apoptotic tumor cells ($P < 0.01$; Fig. 5A). Furthermore, vessels in liver metastases from mice treated with S247 also demonstrated significantly more apoptotic ECs than did controls (Fig. 5B). Interestingly, no apparent effect of S247 was seen in normal liver tissue or liver sinusoidal ECs (data not shown).

Effect of S247 on Angiogenesis in Colon Cancer Liver Metastases. On CD31 immunohistochemical analysis, liver metastases from S247-treated mice demonstrated significantly fewer vessels than did liver metastases from control mice ($P < 0.001$; Figs. 4 and 6A). In addition, concurrent staining for CD31 and α -SMA was performed to quantify the percentage of tumor vessels that were covered by pericytes because these cells are hypothesized to be stabilizing factors for ECs (25). S247-treated tumors demonstrated significantly less pericyte coverage of vessels compared with control tumors ($P < 0.0001$; Fig. 6B). Representative photomicrographs are displayed in Fig. 4.

Effect of S247 on Cell Growth and $\alpha_{v\beta_3}$ Signaling *in Vitro*. Because of the profound impact of S247 on tumor cells and ECs *in vivo*, we additionally evaluated S247's effects *in vitro* on HUVECs, hVSMCs, HT29 cells, and CT26 cells. In monolayer cell cultures on vitronectin-coated surfaces, S247 significantly decreased the growth of HUVECs and hVSMCs after 48 h compared with medium only

Fig. 3. Effect of therapy with S247 on the survival of mice with liver metastases. CT26 colon cancer cells were injected into the spleens of BALB/c mice. Continuous treatment with 70 mg/kg/day S247 or saline was started on day 7 via osmotic pump, and a bolus of 10.75 mg/kg S247 or saline was given i.p. to accelerate therapeutic serum concentrations. Mice were sacrificed when moribund as determined by an observer blinded to the treatment given. On day 36, the experiment was stopped because the osmotic pumps ran empty. *A*, therapy with S247 significantly improved the overall survival of mice with liver metastases (* $P < 0.05$; log-rank test). *B*, representative photographs of livers from mice in each group are shown. Mice treated with S247 developed significantly fewer liver metastases compared with the control group. In contrast, the weight and volume of the primary splenic tumors were not significantly different.



