Integrin $\alpha_4\beta_1$–VCAM-1–mediated adhesion between endothelial and mural cells is required for blood vessel maturation

Barbara Garmy-Susini,[1] Hui Jin,[1] Yuhong Zhu,[1] Rou-Jia Sung,[1] Rosa Hwang,[1,2] and Judy Varner[1,3]

[1]John and Rebecca Moores Comprehensive Cancer Center, [2]Department of Surgery, and [3]Department of Medicine, University of California, San Diego, La Jolla, California, USA.

Neovascularization depends on vascular cell proliferation and on the stabilization of vessels by association of vascular smooth muscle–like pericytes with ECs. Here we show that integrin $\alpha_4\beta_1$ (VLA-4) and VCAM-1 promote close intercellular adhesion between ECs and pericytes and that this interaction is required for blood vessel formation. Integrin $\alpha_4\beta_1$ is expressed by proliferating but not quiescent ECs, while its ligand VCAM-1 is expressed by proliferating but not quiescent mural cells. Antagonists of this integrin-ligand pair block the adhesion of mural cells to proliferating endothelia in vitro and in vivo, thereby inducing apoptosis of ECs and pericytes and inhibiting neovascularization. These studies indicate that integrin $\alpha_4\beta_1$ and VCAM-1 facilitate a critical cell-cell adhesion event required for survival of endothelial and mural cells during vascularization.

Introduction

Neovascularization, or the development of new blood vessels, promotes embryonic development as well as the healing of injured tissues and ovulation (1–3). It also plays a critical role in pathologies such as tumor growth and inflammatory diseases (1–4). While it is well known that blood vessels are composed of 2 cell types, ECs and mural cells (VSMCs and pericytes), little is known about the mechanisms by which these 2 cell types associate with each other during developmental and pathological vascularization. Most studies of the molecular mechanisms regulating neovascularization have focused on the roles of ECs in the sprouting and extension of new vessels from parent vessels (1–4). However, it has recently become clear that mural cells also play critical roles in vascularization (5–10). These desmin- and smooth muscle actin–positive (SMA-positive) cells surround the endothelia and provide structural support and regulate blood flow (8, 9). While larger vessels such as arteries and veins are lined by VSMCs, capillaries and postcapillary venules of normal tissues are lined by a sparse covering of pericytes (9). Pericytes also associate with tumor vessels, although they are often more loosely associated with endothelia in tumors than in normal tissues (7–10).

Recent studies demonstrated that pericytes are attracted to proliferating endothelia by EC-derived PDGF and that both PDGF and its receptor are critical for the proper formation of stable blood vessels during development and tumorigenesis (7–8). Blood vessels in PDGF$^{-/-}$ animals are characterized by dilation, rupture, leakage, and hemorrhage and contribute to embryonic lethality (5–6). Importantly, PDGF and PDGF-receptor inhibitors disrupt mural cell association with ECs and block angiogenesis and tumor growth (10). Thus, current studies indicate that both ECs and mural cell layers are critical for the formation of functioning blood vessels and the support of growing tissues, including tumors.

Nevertheless, it remains unclear in what way ECs and mural cells closely associate to form a single functional unit, the blood vessel (8). Our studies on the roles of integrins and their ligands in vascular development revealed surprising roles for integrin $\alpha_4\beta_1$ (VLA-4) and its ligand VCAM-1 in this process. Integrin $\alpha_4\beta_1$ is best known as a lymphocyte integrin that mediates adhesion of circulating lymphocytes to VCAM-1 expressed on activated endothelia in inflamed tissues, thereby promoting extravasation of lymphocytes into inflamed tissue (11). Although some studies have suggested roles for integrin $\alpha_4\beta_1$ in angiogenesis, particularly in inflammatory angiogenesis, little is known about how this integrin might contribute to vascularization in vivo (12–15).

Integrin $\alpha_4\beta_1$ and VCAM-1 have been shown to regulate embryonic development, as loss of either gene causes embryonic lethality by E11.5–E12.5 from a failure of the endocardium to fuse with the myocardium (16–18) and a failure of the chorion to fuse with the allantois (16, 17). In addition, loss of either gene results in abortive coronary artery formation, which results in cardiac hemorrhage (16, 17). Nevertheless, little is known about the mechanisms by which VCAM-1 and integrin $\alpha_4\beta_1$ contribute to the formation of blood vessel development in vivo. In this report, we demonstrate that this receptor-ligand pair mediates the adhesion of endothelia and mural cells of developing vessels, an event that is required for the survival of proliferating endothelial *and* mural cells and, thereby, for neovascularization.

Results

Integrin $\alpha_4\beta_1$ is expressed by proliferating but not mature ECs in vivo. To evaluate potential roles for integrin $\alpha_4\beta_1$ and its ligand VCAM-1 in neovascularization, we first determined the expression of these proteins on vascular cells during neovascularization in vivo. We found that integrin $\alpha_4\beta_1$ was strongly expressed on endothelia of developing vessels but not on endothelia of quiescent vessels. In initial studies, we stimulated the chorioallantoic membranes (CAMs) of 10-day-old chicken embryos with saline or with a one of a variety of factors that promote neovascularization, such as

Nonstandard abbreviations used: CAM, chorioallantoic membrane; CMTMR, 5-(and-6)-(4-chloromethyl(benzoyl)amino)tetramethylrhodamine; EILDV, glutamic acid–isoleucine-leucine-asparctic acid–valine; HUVEC, human umbilical vein EC; PS/2, rat anti–murine $\alpha_4\beta_1$; rsVCAM-1, recombinant soluble VCAM-1; SMA, smooth muscle actin; TSP, thrombospondin.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **115**:1542–1551 (2005). doi:10.1172/JCI23445.

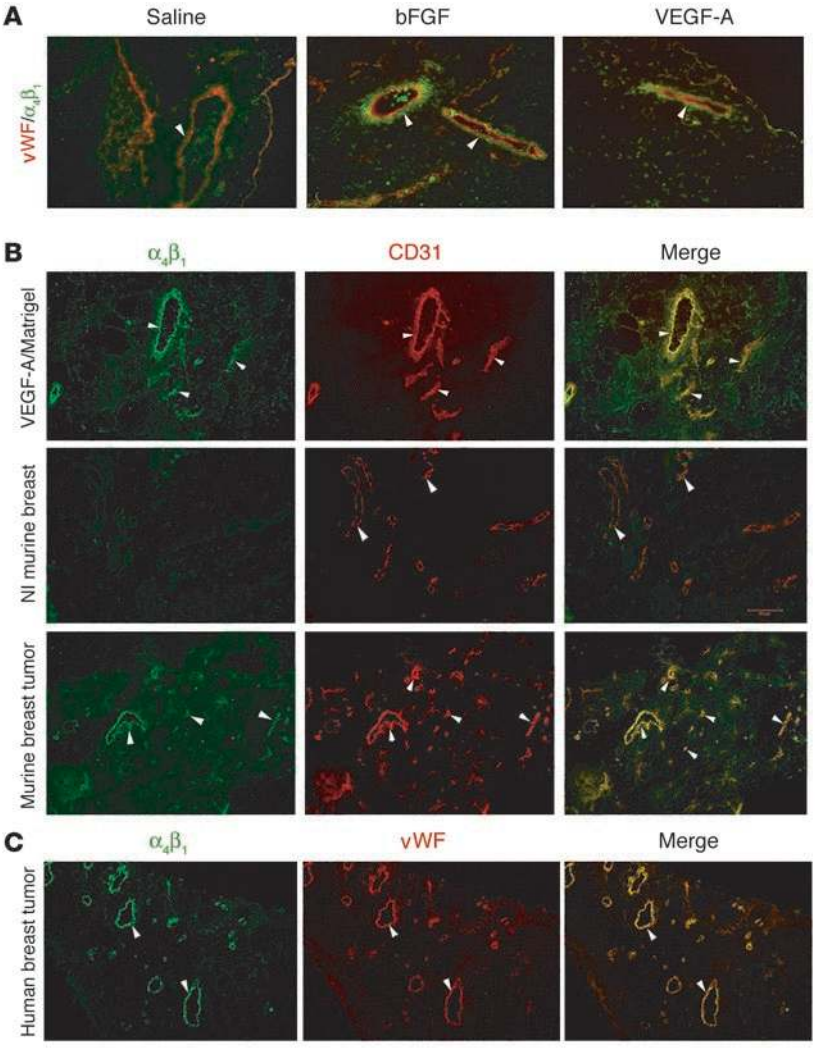

Figure 1
ECs in developing vessels express integrin $\alpha_4\beta_1$. (**A**) Cryosections of saline-, bFGF-, or VEGF-A–stimulated chick CAMs were immunostained to detect vWF (red) and integrin $\alpha_4\beta_1$ ($\alpha_4\beta_1$) (green). Yellow color indicates overlap of expression patterns. (**B**) Cryosections of normal mouse breast, growth factor–reduced Matrigel supplemented with 400 μg/ml recombinant human VEGF-A, and MMTV-PyV-mT spontaneous breast carcinoma were immunostained to detect vascular EC CD31 (red) and integrin $\alpha_4\beta_1$ (green). Yellow color indicates overlap of expression patterns. (**C**) Cryosections of human ductal breast carcinoma in situ were immunostained to detect vWF (red) and integrin $\alpha_4\beta_1$ (green). Yellow color indicates overlap of expression patterns. In **A**–**C**, Arrowheads indicate blood vessels. NI, normal. Magnification, ×200. Scale bar: 100 μm.

