# Antiintegrin $\alpha v\beta 3$ blocks human breast cancer growth and angiogenesis in human skin

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## Abstract

**Angiogenesis plays a fundamental role in human breast tumor progression. In fact, recent findings indicate that vascular density is a prognostic indicator of breast cancer disease status. Evidence is presented that the integrin $\alpha v\beta 3$ is not only a marker of human breast tumor–associated blood vessels, but that it plays a significant role in human angiogenesis and breast tumor growth. To assess the role of $\alpha v\beta 3$-dependent angiogenesis in the progression of human breast cancer, we examined a SCID mouse/human chimeric model with transplanted full thickness human skin containing $\alpha v\beta 3$-negative human breast tumor cells. This tumor induced a human angiogenic response as measured by vascular cell immunoreactivity with monoclonal antibodies LM609 and P2B1 directed to human $\alpha v\beta 3$ and CD31, respectively. Intravenous administration of LM609 either prevented tumor growth or markedly reduced tumor cell proliferation within the microenvironment of the human skin. These LM609-treated tumors not only contained significantly fewer human blood vessels but also appeared considerably less invasive than tumors in control animals. These findings demonstrate that $\alpha v\beta 3$ antagonists may provide an effective antiangiogenic approach for the treatment of human breast cancer. (*J. Clin. Invest.* 1995. 96:1815–1822.)**
**Key words: tumor • invasion • malignant • chimera • therapy**

## Introduction

The invasive progression of tumors is a major impediment to the successful treatment of many neoplastic diseases. Therefore, a more complete understanding of the factors contributing to tumor proliferation and malignancy is of paramount importance. The growth and metastatic dissemination of human breast cancer has been functionally linked to the process of angiogenesis (1–4). In fact, the extent of vascularization of primary breast tumors has been shown to directly correlate with disease status and thus serves as a prognostic indicator of breast cancer progression (5–8).

Tumor-induced angiogenesis is initiated by tumor and inflammatory cell release of angiogenic cytokines (9–12). However, this process also depends on vascular cell migration and invasion, processes regulated by cellular adhesion receptors (10, 13, 14). To this end, we recently demonstrated that blood vessels in human granulation tissue and cytokine-stimulated chick embryonic vessels show enhanced expression of integrin $\alpha v\beta 3$ (15). More importantly, antagonists of this integrin block cytokine or tumor-induced angiogenesis on the chick chorioallantoic membrane (15, 16). However, at present, no evidence exists supporting a biological role for adhesion receptors in human angiogenesis. We therefore examined the functional role of vascular integrin $\alpha v\beta 3$ in human breast tumor–induced angiogenesis in the microenvironment of the human skin. A severe combined immunodeficient (SCID)[1] mouse/human model (17, 18) was established to monitor the progression of an $\alpha v\beta 3$-negative human breast carcinoma growing within human skin, allowing us to specifically examine the role of vascular cell integrin $\alpha v\beta 3$ in human angiogenesis.

In this report we provide evidence that integrin $\alpha v\beta 3$ is highly expressed on angiogenic vessels associated with biopsies of malignant human breast carcinoma, suggesting that integrin $\alpha v\beta 3$ may be a useful marker of blood vessels associated with human breast cancer tissue. Furthermore, systemic administration of mAb LM609 directed to integrin $\alpha v\beta 3$ not only disrupts human angiogenesis but reduces the growth and invasive properties of human breast carcinoma in the SCID mouse/human chimeric model. Therefore, antagonists of integrin $\alpha v\beta 3$ may provide a novel approach for the treatment of malignant breast tumors.

## Methods

*Antibodies, reagents and chemicals.* mAbs LM609 (anti-$\alpha v\beta 3$) has been described previously (19). W6/32 hybridoma (anti-MHC class I) was obtained from American Type Culture Collection (ATCC, Rockville, MD). mAbs were purified from acites fluid on a protein A Sepharose column. Polyclonal rabbit antifactor VIII antibody 016P was obtained from Biogenex (San Ramon, CA). mAb P2B1 (anti-CD31) was obtained from Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, University of Iowa (Iowa City, IA). FITC and rhodamine-labeled secondary antibodies were obtained from Biosource International, (Camarillo, CA). OCT embedding medium was obtained from Baxter (McGraw Park, IL). Fluoromount G was obtained from Southern Biotechnology Associates (Birmingham, AL).