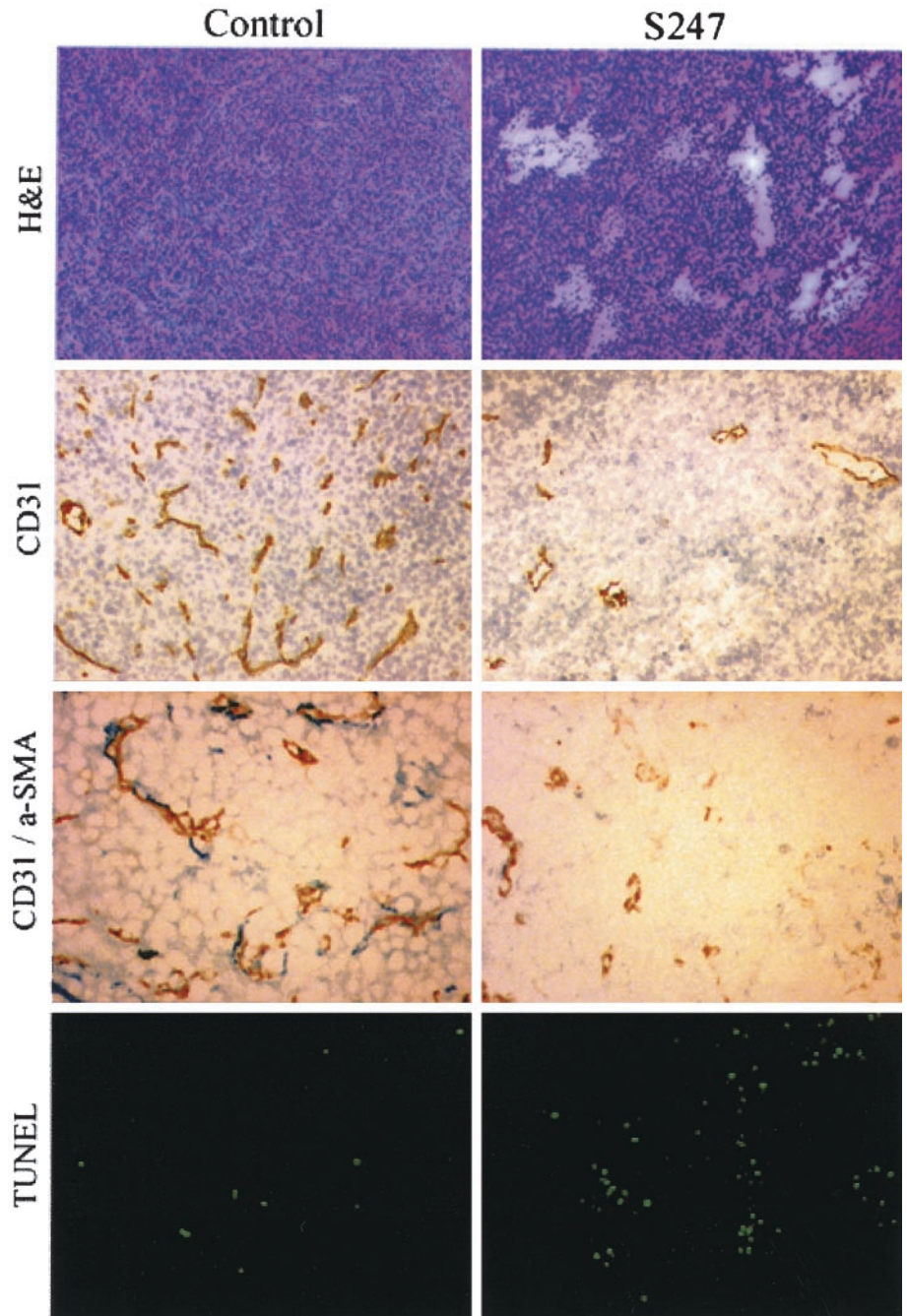


Fig. 4. Immunohistochemical analysis of liver metastases treated with S247. CT26 colon cancer cells were injected into the spleens of BALB/c mice, and 7 days later, mice began continuous treatment with S247 or saline for 14 days. Liver metastases were harvested, and frozen sections were stained by immunohistochemistry. Representative areas are shown for H&E ($\times 50$), CD31 ($\times 100$), CD31/ α -SMA ($\times 200$; brown/blue, respectively), and TUNEL staining ($\times 100$).

($P < 0.001$; Fig. 7A). In addition, S247 significantly decreased the number of CT26 cells within 48 h ($P < 0.05$; data not shown). There was no significant decrease in the number of HT29 cells within 48 h, but after 96 h, most HT29 cells underwent anoikis (data not shown). In a separate experiment, addition of S286, an inactive analogue to S247, showed no activity in a proliferation assay using HUVECs incubated with 10% FBS-containing medium with increasing doses of S286 up to 100 μM (data not shown).

Studying the three-dimensional growth in an ECM-like environment, S247-treated HUVECs and hVSMCs formed significantly fewer colonies ($P < 0.05$) compared with cells treated with 10% FBS-containing medium only (data not shown). The inhibitory effect of S247 on colony formation by HUVECs and hVSMCs could not be overcome by the addition of VEGF or PDGF-BB, respectively (not significant; data not shown).

We additionally sought to determine the effect of S247 on $\alpha_v\beta_3$ signaling. By Western blot analysis, incubation with S247 for 30 min did not decrease the phosphorylation of FAK of HUVECs in suspension or as attached cells in the presence of vitronectin (data not shown). Similarly, S247 did not affect the phosphorylation of Akt in HUVECs that have been prestimulated with VEGF for 1 h. (data not shown).

Effect of S247 on Cell Attachment and Apoptosis *in Vitro*. In the cell attachment assay, most HUVECs and hVSMCs in 10% FBS-containing medium with or without S286 attached within 30 min. In contrast, the presence of S247 completely blocked cell attachment ($P < 0.0001$; Fig. 7B). We also evaluated the effect of S247 on cell death in monolayer cultures on vitronectin-coated flasks. Within 24 h, S247 caused six to nine times more detachment and subsequent anoikis of HUVECs and hVSMCs compared with controls, whereas

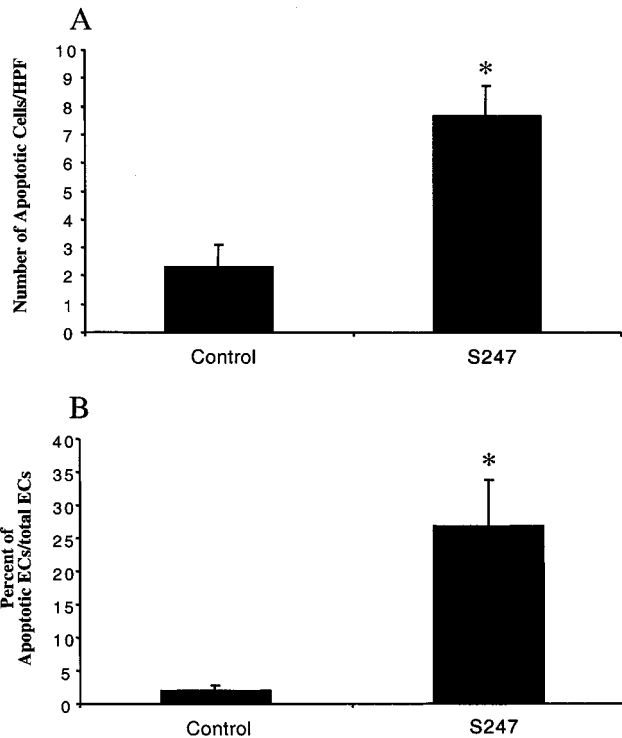


Fig. 5. Quantification of TUNEL-positive tumor cells and ECs in liver metastases from S247-treated mice. Tissue sections were double stained for TUNEL-positive or CD31-positive cells by fluorescent immunohistochemistry. Single TUNEL-positive cells (apoptotic tumor cells) and both TUNEL- and CD31-positive cells (apoptotic ECs) were counted in four distinct areas where there was a high density of CD31- or TUNEL-positive cells (hot spots). A, liver metastases from S247-treated mice contained significantly more apoptotic tumor cells than controls (*, $P < 0.01$). B, in S247-treated mice, vessels in liver metastases contained more apoptotic ECs compared with controls, as indicated by the ratio of apoptotic ECs/total ECs (* $P < 0.05$).