bFGF or VEGF. Cryosections of these CAMs were immunostained to detect expression of the EC marker vWF (Figure 1A, red) and integrin $\alpha_4\beta_1$ (green). While the established blood vessels (Figure 1A, arrowheads) that are present in saline-treated tissues did not express integrin $\alpha_4\beta_1$, those in bFGF- or VEGF-A–stimulated CAM tissues did express abundant integrin $\alpha_4\beta_1$ within 24 hours after growth factor stimulation. Integrin $\alpha_4\beta_1$ expression first appeared after 24 hours of stimulation and remained elevated for up to 72 hours after stimulation. These studies indicate that this integrin is expressed on neovessels but not on quiescent normal vessels.

We next evaluated the expression of integrin $\alpha_4\beta_1$ on endothelia in murine vessels. Murine tissues including normal breast, liver, lungs, ovary, skin, and colon as well as VEGF-A–stimulated tissues were immunostained with anti–integrin $\alpha_4\beta_1$ antibodies (Figure 1B, green) and anti-CD31 (red), a marker of endothelia. While vessels in normal, unstimulated tissues such as mouse breast (Figure 1B), liver (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI23445DS1), or skin (not shown) did not express integrin $\alpha_4\beta_1$, VEGF-A–stimulated vessels (arrowheads) were uniformly integrin $\alpha_4\beta_1$ positive (Figure 1B, yellow). To determine whether integrin $\alpha_4\beta_1$ is expressed on tumor vessels, we immunostained spontaneous murine breast carcinomas from MMTV-PyV-mT mice and human breast carcinomas (dysplastic carcinoma in situ) to detect integrin $\alpha_4\beta_1$ and markers of the vascular endothelia. We detected strong integrin $\alpha_4\beta_1$ expression on virtually all of the blood vessels (arrowheads) in both murine breast carcinomas (Figure 1B) and human breast carcinomas (Figure 1C). Integrin $\alpha_4\beta_1$ expression was detected on larger vessels (50–100 μm in diameter) as well as on small capillaries (>10 μm in diameter). No expression of integrin $\alpha_4\beta_1$ was observed on blood vessels in any normal human tissues (not shown).

We also observed integrin $\alpha_4\beta_1$ expression on virtually all of the vascular endothelia in experimental subcutaneous HT29 colon carcinoma, M21 melanoma, and Lewis lung carcinoma tumors (Figure 2A). In addition, we were able to demonstrate integrin $\alpha_4\beta_1$ expression on neovessels by intravenously injecting rat anti–murine integrin $\alpha_4\beta_1$ (PS/2) antibodies into animals undergoing VEGF-A–stimulated angiogenesis. These antibodies but not isotype-matched control antibodies selectively bound to new vessels in Matrigel (Figure 2B) but not to preexisting vessels in unstimulated tissues (not shown). These studies demonstrate that integrin $\alpha_4\beta_1$ is expressed on neovessels in both murine and avian tissues in response to angiogenic factors.

VCAM is expressed by pericytes. The finding that integrin $\alpha_4\beta_1$ is strongly expressed only on neovessels prompted us to examine the localization of integrin $\alpha_4\beta_1$ ligands, VCAM-1, and fibronectin in angiogenic tissues. We had previously shown that fibronectin is strongly expressed in association with the vascular endothelia of tumors but not of normal tissues (19). Again we observed that fibronectin is expressed by neovessels in mouse tumors but rarely in normal mouse tissues (Supplemental Figure 2). Surprisingly, however, we also found that VCAM-1 is strongly expressed on vascular smooth muscle–like cells of neovessels but not of quiescent vessels. In contrast to integrin $\alpha_4\beta_1$, its expression is primarily on cells surrounding the endothelia and only rarely on the endothelia. Immunostaining of spontaneous murine breast carcinomas with antibodies directed against VCAM-1 (Figure 3A, red) or the endothelial marker CD31 (green) demonstrated that VCAM-1 is strongly expressed by cells that are closely aligned with

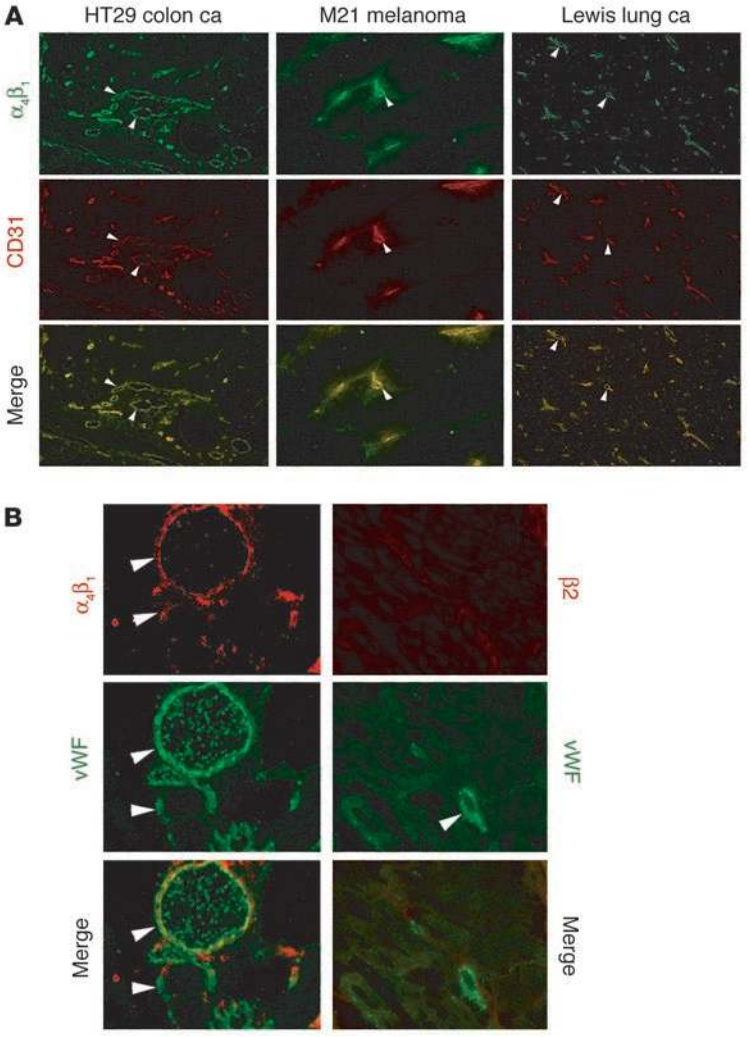

Figure 2
Integrin $\alpha_4\beta_1$ is accessible to the circulation in developing vessels. (**A**) Cryosections of subcutaneous xenograft HT29 colon carcinoma, M21 melanoma, and Lewis lung carcinoma tumors in nude mice were immunostained to detect integrin $\alpha_4\beta_1$ (green) and CD31 (red). Overlap of expression is indicated by yellow (arrowheads). Ca, carcinoma. (**B**) Frozen sections of VEGF-A Matrigel plugs from mice injected intravenously with 50 μg doses of either PS/2 (left panels) or control rat anti–murine CD18 (M18/2, right panels) immunostained to detect anti-vWF (green) and bound rat anti-mouse antibodies (red). Yellow color in merged images indicates bound anti–integrin $\alpha_4\beta_1$ or anti–integrin β2 (β2) antibody on endothelia. Magnification, ×200.

tumor endothelia (arrows). Immunostaining of human breast carcinomas also shows that VCAM-1 is strongly expressed by cells that surround human endothelia (Figure 3A, arrows). In some vessels, VCAM-1$^+$ cells are intertwined with and adjacent to CD31$^+$ cells (Figure 3A, right panel, arrow). VCAM-1 is also occasionally expressed on the vascular endothelia of a small subset of tumor vessels (Figure 3A, right panel, arrowheads), as previously shown (19). In contrast, VCAM-1 is not expressed by vascular cells of normal tissues such as mouse breast (Figure 3A), mouse liver (Supplemental Figure 1), or human breast (Figure 3A). These studies thus indicate that VCAM-1 is expressed by a distinct population of neovascular cells that are similar to mural cells, called pericytes.