*Cell culture and human tissues.* The human breast carcinoma cell line MCF-7 was obtained from ATCC. A variant of MCF-7 (MCF-7PB) was selected in culture for growth in the absence of added estrogen and was used in all tumor growth assays. MCF-7PB cells were cultured

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1. *Abbreviation used in this paper:* SCID, severe combined immunodeficient.

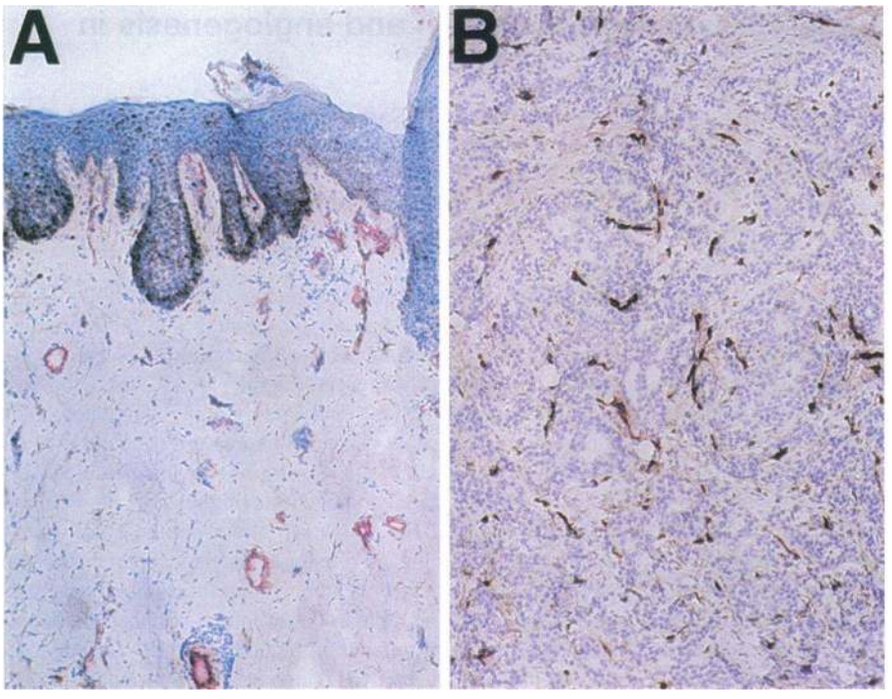



*Figure 1.* Vascular expression of integrin $\alpha v\beta 3$ in human breast tissue biopsies. 4 $\mu$m cryostat sections from breast tissue biopsies were stained with murine anti-$\alpha v\beta 3$ (LM609) and rabbit antifactor VIII antibodies. Immunoreactivity was detected by immunoperoxidase and alkaline phosphatase as described in Methods. (*A*) Normal breast tissue; and (*B*) adjacent malignant breast carcinoma from the same patient costained with anti-$\alpha v\beta 3$ (brown) and anti-factor VIII (red) antibodies. Tissue sections were photographed at a magnification of 100.

in DME (GIBCO BRL, Grand Island, NY) supplemented with 10% FBS, and 1% sodium pyruvate. MCF-7PB cells were shown to be $\alpha v\beta 3$ negative by FACS® analysis, ELISA and immunohistochemical staining anti-$\alpha v\beta 3$–directed monoclonal antibodies (data not shown). 10 paired normal human breast and breast carcinoma in situ biopsies were provided by the pathology department tissue bank at Wayne State University School of Medicine, Detroit, MI. Fresh human neonatal foreskins were obtained from the Cooperative Human Tissue Network (CHTN) Cleveland, OH, and were stored in sterile RPMI-1640 media supplemented with 2% FBS and 1% gentamycin.