S286 had no significant effect on cell death (Fig. 7C). S247 caused about a 3-fold increase in anoikis in CT26 cells but had no effect on HT29 cells within 24 h (data not shown).

Migration and Invasion of HUVECs and hVSMCs Treated with S247 *in Vitro*. To minimize the effect of S247 on detachment, HUVECs and hVSMCs were plated in Matrigel-coated invasion chambers with HT29-conditioned medium as a chemoattractant. After attachment of the cells, S247 was added. S247 had no effect on cell morphology or attachment in any cell line studied. However, the addition of S247 significantly reduced the migration of HUVECs compared with controls ($P < 0.05$). The effect of S247 was even more distinct in hVSMCs ($P < 0.01$; Fig. 8). In contrast, treatment with S286 did not impair cell migration in either cell line.

DISCUSSION

The liver is the most common and critical site for the development of colorectal cancer metastases. The development of metastases results from a series of interlinked steps, described in detail elsewhere (26, 27). Integrins, in particular $\alpha_v\beta_3$, mediate the binding of ECs (and tumor cells) to the ECM, thereby affecting EC adhesion, migration, proliferation, and survival. However, the importance of $\alpha_v\beta_3$ in mediating colon cancer growth and metastasis is poorly understood. We therefore studied the effect of a novel $\alpha_v\beta_3$ integrin antagonist on colon cancer metastasis in a murine model. In our study, continuous therapy with S247 significantly decreased the development of liver metastases. Although control mice developed liver metastases within 14 days, S247-treated mice were free of macroscopically visible metastases at 14 days. After 21 days, when control mice became

moribund, S247-treated animals demonstrated significantly fewer liver metastases. In addition, treatment with S247 significantly improved survival. However, despite its favorable effects in inhibiting liver metastasis development, the effect of S247 on growth of the primary splenic tumor was minimal. Several authors have demonstrated that ECs in different organs are phenotypically distinct as the regulation of angiogenesis itself is also organ dependent (23, 28, 29). Furthermore, integrin expression and their ECM ligands vary in different organs (30–32). Likewise, the response to antiangiogenic therapy, including integrin antagonists, potentially may therefore depend on the site of tumor growth. In the survival experiment, all S247-treated mice developed a splenic tumor that was not significantly different from the splenic tumors of the animals from the control group. We believe the primary tumor led to death in the S247-treated animals for two reasons: (a) the primary splenic tumor distorted the stomach, which likely led to proximal bowel obstruction; and (b) mice in the S247-treated group had very little hepatic tumor burden.

Only a few publications have previously reported on the effect of anti- $\alpha_v\beta_3$ therapy on the development of liver metastases. In a study of $\alpha_v\beta_3$ -positive murine RAW117 large cell lymphoma sublines injected i.v. into mice, treatment with an anti- β_3 monoclonal antibody inhibited the formation of liver metastases (33). In a landmark study, Brooks *et al.* (7) implanted tumor fragments derived from various human carcinomas on chick chorioallantoic membranes. Application of an $\alpha_v\beta_3$ antibody (LM609) not only prevented the growth of these tumors but induced regression in most cases. Because $\alpha_v\beta_3$ staining was not detectable on tumor cells, the major effect of the antibody was hypothesized to be through the inhibition of angiogenesis. In fact, the anti- $\alpha_v\beta_3$ antibody induced apoptosis of proliferating angiogenic vas-

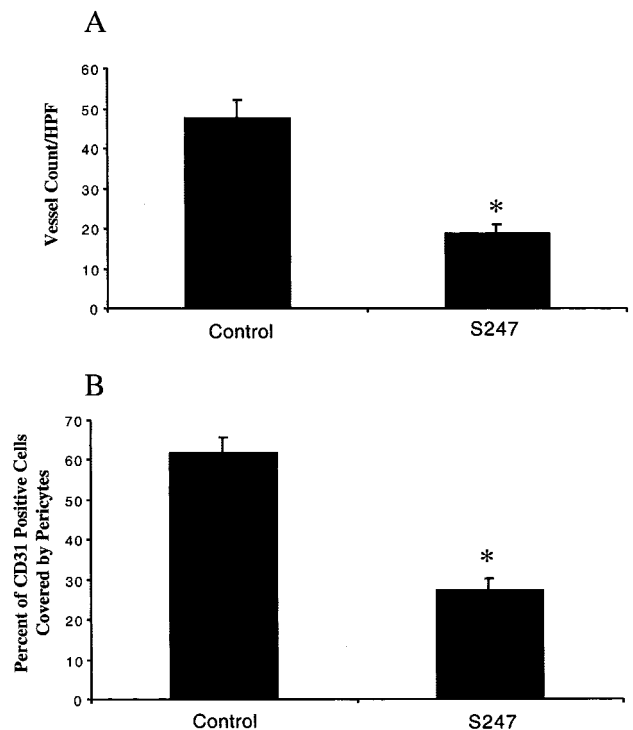


Fig. 6. Effect of S247 on vessel development and pericyte coverage in liver metastases. Frozen sections from liver metastases of S247-treated and control mice were stained for CD31 or CD31/ α -SMA. Four distinct areas at the tumor edge containing the most strongly stained vessels (hot spots) were evaluated. A, liver metastases from S247-treated mice developed significantly fewer vessels compared with control mice (* $P < 0.001$). B, vessels in liver metastases from S247-treated mice had significantly less pericyte coverage (* $P < 0.0001$).