To determine whether VCAM-1 is expressed by mural cells such as vascular smooth muscle cells or pericytes, we asked whether there was similarity in the expression patterns of VCAM-1 and SMA or desmin, proteins which are expressed by pericytes and VSMCs (9). Indeed, desmin-positive tumor vessels (Figure 3B, arrowheads) expressed VCAM-1 as did SMA-positive tumor vessels (Figure 3C, arrowheads). Importantly, our studies show that VCAM-1 is expressed by mural cells of tumor vessels but not by those of normal tissues (Figure 3, A–C). These studies therefore indicate that VCAM-1 is a marker of the mural cells of the neovasculature but not of normal vessels. Importantly, integrin $\alpha_4\beta_1$ is expressed on the endothelia (Figure 4, arrowheads), and VCAM-1 is expressed on the surrounding mural cells (arrows) of the same tumor vessels in both mouse and human tumors.

Integrin $\alpha_4\beta_1$ and VCAM-1 mediate adhesion of ECs and pericytes in vitro and in vivo. As VCAM-1 is a ligand for integrin $\alpha_4\beta_1$ and is expressed by tumor pericytes, we determined whether VCAM-1 and integrin $\alpha_4\beta_1$ contribute to a physical interaction between endothelia and mural cells. Cultured human vascular SMCs were labeled with a fluorescent vital dye, CellTracker Orange (5-(and-6)-(4-chloromethyl(benzoyl)amino)tetramethylrhodamine (CMTMR), and were plated on monolayers of cultured human ECs. SMCs rapidly adhered to and spread on ECs, maintaining stable adhesive contacts for at least 24 hours (Figure 5A). This adhesive interaction depended upon integrin $\alpha_4\beta_1$, because antibody antagonists of integrin $\alpha_4\beta_1$ as well as recombinant soluble VCAM-1 (rsVCAM-1), a competitive inhibitor of integrin $\alpha_4\beta_1$ function, strongly blocked SMC attachment to endothelia in vitro (Figure 5, B and C). In contrast, antibody antagonists of integrin $\alpha_v\beta_5$, another SMC integrin, did not affect this adhesion (Figure 5, B and C). Thus, integrin $\alpha_4\beta_1$–VCAM-1 interactions promote EC–mural cell adhesion in vitro.

To determine whether integrin $\alpha_4\beta_1$–VCAM-1 interactions regulate pericyte/EC associations in vivo, chick CAMs were stimulated with the angiogenic growth factor bFGF and were treated with saline, control anti–integrin $\alpha_v\beta_5$ antibodies, which do not inhibit bFGF-induced angiogenesis, or anti–integrin $\alpha_4\beta_1$ antibodies. Frozen sections of CAMs were immunostained with anti–integrin $\alpha_v\beta_3$ to detect proliferating endothelia and with antidesmin to detect mural cells. Treatment with anti–integrin $\alpha_4\beta_1$ significantly reduced the association of mural cells with endothelia in growth factor–stimulated tissues in vivo (Figure 5, D and E). CAMs that had been treated with anti–integrin $\alpha_4\beta_1$ antagonists exhibited fewer pericyte-coated vessels, that is, fewer desmin-positive blood vessels, than control-treated CAMs ($P = 0.0003$). In fact, tissues treated with integrin $\alpha_4\beta_1$ antagonists exhibited not only greatly reduced numbers of intact vessels (Figure 5, F and G) but also reduced numbers of mural and ECs (Figure 5, D and E). These studies suggest that integrin $\alpha_4\beta_1$ antagonists perturb blood vessel stabilization by blocking the adhesion of mural cells and ECs in developing vessels.

Blockade of integrin $\alpha_4\beta_1$ induces EC and pericyte apoptosis. Integrin ligation by extracellular matrix proteins has been shown to promote the survival of ECs during angiogenesis (15–17). Therefore, we considered whether loss of endothelial and mural cells in tis-

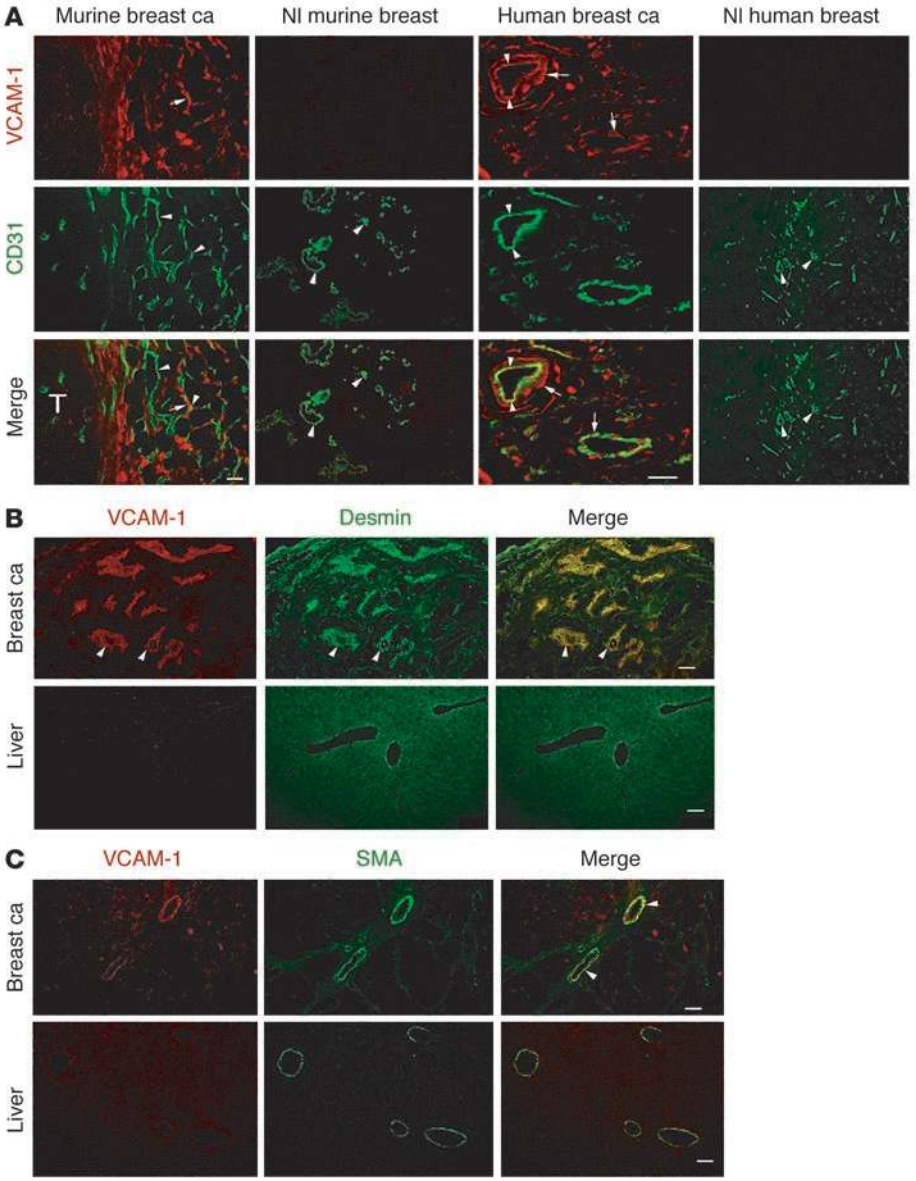

Figure 3
Mural cells in developing vessels express VCAM. (**A**) Cryosections of murine MMTV-PyV-mT spontaneous breast carcinoma, human ductal breast carcinoma in situ, normal human breast, and normal murine breast were immunostained with anti-CD31 (green, arrowheads) and anti–VCAM-1 (red, arrows) antibodies. T, tumor. (**B**) Cryosections of murine MMTV-PyV-mT spontaneous breast carcinoma and normal mouse liver were immunostained to detect VCAM-1 (red) and desmin (green). Expression of VCAM-1 on mural cells is indicated by yellow (arrowheads). (**C**) Cryosections of murine MMTV-PyV-mT spontaneous breast carcinoma and normal mouse liver were immunostained to detect VCAM-1 (red) and SMA (green). Expression of VCAM-1 on mural cells is indicated by yellow (arrowheads). Scale bars: 50 μm.

sues treated with integrin $\alpha_4\beta_1$ antagonists might be explained by an increase in the vascular cell apoptotic rate. Chick CAMs were stimulated with bFGF and treated with anti–integrin $\alpha_4\beta_1$ or control (anti–integrin $\alpha_v\beta_5$) antibodies. Both of these integrins are expressed by ECs in bFGF-stimulated CAMs. Frozen sections of these CAMs were immunostained with either anti-SMA (Figure 6, red) to detect mural cells or anti-vWF (red) to detect endothelia as well as by TUNEL staining to detect apoptotic cells (green). While little evidence of vascular cell apoptosis was detected in control-treated embryos (Figure 6, left panels), both ECs and mural cells (arrowheads) in anti–integrin $\alpha_4\beta_1$–treated embryos were highly positive for TUNEL staining (Figure 6, middle and right panels). These results suggest that integrin $\alpha_4\beta_1$ antagonists, which bind to ECs but not to mural cells, induced not only apoptosis of ECs but also, indirectly, apoptosis of the mural cells by blocking the adhesion of the 2 cell types. Thus, pericyte-EC adhesion appears to promote both EC and mural cell survival during angiogenesis in vivo. Taken together, these results indicate for the first time, to our knowledge, that proliferating EC–pericyte intercellular adhesion may be required for the generation of survival signals by both cell types during angiogenesis. These studies also suggest that integrin $\alpha_4\beta_1$ might regulate angiogenesis and/or vasculogenesis in vivo by promoting endothelial and mural cell intercellular adhesion.

Integrin $\alpha_4\beta_1$ blockade inhibits angiogenesis and tumor growth. To determine whether integrin $\alpha_4\beta_1$ plays a requisite role in angiogenesis, function-blocking anti–integrin $\alpha_4\beta_1$ or control isotype-matched antibodies were applied to chick CAMs stimulated with several distinct angiogenesis inducers, including bFGF, VEGF-A, TNF-α, and IL-8. Importantly, anti–integrin $\alpha_4\beta_1$ was shown to potently block angiogenesis in chicken embryos induced by each of these angiogenic factors whether analyzed by counting blood vessel branchpoints macroscopically (Figure 7A, $P < 0.05$) or by quantifying microvessels in cryosections of treated CAMs (data not shown). Strikingly, these results indicate that integrin $\alpha_4\beta_1$ regulates angiogenesis induced by a wide range of distinct stimuli, consistent with a general role in neovascularization.

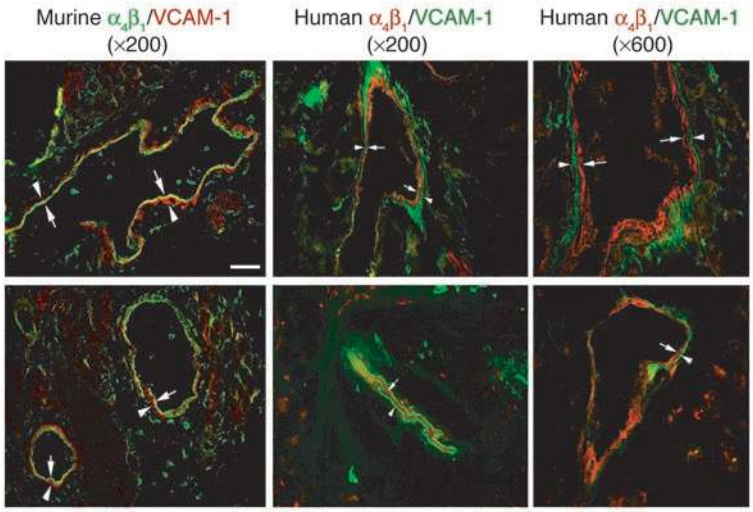

Figure 4
VCAM-1 is expressed on pericytes and integrin $\alpha_4\beta_1$ on the endothelia of the same tumor vessels. Cryosections of murine MMTV-PyV-mT spontaneous breast carcinoma (left) and human breast carcinoma (middle and right) were immunostained with anti–integrin $\alpha_4\beta_1$ (arrows) and anti–VCAM-1 (arrowheads) antibodies. Magnification, ×200 (left and middle panels); ×600 (right panels). Scale bar: 50 μm. Integrin $\alpha_4\beta_1$ expression is generally restricted to the inner EC layer, and VCAM-1 expression is restricted to the outer cell layer of both small and large vessels.

Our studies also show that integrin $\alpha_4\beta_1$ generally promotes mammalian angiogenesis. Mice were stimulated to undergo corneal angiogenesis induced by implantation of a pellet containing bFGF and were treated with function-blocking anti–integrin $\alpha_4\beta_1$ antibodies or control anti–integrin β2 antibodies. We found that $\alpha_4\beta_1$ antagonists but not control antibodies significantly suppressed blood vessel formation in this model (Figure 7B, $P < 0.05$). In addition, these antagonists blocked angiogenesis induced by either bFGF or VEGF-A in mouse skin (Figure 7C). Importantly, antibody and peptide antagonists of integrin $\alpha_4\beta_1$ (glutamic acid–isoleucine-leucine-asparctic acid–valine [EILDV], from CS-1 fibronectin) similarly blocked neovascularization in mouse skin (Figure 7, D and E), providing further support for a role for integrin $\alpha_4\beta_1$ in neovascularization.

VCAM-1 blockade inhibits angiogenesis. To determine whether VCAM-1, a key integrin $\alpha_4\beta_1$ ligand, also plays a role in angiogenesis, neovascularization was initiated in mice by subcutaneous injection of Matrigel containing bFGF or VEGF-A and saline, function-blocking

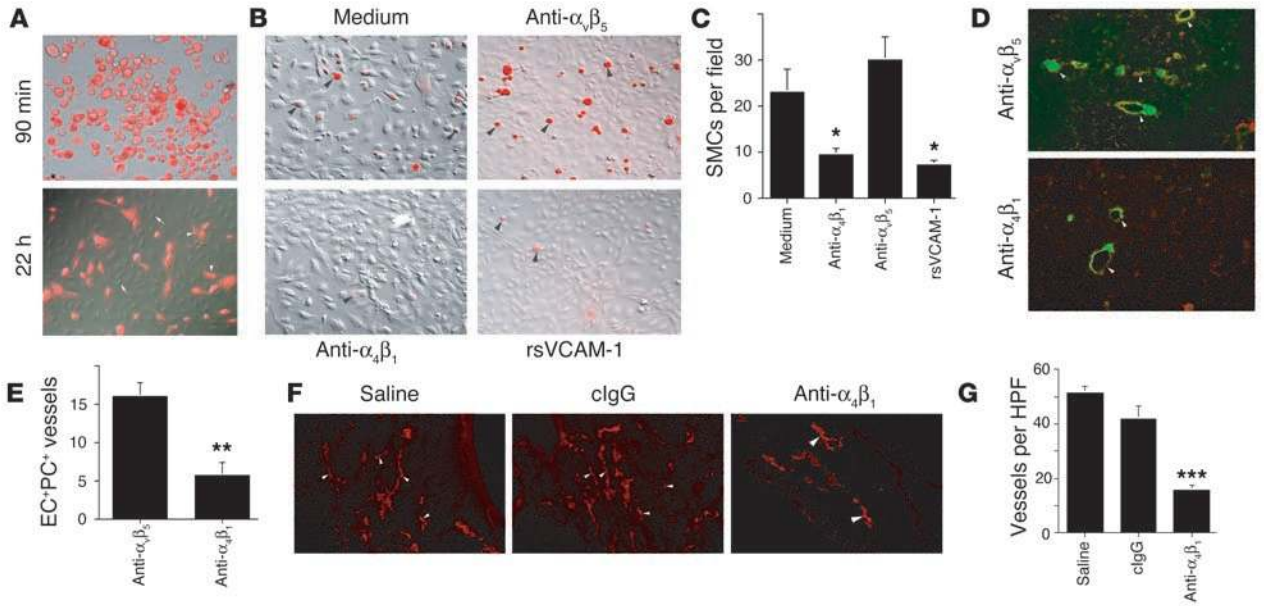

Figure 5
VCAM-1–integrin $\alpha_4\beta_1$ interactions govern EC–mural cell interactions. (**A**) CMTMR-labeled (red) SMCs adherent on endothelial monolayers after 90 minutes or 22 hours of coincubation. Arrows indicate ECs. (**B**) CMTMR-labeled VSMCs (red) adherent on endothelial monolayers in the presence of culture medium, anti–integrin $\alpha_4\beta_1$ (HP1/2), anti–integrin $\alpha_v\beta_5$ (anti-$\alpha_v\beta_5$) (P1F6), or rsVCAM-1. Arrowheads indicate SMCs. (**C**) Quantification of attached SMCs per microscopic field. (**D**) Cryosections of chick CAMs treated with anti–integrin $\alpha_v\beta_5$ or anti–integrin $\alpha_4\beta_1$ antibodies were immunostained to detect endothelia (anti–integrin $\alpha_v\beta_3$, green) and pericytes (anti-desmin, red). Representative vessels coated with mural cells are indicated by arrowheads. (**E**) Pericyte-coated vessels were quantified by counting integrin $\alpha_v\beta_3^+$ desmin$^+$ vessels per field (EC$^+$PC$^+$ vessels ± SEM). (**F**) Cryosections from bFGF-stimulated CAMs that were treated with saline, PS/2, or control antibodies (cIgG, M1/70). CAMs were immunostained to detect vWF expression (red) in blood vessels. Blood vessels are indicated by arrowheads. (**G**) vWF-positive vessels ± SEM per microscopic field quantified in replicate samples. Magnification in **A**, **B**, **D**, and **F**: ×200. *P = 0.001; **P = 0.0003; ***P = 0.0035.