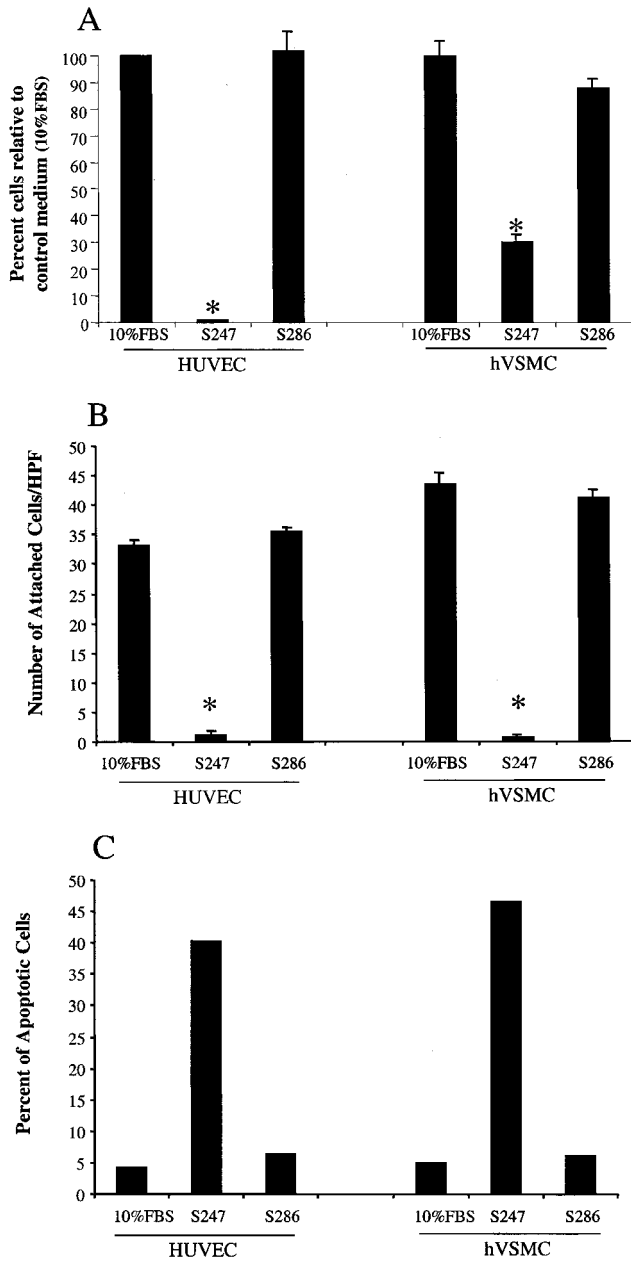


Fig. 7. Effect of S247 on growth, attachment, and apoptosis of HUVECs and hVSMCs. A, HUVECs and hVSMCs were plated on vitronectin-coated flasks at equal densities and incubated with or without S247 or S286 (10 μ M) for 48 h. A MTT or alamarBlue assay was performed, and the absorbance was read to provide an estimate of cell count based on metabolic growth rate. Data are presented as percent maximal proliferation to normalize the two assays. S247 significantly reduced the growth of both HUVECs and hVSMCs compared with controls as indicated by the decreased cell number relative to cells in 10% FBS as control medium ($*P < 0.001$). B, cells were suspended in 10% FBS-containing medium and then seeded on vitronectin-coated flasks with or without S247 or S286 (10 μ M). After 30 min, cells were washed and counted at $\times 200$ magnification. The addition of S247 completely abolished cell attachment, whereas treatment with S286 had no effect ($*P < 0.0001$). C, cells were plated on vitronectin-coated flasks and incubated for 48 h to a final density of 60%. Then, the medium was replaced with 10% FBS-containing medium with or without S247 or S286 (10 μ M). Floating and attached cells were harvested after 24 h and stained with propidium iodide, and the fraction of apoptotic cells was determined by flow cytometry (subdiploid population). After 24 h, S247 caused a 6–9-fold increase in apoptosis compared with controls.

cular cells without affecting quiescent blood vessels (7). Therefore, anti- $\alpha_v\beta_3$ therapy seems to target newly sprouting blood vessels without affecting ECs in mature vessels (7). Similarly, HT29 and CT26 colon cancer cells lack $\alpha_v\beta_3$ expression (12, 18, 19). However, our data suggest that anti- $\alpha_v\beta_3$ therapy alone might be very effective

in inhibiting the development and growth of metastasis of human colon cancer.