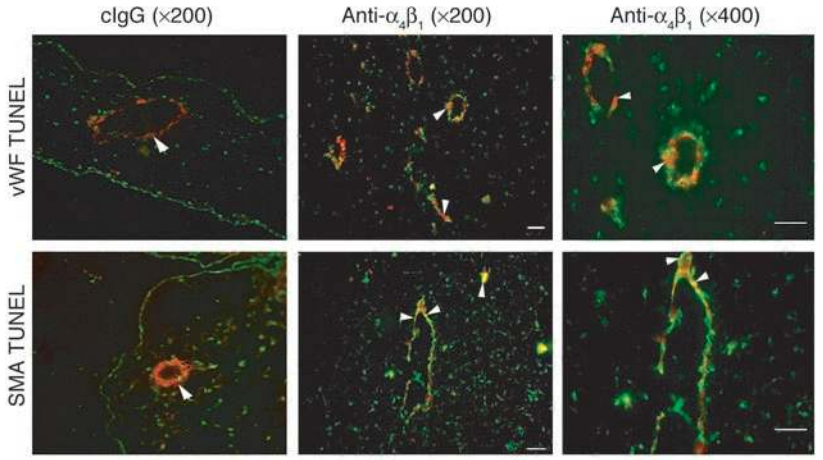

Figure 6
Antagonists of integrin $\alpha_4\beta_1$ induce apoptosis in ECs and pericytes. Cryosections from bFGF-stimulated CAMs that were treated for 24 hours with either anti–integrin $\alpha_4\beta_1$ (HP1/2) or cIgG (anti–integrin $\alpha_v\beta_5$, P1F6) antibodies were immunostained to detect vWF (red) and SMA (red). Sections were also treated to detect apoptotic cells by TUNEL analysis (green). Representative blood vessels are indicated by arrowheads. Magnification, ×200 (left and center panels); ×400 (right panels). Scale bars: 50 μm.

anti–murine VCAM-1 antibodies, or control isotype-matched antibodies. Importantly, VCAM-1 antagonists substantially blocked both bFGF- and VEGF-A–induced neovascularization ($P < 0.0001$) while control antibodies had little effect (Figure 8).

As integrin $\alpha_4\beta_1$ promotes EC and mural cell association during angiogenesis, we considered whether integrin $\alpha_4\beta_1$ antagonists might inhibit tumor growth by blocking tumor EC and mural cell association. We next found that antagonists of integrin $\alpha_4\beta_1$ significantly inhibited tumor angiogenesis and growth. Nude mice with HT29 colon carcinoma xenograft tumors were treated with function-blocking anti–murine integrin $\alpha_4\beta_1$ and isotype matched anti–murine CD11b antibodies. The tumor cells themselves did not express integrin $\alpha_4\beta_1$. Within 12 days after initiation of treatment, tumor volumes in anti–integrin $\alpha_4\beta_1$–treated mice were significantly less than those in control-treated mice. After 1 month of therapy, anti–integrin $\alpha_4\beta_1$–treated tumors ceased to increase in size while control-treated tumors continued to grow at an exponential rate (Figure 9A, $P < 0.01$). The average tumor mass of anti–integrin $\alpha_4\beta_1$–treated tumors at necropsy was 50% that of control-treated tumors (Figure 9B, $P < 0.02$). In fact, anti–integrin $\alpha_4\beta_1$–treated tumors exhibited considerably more necrosis than was exhibited by control-treated tumors (Figure 9C, arrowheads). Anti–integrin $\alpha_4\beta_1$–treated tumors also exhibited significantly reduced

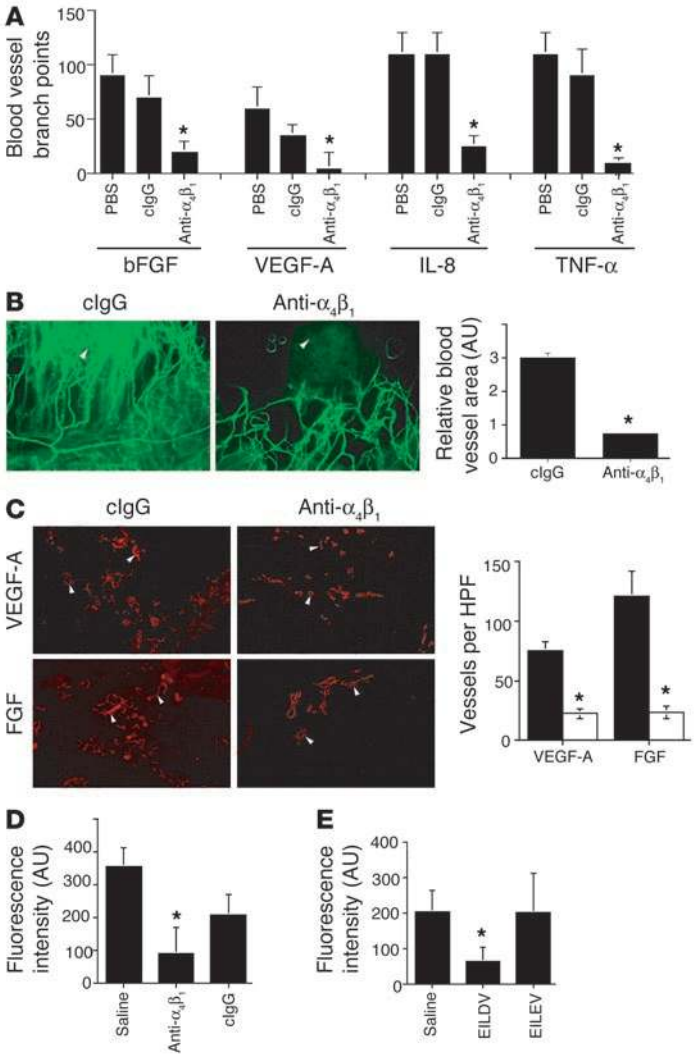

Figure 7
Integrin $\alpha_4\beta_1$ is required for angiogenesis. (**A**) Blood vessel branch points ± SEM in bFGF-, VEGF-A–, TNF-α–, or IL-8–stimulated CAMs treated with saline, anti–integrin $\alpha_4\beta_1$ (P1H4), or control isotype-matched antibodies (P1F6, anti–integrin $\alpha_v\beta_5$). (**B**) Mice with 400 μg/ml VEGF-A implanted in the cornea were treated systemically with PS/2 or control IgG (rat anti-CD18, M18/2). Blood vessels identified by i.v. injection with FITC-labeled *B. simplicifolia* lectin. Magnification, ×200. Graph, area of neovascularization quantified by densitometry. Arrowheads indicate source of growth factor. (**C**) Mice subcutaneously injected with GF-reduced Matrigel containing 400 μg/ml rVEGF-A or bFGF were treated systemically for 5 days with PS/2 or control antibodies (rat anti-CD18, M18/2). Graph, CD31 positive vessels (arrowheads) per microscopic field in anti–integrin $\alpha_4\beta_1$ (white bars) or control-treated tissues (black bars). Magnification, ×200. (**D**) Angiogenesis was initiated with GF-reduced Matrigel containing 400 ng/ml bFGF and 200 μg PS/2 or isotype-matched control antibodies. (**E**) Angiogenesis initiated as in **D** but with 50 μM EILDV or glutamic acid–isoleucine-leucine-glutamic acid–valine (EILEV) peptides incorporated in the Matrigel. (**D** and **E**) After 5 days, mice were injected intravenously with FITC-labeled *B. simplicifolia*; Matrigel plugs were homogenized in RIPA buffer, and fluorescence intensity was determined. *$P < 0.05$.

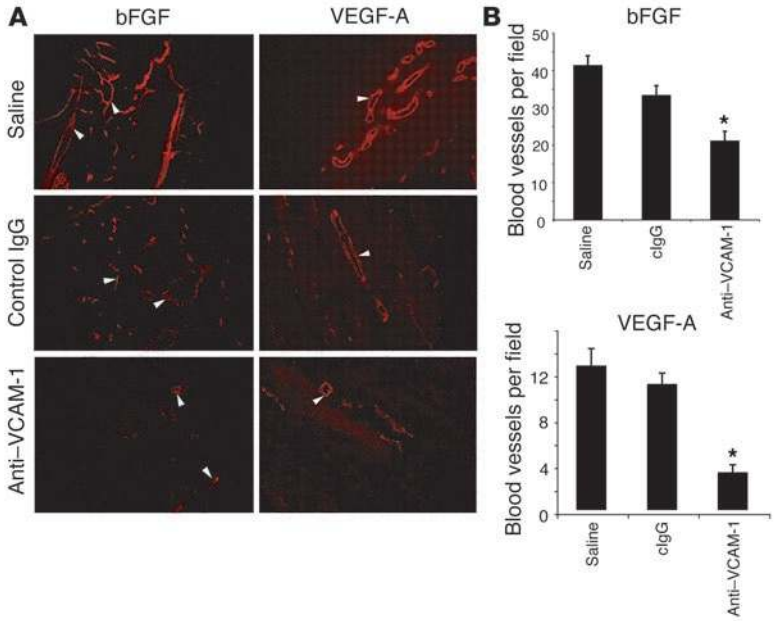

Figure 8
Antagonists of VCAM-1 inhibit angiogenesis. (**A**) Angiogenesis was initiated in nude mice by subcutaneous injection of 400 μl growth factor–reduced Matrigel supplemented with 400 ng/ml of bFGF or VEGF-A containing saline, 200 μg rat anti–murine VCAM-1 (M/K-2), or 200 μg isotype-matched control antibodies (normal rat IgG1). After 5 days, tissues were resected and cryosections were immunostained with rat anti–murine CD31 (red structures indicated by arrowheads). Magnification, ×200. (**B**) CD31-positive vessels per microscopic field were quantified and graphed. Magnification, ×200. *$P = 0.0001$.

microvascular density compared with control-treated tumors, as determined by anti-CD31 immunostaining of frozen sections of tumors (Figure 9D, arrowheads, $P < 0.003$). In additional studies, we found that anti–integrin $\alpha_4\beta_1$ antibodies prevented the outgrowth of pancreatic tumors that were orthotopically transplanted into the pancreata of nude mice (Figure 9E, $P < 0.02$). These antagonists also inhibited the growth of Lewis lung carcinomas in nude mice (data not shown).

Tumors from mice treated with integrin $\alpha_4\beta_1$ inhibitors exhibited reduced mural cell density (Figure 9F). Frozen sections of tumors from mice that had been inoculated with HT29 tumor cells and treated with anti–murine integrin $\alpha_4\beta_1$ or control antibodies were immunostained with anti-CD31 to detect endothelia and with anti-SMA or anti-desmin to detect mural cells. Treatment with anti–integrin $\alpha_4\beta_1$ significantly reduced the association of mural cells with endothelia in vivo. Tumors that had been treated with anti–integrin $\alpha_4\beta_1$ antagonists exhibited far fewer intact vessels with lumens as well as fewer SMA-positive (Figure 9, F and G, $P < 0.002$) or desmin-positive (Figure 9, F and H, $P < 0.05$) blood vessels than control-treated tumors. These studies indicate that integrin $\alpha_4\beta_1$ antagonists perturb blood vessel stabilization by blocking the adhesion of mural cells and ECs in developing tumor vessels. Thus, our studies show that integrin $\alpha_4\beta_1$ plays an important role in the control of tumor-induced neovascularization and, thereby, tumor growth, at least in part by blocking the requisite adhesion of mural cells and ECs during neovascularization.

Discussion

Our studies demonstrate that integrin $\alpha_4\beta_1$ and its ligand VCAM-1 facilitate a critical physical interaction between ECs and mural cells during blood vessel formation. Suppression of this interaction prevents blood vessel maturation and stabilization by directly inducing EC apoptosis and indirectly inducing mural cell apoptosis. It is important to note that neither integrin $\alpha_4\beta_1$ nor VCAM-1 are expressed on the cells of quiescent vessels, suggesting that once vessel formation is complete, these molecules are downregulated and not necessary for the proper functioning of normal quiescent blood vessels. Although a number of adhesion molecules, such as integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ (20–22) regulate angiogenesis, our studies show that integrin $\alpha_4\beta_1$ plays a unique role during angiogenesis by mediating intercellular adhesion and survival of both of the adhesive cell types.

As integrin $\alpha_4\beta_1$ and VCAM-1 play critical roles in embryonic vascular development (16–18), our studies suggest that loss of either molecule may perturb the normal association of endothelia and mural cells in the developing organism. In fact, the integrin $\alpha_4\beta_1$– and VCAM-1–deficient embryos exhibit similar defects during development, including defects in coronary vessel formation (16–17). These defects in coronary vessel formation are similar to defects in PDGF- and PDGF receptor–deficient embryos, suggesting that each of these molecules may be required for the coordinated association of mural and ECs during embryogenesis (5–8, 16–17). In fact, PDGF released by ECs is required to stimulate chemotaxis of pericytes toward the endothelia during blood vessel maturation (7–8). It is possible that PDGF regulates VCAM-1 expression in pericytes, thereby promoting cell guidance cues. Some studies also suggest that VEGF-A released by pericytes may promote EC survival during angiogenesis (23). It is thus possible that the integrin $\alpha_4\beta_1$–VCAM-1–mediated intercellular adhesion of endothelial and mural cells allows the cells to receive survival stimuli from growth factors secreted by their heterotypic partners. Our studies are the first, to our knowledge, to indicate that intercellular adhesion of these 2 cell types is required during angiogenesis.

Our studies indicated that integrin $\alpha_4\beta_1$ plays a role in all forms of angiogenesis investigated. We found that angiogenesis induced by a variety of angiogenic growth factors (IL-8, TNF-α, VEGF-A, and bFGF) could be blocked by integrin $\alpha_4\beta_1$ antagonists. Angiogenesis in a variety of tissues such as skin and eyes as well as in subcutaneous and orthotopic tumors was blocked by integrin $\alpha_4\beta_1$ inhibitors. These results are consistent with a role for the integrin in an essential process of neovascularization, that is, blood vessel stabilization by mural cells. Our studies thus extend the observations of previous studies that suggested a role for integrin $\alpha_4\beta_1$ in angiogenesis induced by select inflammatory mediators or by recombinant fragments of thrombospondin (TSP) (12–15) by identifying a key mechanism of action and by demonstrating a role for integrin $\alpha_4\beta_1$ in diverse forms of angiogenesis.

Our studies identified a new role for the integrin $\alpha_4\beta_1$ ligand VCAM-1 in angiogenesis. Although previous studies suggested a role for VCAM-1 in angiogenesis (12–13), our studies demonstrate for what we believe is the first time that VCAM-1 promotes intercellular adhesion of ECs and mural cells during angiogenesis.