The delay in metastasis growth caused by anti- $\alpha_v\beta_3$ therapy in our study is in agreement with findings in studies investigating antiangiogenic therapy. Most antiangiogenic therapy is intended to decrease the development of new blood vessels. Although rare reports on tumor regression exist (34), the vast majority of studies demonstrate that antiangiogenic therapy leads to inhibition of tumor growth rather than regression of established tumors (21, 35). Thus, therapy with a combination therapy of antiangiogenic and antitumor agents might lead to tumor regression and thereby improve therapeutic results (36).

Using immunohistochemical analysis, we studied the mechanism of the inhibition of tumor growth by the $\alpha_v\beta_3$ antagonist S247 in mice in which treatment did not completely suppress liver metastasis formation. On average, the vessel count was decreased by 60% in S247-treated mice compared with controls. This finding is supported by our additional finding of a significant increase in apoptotic ECs. In addition, S247 caused an increase in tumor cell apoptosis. Furthermore, morphological evaluation of metastases from S247-treated mice revealed vast necrotic regions in comparison to control tumors. The effect of S247 on tumor cell apoptosis might be secondary to its effect on tumor vessels; still, a possible direct effect of S247 on the CT26 cells used in our experiments cannot be excluded because of effects of S247 on both $\alpha_v\beta_3$ and $\alpha_v\beta_5$. The absence of the integrin $\alpha_v\beta_3$ has been demonstrated in a variant of CT26 cells (12); however, this tumor cell line adheres to vitronectin-coated plates and expresses the integrin $\alpha_v\beta_5$ (18, 19, 37), the most widely expressed vitronectin receptor integrin in malignant tumor cells (31). S247 is a potent antagonist of $\alpha_v\beta_5$ (1.5 nM), as well as $\alpha_v\beta_3$, as determined in a cell binding assay using HEK 293 cells transfected with recombinant human $\alpha_v\beta_5$. $\alpha_v\beta_5$ mediates cell adhesion and acts coordinately with growth factor-derived signaling events to promote and enable cell migration (38, 39). Thus, tumor cell metastasis can be regulated by $\alpha_v\beta_5$ in a manner independent of tumor cell growth (39). In our *in vitro* studies, we did observe an effect of S247 on CT26 cell adhesion to vitronectin, suggesting an effect of S247 directly on $\alpha_v\beta_5$ function. However, this effect was much less pronounced than that seen on HUVECs or VSMCs ($\sim 33\%$). Taken together with the fact that S247 did not affect primary tumor growth *in vivo*, we theorize that the major antitumor effect was its ability to inhibit angiogenesis of metastasis.

Morphologically, we noted S247 led to smaller, less clearly defined vessels in metastases compared with controls. Therefore, we sought to

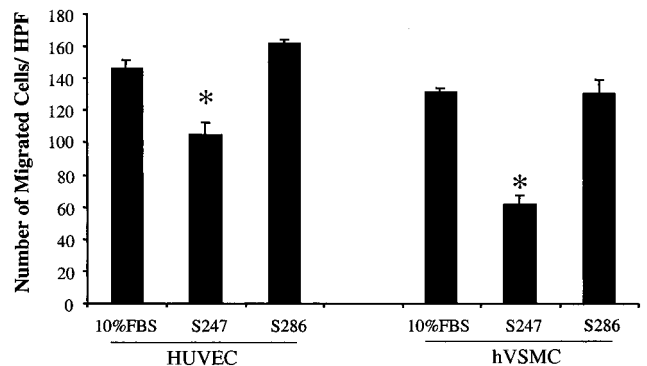


Fig. 8. Effect of S247 on invasion of HUVECs and hVSMCs. Cells were plated on Matrigel-coated invasion chambers in 10% FBS-containing medium. After 2 h, the medium was changed to 10% FBS-containing medium (chambers) or human HT29-derived conditioned medium (wells) with or without S247 or S286 (5 μ M). After 20 h, migrated cells were fixed and stained with H&E and then counted in five distinct areas at $\times 100$ magnification. Treatment with S247 significantly inhibited invasion of both HUVECs and hVSMCs compared with controls [$*P < 0.05$ (HUVECs), $P < 0.01$ (hVSMCs)].

determine the effect of S247 on pericyte coverage because pericytes are EC-supportive structures. Pericytes are known to be closely associated with ECs and seem to be critical for proper vascular development and maintenance (40). Colocalized ECs and pericytes have recently been used to define mature blood vessels (23). We have previously shown that in addition to pericytes providing a direct mechanical stabilizing function on ECs, pericytes are also likely to protect ECs from apoptosis by a paracrine mechanism via VEGF secretion (25). Because pericytes are present in tumor vessels to varying degrees (23), we developed an immunohistochemical method to study the colocalization of perivascular cells and ECs. In S247-treated metastases, pericyte coverage was significantly decreased. Therefore, anti- $\alpha_v\beta_3$ therapy affects not only ECs but also perivascular cells. These novel *in vivo* findings are supported by previous *in vitro* studies showing that $\alpha_v\beta_3$ binding to the ECM is necessary for hVSMCs to migrate in response to insulin-like growth factor I (41, 42).

To better understand the mechanism of S247 therapy on the cells that comprise the tumor vasculature, we evaluated S247's effect on proliferation, adhesion, migration, and apoptosis of ECs and hVSMCs *in vitro*. Cell growth and colony formation of HUVECs and hVSMCs were significantly reduced by S247 in monolayer or three-dimensional cell cultures, respectively. With prolonged exposure of attached monolayer HUVEC and hVSMC cultures to S247, most cells became round, detached from the vitronectin-coated flask surface, and underwent anoikis. Anoikis describes the subset of apoptosis triggered by loss of anchorage. The preceding change in cell shape (round) governs the subsequent anoikis (43) and seems to indicate one mechanism of the activity of S247. In contrast, treatment with S286 (an inactive analogue of S247) had no effect on cell shape, and cells remained attached. Furthermore, the addition of S247 completely inhibited new attachment of HUVECs and hVSMCs, whereas S286 had no effect on cell attachment in either cell line. There was also a less prominent direct effect of S247 on cell growth of CT26 and HT29 colon cancer cells, possibly attributable to the inhibitory action of S247 on $\alpha_v\beta_5$.

To date, the molecular mechanisms of S247's interfering with adhesion and apoptosis remain unknown. Among a variety of signaling cascades, the phosphorylation of FAK and activation of the phosphatidylinositol 3'-kinase/Akt pathway have been demonstrated to be involved in $\alpha_v\beta_3$ -mediated adhesion, migration, and cell survival (44–46). However, incubation of HUVECs with S247 did not decrease the phosphorylation of either FAK or Akt. Because integrins are involved in numerous pathways, S247 is likely to inhibit signaling independently of FAK or Akt. For example, the integrin-mediated activation of Ras has recently been implicated in preventing anoikis in a manner that is independent of phosphatidylinositol 3'-kinase (47). Furthermore, there is evidence of cross-signaling between different integrin receptors. In macrophages that were transfected with $\alpha_v\beta_3$ integrin, the inhibition of $\alpha_v\beta_3$ also partially blocked $\alpha_5\beta_1$ -mediated phagocytosis (48). Moreover, cell behavior may be dependent on different integrins that are expressed secondary to alterations in culture conditions (43).

Finally, we determined the effect of S247 on migration and invasion of HUVECs and hVSMCs. The addition of S247 significantly decreased migration of both HUVECs and hVSMCs in Matrigel. Interestingly, the effect of S247 was more prominent on hVSMCs, suggesting that these cells are as important and relevant targets for anti $\alpha_v\beta_3$ integrin therapy.

In conclusion, the novel integrin antagonist S247 targets ECs and perivascular cells both *in vitro* and *in vivo*. In cell cultures, S247 caused detachment and apoptosis and inhibited cell growth, attachment, and migration. In a murine model, S247 therapy significantly inhibited the development of liver metastases from colon cancer and

increased survival. The major effect of S247 appeared to be related to its ability to inhibit tumor angiogenesis. The vast decrease in vessel numbers seen might be due not only to an increase in apoptotic ECs but also to reduced coverage of vessels by perivascular cells (pericytes). Therefore, anti- $\alpha_v\beta_3$ treatment may also affect the stabilization of ECs through a decrease in pericyte coverage, in addition to its known apoptotic effect on activated ECs. Studies are needed to additionally address perivascular cells as targets for anti- $\alpha_v\beta_3$ therapy. S247 seems to be a very promising antiangiogenic compound that may also interfere with integrins other than $\alpha_v\beta_3$, thereby increasing its therapeutic effect. For clinical studies, combination therapy with S247 and known cytotoxic agents may be promising as a strategy to inhibit the growth of primary tumors and metastases.

ACKNOWLEDGMENTS

We thank Melissa G. Burkett of the Department of Scientific Publications for editorial assistance.