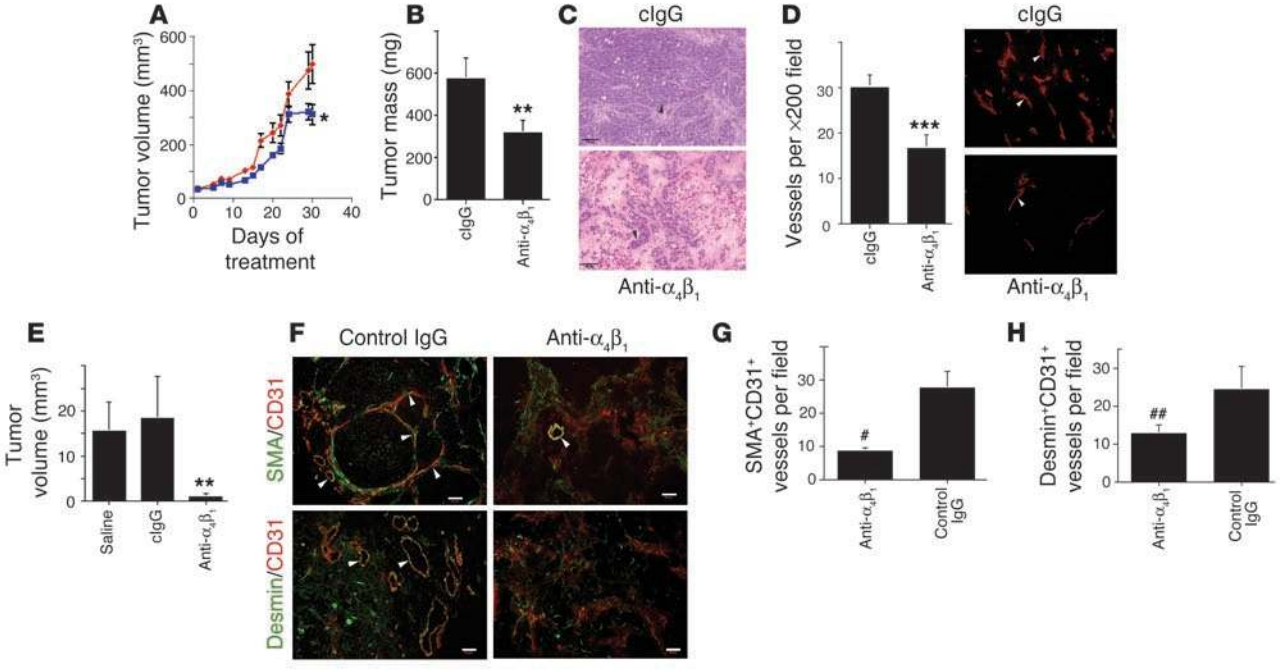

Figure 9

Antagonists of integrin $\alpha_4\beta_1$ block tumor growth, angiogenesis, and association of endothelia with mural cells. (**A**) Tumor volume $(l \times w^2)/2$ was determined every other day for 4 weeks for HT29 human integrin $\alpha_4\beta_1$–negative colon carcinoma tumors treated with saline, PS/2, or isotype-matched control antibody (anti–CD11b integrin). (**B**) Mean tumor mass ± SEM after 32 days of treatment. (**C**) H&E staining of tumor sections. Arrowheads indicate healthy tumor cells. Scale bars: 50 μm. (**D**) Graph, $CD31^+$ microvessels in frozen sections of tumors (arrowheads) were quantified per field. Representative micrographs are shown. Arrowheads indicate blood vessels. Magnification, ×200. (**E**) Mean tumor mass ± SEM for orthotopic pancreatic carcinoma tumors treated with saline, PS/2, or isotype-matched control antibody (cIgG, anti-CD11b) for 2 weeks. (**F**) Cryosections of control IgG antibody–treated (anti-CD11b, M1/70) and PS/2 antibody–treated integrin $\alpha_4\beta_1$ negative HT29 colon carcinoma tumors were immunostained to detect CD31 (red) and SMA (green) or desmin (green). Representative vessels coated with SMA or desmin-positive mural cells are indicated by arrowheads. (**G**) SMA-coated vessels were quantified by counting $CD31^+SMA^+$ vessels per field. Magnification, ×200. (**H**) Desmin-coated vessels were quantified by counting $CD31^+desmin^+$ vessels. $^*P = 0.01$; $^{**}P = 0.02$; $^{***}P < 0.003$; $^{\#}P < 0.002$; $^{\#\#}P < 0.05$.

Integrin $\alpha_4\beta_1$ can also interact with extracellular matrix proteins expressed by ECs during angiogenesis, such as cellular fibronectin (20) and TSP (15). We have previously shown that fibronectin supports angiogenesis by promoting integrin $\alpha_5\beta_1$-dependent EC migration and survival during angiogenesis (20). We also observed that inhibition of the integrin $\alpha_4\beta_1$-binding site on fibronectin suppresses angiogenesis and EC migration and that antagonists of integrin $\alpha_4\beta_1$ inhibit EC attachment to and migration on cellular fibronectin (Supplemental Figure 2). These studies suggest that integrin $\alpha_4\beta_1$ may participate in angiogenesis by interacting with several ligands. Recent studies also showed that integrin $\alpha_4\beta_1$ binds to the N terminus of TSP and mediates EC migration on recombinant fragments of TSP (15). However, several studies have shown that TSP is an angiogenesis suppressor in vivo (24–26). It remains unclear whether TSP and integrin $\alpha_4\beta_1$ interactions occur in vivo. Our studies however, identify a novel role for a key integrin $\alpha_4\beta_1$ ligand, VCAM-1, in regulating blood vessel formation.

Our findings suggest that antagonists of integrin $\alpha_4\beta_1$ may be useful therapeutics for solid tumors, as they prevent tumor blood vessel maturation and stabilization. Integrin $\alpha_4\beta_1$ antagonists are currently in clinical trials for treatment of inflammatory disorders (27–29) where they may influence neovascularization in addition to reducing leukocyte trafficking. Our studies indicate that antagonists of the integrin $\alpha_4\beta_1$/VCAM-1 interaction may be beneficial in cancer or inflammatory disease therapy due to their antiangiogenic properties.

Methods

Reagents. bFGF, VEGF-A, TNF-α and IL-8 were purchased from Genzyme. FITC-annexin V was purchased from BD Biosciences — Pharmingen. Human umbilical vein ECs (HUVECs) and human pulmonary VSMCs as well as culture media were from Cambrex Bioproducts. Poly-L-lysine was purchased from Sigma-Aldrich. Ten-day-old chicken eggs were purchased from McIntyre Poultry. W6/32 (mouse anti–human MHC) and P1F6 (anti–integrin $\alpha_v\beta_5$) were from David Cheresh (The Scripps Research Institute). HP2/1, P4G9, and P1H4 (mouse anti–human integrin $\alpha_4\beta_1$) were from Chemicon International. rsVCAM-1 was purchased from R&D Systems. PS/2 hybridoma was acquired from The American Type Culture Collection, and purified antibody was prepared under contract with Chemicon International.

Immunohistochemistry. To detect expression of integrin $\alpha_4\beta_1$, vWF, VCAM-1, CD31, SMA, and desmin in tissues, glass slides with frozen tissue sections were fixed in cold acetone for 2 minutes, air dried, and rehydrated in PBS for 5 minutes. Slides were washed in 0.05–0.1% Triton X-100 in PBS for 2 minutes, then blocked in 5% BSA in PBS overnight at 4°C. Slides were incubated in primary antibody at 5–10 μg/ml for 2 hours at room temperature. Slides were washed 3 times in PBS, incubated in secondary antibody (Alexa 499 or Alexa 594 conjugated goat anti–rat, goat anti–mouse, goat anti–rabbit, or donkey anti–goat IgG) at 1 μg/ml for 1 hour at room tem-

perature. Slides were washed 3 times in PBS, then stained with DAPI, after which coverslips were mounted. The following primary antibodies were used: goat anti–integrin $\alpha_4\beta_1$ (sc-6590; Santa Cruz Biotechnology Inc.), rat anti–mouse VCAM-1 (M/K-2; Chemicon International), mouse anti–human VCAM-1 (P8B1; Chemicon International), goat anti–pan species VCAM-1 (H276, sc-8304; Santa Cruz Biotechnology Inc.), mouse anti-SMA (Chemicon International), goat anti-desmin (sc-7559; Santa Cruz Biotechnology Inc.), rat anti–mouse CD31 (MEC 13.3; BD), or mouse anti–human CD31 (HEC–7; Pierce). Alexa 568– or 488–conjugated secondary antibodies were from Invitrogen Corp. TUNEL staining was performed on frozen sections according to manufacturer's recommendations (Chemicon International). Tissues immunostained included murine spontaneous breast carcinomas from the MMTV-PyV-mT mouse model, normal mouse tissues from healthy 6-week-old nude mice, HT29 colon carcinoma experimental tumors grown in nude mice, human breast carcinomas (DCIS), and normal human tissues obtained from the Cooperative Human Tissue Network. Detection of integrin $\alpha_4\beta_1$ in murine and human tissues was also observed with sc-14008, sc-6589, sc-6591 (Santa Cruz Biotechnology Inc.), and monoclonal P1H4 (Chemicon International). Appropriate immunoreactivity of sc-6590 with integrin $\alpha_4\beta_1$ in tissues was verified with the use of selective blocking peptide sc-6590P (Santa Cruz Biotechnology Inc.).

Angiogenesis assays. CAMs (n = 10) stimulated with 1 μg/ml bFGF, VEGF-A, TNF-α, or IL-8 (R&D Systems) were treated with saline, anti-integrin $\alpha_4\beta_1$ (HP2/1, P4G9, P1H4, or PS/2 were all tested with equal efficacy; data for PS/2 and P1H4 are shown), or isotype-matched control antibodies (W6/32, P1F6, anti-CD11b, or M1/70) as previously described (17). Three days later, blood vessel branch points were counted, or vWF$^+$ and integrin $\alpha_v\beta_3^+$ vessels were quantified in 5 microscopic fields at ×200 magnification. Experiments were repeated 4 times; results from representative experiments are shown. Statistical significance was determined using Student's t test.

Inbred mice of the strain FVB/N were subcutaneously injected with 400 μl growth factor–reduced Matrigel supplemented with 400 ng/ml of bFGF or VEGF-A and treated on days 1 and 3 by intravenous injection of 200 μg in 100 μl of endotoxin-free PS/2 antibody or control isotype matched rat anti–murine integrin β2 antibody (rat-anti–mouse CD11b, low endotoxin M1/70; BD) on days 1 and 4 (n = 5). After 5 days, Matrigel plugs were excised, embedded in OCT, frozen, and sectioned. Thin sections (5 μm) were immunostained with rat anti–murine CD31 followed by Alexa 565–conjugated goat anti–rat immunoglobulin. CD31-positive vessel density per microscopic field at ×200 magnification was determined in 10 randomly selected microscopic fields per Matrigel plug. Mean CD31$^+$ vessel density per field ± SEM for each treatment group was graphed. Photographs were taken of representative fields, with red indicating CD31-positive blood vessels. Experiments were performed 3 times and results from representative experiments are shown. Statistical significance was determined using Student's t test.