REFERENCES

- Ivaska, J., and Heino, J. Adhesion receptors and cell invasion: mechanisms of integrin-guided degradation of extracellular matrix. *Cell. Mol. Life Sci.*, 57: 16–24, 2000.
- Hynes, R. O. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, 69: 11–25, 1992.
- Ruoslahti, E., and Pierschbacher, M. D. New perspectives in cell adhesion: RGD and integrins. *Science (Wash. DC)*, 238: 491–497, 1987.
- Friedlander, M., Theesfeld, C. L., Sugita, M., Fruttiger, M., Thomas, M. A., Chang, S., and Cheresch, D. A. Involvement of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in ocular neovascular diseases. *Proc. Natl. Acad. Sci. USA*, 93: 9764–9769, 1996.
- Brooks, P. C., Clark, R. A., and Cheresch, D. A. Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science (Wash. DC)*, 264: 569–571, 1994.
- Gasparini, G., Brooks, P. C., Biganzoli, E., Vermeulen, P. B., Bonoldi, E., Dirix, L. Y., Ranieri, G., Miceli, R., and Cheresch, D. A. Vascular integrin $\alpha_v\beta_3$: a new prognostic indicator in breast cancer. *Clin. Cancer Res.*, 4: 2625–2634, 1998.
- Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresch, D. A. Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell*, 79: 1157–1164, 1994.
- Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresch, D. A. Definition of two angiogenic pathways by distinct α_v integrins. *Science (Wash. DC)*, 270: 1500–1502, 1995.
- Kerr, J. S., Wexler, R. S., Mousa, S. A., Robinson, C. S., Wexler, E. J., Mohamed, S., Voss, M. E., Devenny, J. J., Czerniak, P. M., Gudzelak, A., Jr., and Slee, A. M. Novel small molecule α_v integrin antagonists: comparative anti-cancer efficacy with known angiogenesis inhibitors. *Anticancer Res.*, 19: 959–968, 1999.
- Meyer, D., Nickols, G., Pegg, J., and Westlin, W. An oral $\alpha_v\beta_3$ antagonist, S-247, induces tumor regression and inhibits hypercalcemia of malignancy in a syngeneic mouse colon model. *Proc. Am. Assoc. Cancer Res.*, 42: 825, 2001.
- Griggs, D., Shannon, K., Settle, S., Duffin, T., Nickols, M., Schroeter, S., Nickols, G., and Westlin, W. Anti-metastatic efficacy mediated by peptidomimetic $\alpha_v\beta_3$ integrin antagonists in orthotopic and experimental models. *Proc. Am. Assoc. Cancer Res.*, 42: 263, 2001.
- Lode, H. N., Moehler, T., Xiang, R., Jonczyk, A., Gillies, S. D., Cheresch, D. A., and Reisfeld, R. A. Synergy between an antiangiogenic integrin α_v antagonist and an antibody-cytokine fusion protein eradicates spontaneous tumor metastases. *Proc. Natl. Acad. Sci. USA*, 96: 1591–1596, 1999.
- Bruns, C. J., Liu, W., Davis, D. W., Shaheen, R. M., McConkey, D. J., Wilson, M. R., Bucana, C. D., Hicklin, D. J., and Ellis, L. M. Vascular endothelial growth factor is an *in vivo* survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. *Cancer (Phila.)*, 89: 488–499, 2000.
- Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.*, 13: 281–292, 1988.
- Pytela, R., Pierschbacher, M. D., Argraves, S., Suzuki, S., and Ruoslahti, E. Arginine-glycine-aspartic acid adhesion receptors. *Methods Enzymol.*, 144: 475–489, 1987.
- Charo, I. F., Nannizzi, L., Phillips, D. R., Hsu, M. A., and Scarborough, R. M. Inhibition of fibrinogen binding to GP IIb-IIIa by a GP IIIa peptide. *J. Biol. Chem.*, 266: 1415–1421, 1991.
- Rodbard, D., Munso, P. J., and de Lean, A. Improved curve fitting, parallelism testing, characterization of sensitivity and specificity, validation, and optimization for radioligand assays. Vienna: International Atomic Energy Agency, p. 469, 1977.
- Geng, L., Ali, S. A., Marshall, J. F., Mackay, C. L., Hart, I. R., Delcommence, M., Streuli, C. H., and Rees, R. C. Fibronectin is chemotactic for CT 26 colon carcinoma cells: sub-lines selected for increased chemotaxis to fibronectin display decreased tumorigenicity and lung colonization. *Clin. Exp. Metastasis*, 16: 683–691, 1998.
- Caltabiano, S., Hum, W. T., Attwell, G. J., Galnick, D. N., Budman, L. J., Cannistraci, A. M., and Bex, F. J. The integrin specificity of human recombinant osteopontin. *Biochem. Pharmacol.*, 58: 1567–1578, 1999.