In other studies, mice (n = 10) were injected with 400-μl growth factor–reduced Matrigel supplemented with 400 ng/ml of bFGF containing no antibody, 25 μg/ml PS/2, or anti–integrin β2. Mice (n = 10) were also injected with 400 μl growth factor–reduced Matrigel supplemented with 400 ng/ml of bFGF containing no peptide, 50 μg/ml PS/2, or rat anti–murine CD18 (low endotoxin M18/2; BD). Mice were injected intravenously on day 5 with FITC-labeled *Bandeiraea simplicifolia* and sacrificed 15 minutes later. Matrigel plugs were homogenized in the presence of RIPA buffer, and total fluorescence intensity was determined on a fluorimeter (Lumat LB 9507; EG&G Bechtold). Experiments were performed 3 times, and results from representative experiments are shown. Statistical significance was determined using Student's t test.

Polymerized pellets containing 400 ng/ml of bFGF were implanted in the corneas of FVB/N mice as described previously; mice (n = 3) were treated on days 1 and 3 with intravenous injection of 200 μg in 100 μl of endotoxin-free PS/2 antibody or control antibody (rat anti–mouse CD18; low endotoxin M18/2; BD) on days 1 and 4. Mice were perfused on day 5 with FITC-labeled *B. simplicifolia*, a lectin that selectively binds to all endothelia. Mice were sacrificed, and corneas were excised and cryopreserved. Thin cryosections were then photographed, and mean area of fluorescence in the cornea was determined by confocal microscopy. To establish whether integrin $\alpha_4\beta_1$ integrin was accessible to the circulation in developing vessels, mice inoculated with VEGF-A–saturated Matrigel for 5 days were injected with PS/2 or control rat anti-murine (M18/2) antibodies. Tissues were harvested after 2 hours. Frozen sections were immunostained with anti-vWF, followed by goat anti–murine IgG Alexa 568 and goat anti–rabbit IgG–Alexa 488.

Tumor studies. HT29 human integrin $\alpha_4\beta_1$–negative colon carcinoma cells (2×10^6) were implanted subcutaneously in 7-week-old Balb/c nu/nu mice. After 2 weeks, mice with 30 mm^3 tumors were treated 3 times weekly by i.v. injection of saline, 200 μg PS/2, or 200 μg rat anti–murine CD11b integrin (M1/70; BD). Tumor dimensions were determined every other day for 4 weeks, and tumor mass was determined upon necropsy after 30 days of therapy (n = 10). Microvessel density was quantified by immunostaining frozen sections for CD31 expression and counting CD31$^+$ vessels in 5 randomly selected microscopic fields for each tumor. Tumor cryosections (5 μm) were also stained with H&E. Slide sectioning was performed by the UCSD Cancer Center Histology Core Facility. Experiments were performed 3 times; results from representative experiments are shown. Statistical significance was determined using Student's t test.

Orthotopic pancreatic tumor models were created by implanting 2×10^6 BXP3 human pancreatic tumor cells in the tails of the pancreata of nude mice. After 1 week, animals were treated every third day for 2 weeks by intravenous injection of 100 μl of saline, 2 mg/ml endotoxin-free PS/2 in saline, or 2 mg/ml endotoxin-free anti-CD11b antibody (M1/70) in saline. Tumors were excised and volumes were determined.

Adhesion assays. Adhesion of smooth muscle to ECs was measured as follows: logarithmic phase human pulmonary VSMCs were labeled with CellTracker Orange (CMTMR) according to manufacturer's directions (Invitrogen Corp.) and incubated with monolayers of HUVECs in EC-culture medium in the presence of 25 μg/ml HP2/1, P1F6, or rsVCAM-1 for 2 hours at 37°C. Cell layers were gently washed with warmed culture medium and fixed in 3.7% paraformaldeyde prior to enumeration of bound cells per microscopic field at ×200 magnification. Experiments were performed 4 times; results from representative experiments are shown. Statistical significance was determined using Student's t test.

Ethical approval. Animal care and use for all experiments was approved by the Institutional Animal Care and Use Committee of UCSD prior to the initiation of all studies. Use of human tissue specimens in these studies was approved by the Institutional Review Board of UCSD.

Acknowledgments

This work was supported by grants from the NIH, the UCSD Cancer Center, the Charlotte Geyer Foundation, and the American Heart Association (to J.A. Varner).

Received for publication September 23, 2004, and accepted in revised form March 23, 2005.

Address correspondence to: Judy Varner, John and Rebecca Moores Comprehensive Cancer Center, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0912, USA. Phone: (858) 822-0086; Fax: (858) 822-1325; E-mail: jvarner@ucsd.edu.

Barbara Garmy-Susini and Hui Jin contributed equally to this work.

1. Carmeliet, P. Angiogenesis in health and disease. 2003. *Nat. Med.* **9**:653–660.
2. Stupack, D.G., and Cheresh, D.A. 2003. Apoptotic cues from the extracellular matrix: regulators of angiogenesis. *Oncogene.* **22**:9022–9029.
3. Jin, H., and Varner, J. 2004. Integrins: roles in cancer development and as treatment targets. *Br. J. Cancer.* **90**:561–565.
4. Hanahan, D., and Folkman, J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell.* **86**:353–364.
5. Bjarnegard, M., et al. 2004. Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities. *Development.* **131**:1847–1857.
6. Lindblom, P., et al. 2003. Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. *Genes Dev.* **17**:1835–1840.
7. Abramsson, A., Lindblom, P., and Betsholtz, C. 2003. Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *J. Clin. Invest.* **112**:1142–1151. doi:10.1172/JCI200318549.
8. Jain, R.K., and Booth, M.F. 2003. What brings pericytes to tumor vessels? *J. Clin. Invest.* **112**:1134–1136. doi:10.1172/JCI200320087.
9. Morikawa, S., et al. 2002. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am. J. Pathol.* **160**:985–1000.
10. Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E., and Hanahan, D. 2003. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J. Clin. Invest.* **111**:1287–1295. doi:10.1172/JCI200317929.
11. Elices, M.J., et al. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell.* **60**:577–584.
12. Koch, A.E., Halloran, M.M., Haskell, C.J., Shah, M.R., and Polverini, P.J. 1995. Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. *Nature.* **376**:517–519.
13. Nakao, S., Kuwano, T., Ishibashi, T., Kuwano, M., and Ono, M. 2003. Synergistic effect of TNF-alpha in soluble VCAM-1-induced angiogenesis through alpha 4 integrins. *J. Immunol.* **170**:5704–5711.
14. Fukushi, J., Ono, M., Morikawa, W., Iwamoto, Y., and Kuwano, M. 2000. The activity of soluble VCAM-1 in angiogenesis stimulated by IL-4 and IL-13. *J. Immunol.* **165**:2818–2833.
15. Calzada, M.J., et al. 2004. Alpha4beta1 integrin mediates selective endothelial cell responses to thrombospondins 1 and 2 in vitro and modulates angiogenesis in vivo. *Circ. Res.* **94**:462–470.
16. Yang, J.T., Rayburn, H., and Hynes, R.O. 1995. Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development.* **121**:549–560.
17. Kwee, L., et al. 1995. Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. *Development.* **121**:489–503.
18. Sengbusch, J.K., He, W., Pinco, K.A., and Yang, J.T. 2002. Dual functions of [alpha]4[beta]1 integrin in epicardial development: initial migration and long-term attachment. *J. Cell Biol.* **157**:873–882.
19. Melder, R.J., et al. 1994. During angiogenesis, vascular endothelial growth factor and basic fibroblast growth factor regulate natural killer cell adhesion to tumor endothelium. *Nat. Med.* **2**:992–997.
20. Kim, S., Bell, K., Mousa, S., and Varner, J. 2000. Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. *Am. J. Pathol.* **156**:1345–1362.
21. Brooks, P.C., Clark, R.A., and Cheresh, D.A. 1994. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science.* **264**:569–571.
22. Friedlander, M., et al. 1995. Definition of two angiogenic pathways by distinct alpha v integrins. *Science.* **270**:1500–1502.
23. Darland, D.C., et al. 2003. Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. *Dev. Biol.* **264**:275–288.
24. Good, D.J., et al. 1990. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. U. S. A.* **87**:6624–6628.
25. Dameron, K.M., Volpert, O.V., Tainsky, M.A., and Bouck, N. 1994. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science.* **265**:1582–1584.
26. Jimenez, B., et al. 2000. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat. Med.* **6**:41–48.
27. Miller, D.H., et al. 2003. A controlled trial of natalizumab for relapsing multiple sclerosis. *N. Engl. J. Med.* **348**:15–23.
28. Napier, J., and Wong, R.K. 2003. Natalizumab: a new hope for Crohn's disease? *Am. J. Gastroenterol.* **98**:1197–1199.
29. Vanderslice, P., et al. 2004. Development of cell adhesion molecule antagonists as therapeutics for asthma and COPD. *Pulm. Pharmacol. Ther.* **17**:1–10.