20. Akagi, Y., Liu, W., Xie, K., Zebrowski, B., Shaheen, R. M., and Ellis, L. M. Regulation of vascular endothelial growth factor expression in human colon cancer by interleukin-1 β . *Br. J. Cancer*, *80*: 1506–1511, 1999.
21. Shaheen, R. M., Davis, D. W., Liu, W., Zebrowski, B. K., Wilson, M. R., Bucana, C. D., McConkey, D. J., McMahon, G., and Ellis, L. M. Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer Res.*, *59*: 5412–5416, 1999.
22. Reinmuth, N., Fan, F., Liu, W., Parikh, A. A., Stoeltzing, O., Jung, Y. D., Bucana, C. D., Radinsky, R., Gallick, G. E., and Ellis, L. M. Impact of insulin-like growth factor receptor-I function on angiogenesis, growth, and metastasis of colon cancer. *Lab. Invest.*, *82*: 1377–1389, 2002.
23. Eberhard, A., Kahlert, S., Goede, V., Hemmerlein, B., Plate, K. H., and Augustin, H. G. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. *Cancer Res.*, *60*: 1388–1393, 2000.
24. Weidner, N. Intratumor microvessel density as a prognostic factor in cancer. *Am. J. Pathol.*, *147*: 9–19, 1995.
25. Reinmuth, N., Liu, W., Jung, Y. D., Ahmad, S. A., Shaheen, R. M., Fan, F., Bucana, C. D., McMahon, G., Gallick, G. E., and Ellis, L. M. Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival. *FASEB J.*, *15*: 1239–1241, 2001.
26. Radinsky, R., and Ellis, L. M. Molecular determinants in the biology of liver metastasis. *Surg. Oncol. Clin. N. Am.*, *5*: 215–229, 1996.
27. Terayama, N., Terada, T., and Nakanuma, Y. An immunohistochemical study of tumour vessels in metastatic liver cancers and the surrounding liver tissue. *Histopathology*, *29*: 37–43, 1996.
28. Fidler, I. J., Kumar, R., Bielenberg, D. R., and Ellis, L. M. Molecular determinants of angiogenesis in cancer metastasis. *Cancer J. Sci. Am.*, *4* (Suppl. 1): S58–S66, 1998.
29. Ellerby, H. M., Arap, W., Ellerby, L. M., Kain, R., Andrusiak, R., Rio, G. D., Krajewski, S., Lombardo, C. R., Rao, R., Ruoslahti, E., Bredesen, D. E., and Pasqualini, R. Anti-cancer activity of targeted pro-apoptotic peptides. *Nat. Med.*, *5*: 1032–1038, 1999.
30. Varner, J. A., and Cheresch, D. A. Integrins and cancer. *Curr. Opin. Cell Biol.*, *8*: 724–730, 1996.
31. Pasqualini, R., Bodorova, J., Ye, S., and Hemler, M. E. A study of the structure, function and distribution of $\beta 5$ integrins using novel anti- $\beta 5$ monoclonal antibodies. *J. Cell Sci.*, *105* (Pt. 1): 101–111, 1993.
32. Felding-Habermann, B., and Cheresch, D. A. Vitronectin and its receptors. *Curr. Opin. Cell Biol.*, *5*: 864–868, 1993.
33. Yun, Z., Menter, D. G., and Nicolson, G. L. Involvement of integrin $\alpha v \beta 3$ in cell adhesion, motility, and liver metastasis of murine RAW117 large cell lymphoma. *Cancer Res.*, *56*: 3103–3111, 1996.
34. O'Reilly, M. S., Boehm, T., Shing, Y., Fukui, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*, *88*: 277–285, 1997.
35. Warren, R. S., Yuan, H., Matli, M. R., Gillett, N. A., and Ferrara, N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J. Clin. Invest.*, *95*: 1789–1797, 1995.
36. Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D. J., Bohlen, P., and Kerbel, R. S. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J. Clin. Invest.*, *105*: R15–R24, 2000.
37. Lehmann, M., Rabenandrasana, C., Tamura, R., Lissitzky, J. C., Quaranta, V., Pichon, J., and Marvaldi, J. A monoclonal antibody inhibits adhesion to fibronectin and vitronectin of a colon carcinoma cell line and recognizes the integrins $\alpha v \beta 3$, $\alpha v \beta 5$, and $\alpha v \beta 6$. *Cancer Res.*, *54*: 2102–2107, 1994.
38. Klemke, R. L., Yebra, M., Bayna, E. M., and Cheresch, D. A. Receptor tyrosine kinase signaling required for integrin $\alpha v \beta 5$ -directed cell motility but not adhesion on vitronectin. *J. Cell Biol.*, *127*: 859–866, 1994.
39. Brooks, P. C., Klemke, R. L., Schon, S., Lewis, J. M., Schwartz, M. A., and Cheresch, D. A. Insulin-like growth factor receptor cooperates with integrin $\alpha v \beta 5$ to promote tumor cell dissemination *in vivo*. *J. Clin. Invest.*, *99*: 1390–1398, 1997.
40. Hirschi, K. K., Rohovsky, S. A., Beck, L. H., Smith, S. R., and D'Amore, P. A. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. *Circ. Res.*, *84*: 298–305, 1999.
41. Zheng, B., and Clemmons, D. R. Blocking ligand occupancy of the $\alpha v \beta 3$ integrin inhibits insulin-like growth factor I signaling in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA*, *95*: 11217–11222, 1998.
42. Jones, J. I., Prevette, T., Gockerman, A., and Clemmons, D. R. Ligand occupancy of the $\alpha v \beta 3$ integrin is necessary for smooth muscle cells to migrate in response to insulin-like growth factor. *Proc. Natl. Acad. Sci. USA*, *93*: 2482–2487, 1996.
43. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., and Ingber, D. E. Geometric control of cell life and death. *Science (Wash. DC)*, *276*: 1425–1428, 1997.
44. Cary, L. A., Chang, J. F., and Guan, J. L. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J. Cell Sci.*, *109* (Pt. 7): 1787–1794, 1996.
45. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature (Lond.)*, *377*: 539–544, 1995.
46. Gilmore, A. P., Metcalfe, A. D., Romer, L. H., and Streuli, C. H. Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. *J. Cell Biol.*, *149*: 431–446, 2000.
47. Rosen, K., Rak, J., Leung, T., Dean, N. M., Kerbel, R. S., and Filmus, J. Activated Ras prevents down-regulation of Bcl-X(L) triggered by detachment from the extracellular matrix. A mechanism of Ras-induced resistance to anoikis in intestinal epithelial cells. *J. Cell Biol.*, *149*: 447–456, 2000.
48. Blystone, S. D., Graham, I. L., Lindberg, F. P., and Brown, E. J. Integrin $\alpha v \beta 3$ differentially regulates adhesive and phagocytic functions of the fibronectin receptor $\alpha 5 \beta 1$. *J. Cell Biol.*, *127*: 1129–1137, 1994.