*Chimeric human/mouse model.* Preparation and surgical transplantation of human skin was performed as previously described (17, 18). Briefly, 6-week-old SCID mice were anesthetized and a 2-cm$^2$ section of skin was surgically removed. A precut section of fresh full thickness human neonatal foreskin was sutured into place. The grafts were bandaged securely for 4 wk to allow healing. MCF-7PB human breast carcinoma cells ($3 \times 10^6$) were injected intradermally within the human skin in a total volume of 50 $\mu$l of sterile PBS with a 30-gauge needle. 2 wk later before any visible signs of tumor growth were evident, mice received tail vein injections of either purified mAb LM609 (anti-$\alpha v\beta 3$),

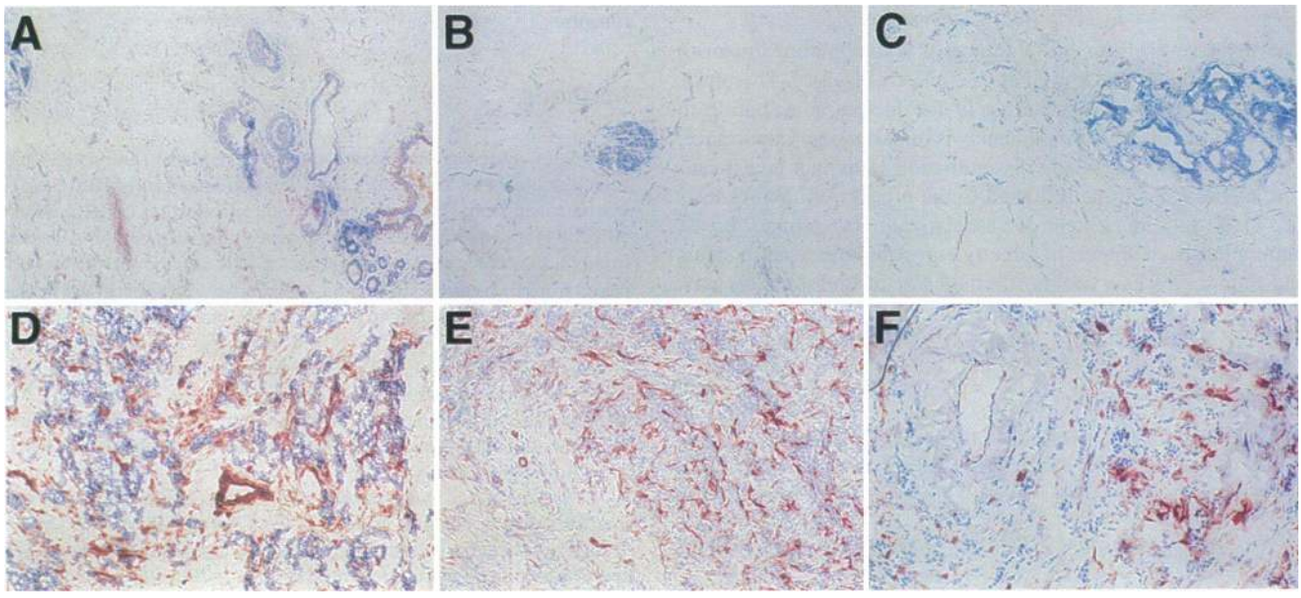



*Figure 2.* Expression of vascular integrin $\alpha v\beta 3$ in human breast tissue biopsies. Cryostat sections of human breast tissue biopsies were stained with mAb LM609 (anti-$\alpha v\beta 3$) as described in Methods. (*A*) fibrocystic breast tissue; (*B*) normal breast tissue; (*C*) benign lobe; (*D–F*) malignant breast carcinomas. Immunoreactivity was detected by alkaline phosphatase staining. Red staining indicates expression of integrin $\alpha v\beta 3$. Tissue sections were photographed at a magnification of 100.

mAb W6/32 (anti-MHC class I), at 250 μg/50 μl, or PBS alone (50 μl). Mice were treated twice weekly for a total of 3 wk.

*Quantification of tumor growth.* At the end of the 3-wk antibody treatment, tumor size was measured with caliper and tumor volumes determined. Next, mice were killed and the well-defined tumors were carefully resected with the use of a stereo microscope and tumor wet weights were determined. Mice with no gross indications of tumor were analyzed microscopically for the presence of human tumor by dissection of the human skin graft and immunohistological staining. Tissue sections were analyzed with a compound microscope (BX60; Olympus Corp., Lake Success, NY). Intact solid tumors were photographed with a stereo microscope (SZH10; Olympus Corp.) at a magnification of 10. Tumor growth assays were completed twice with 5–12 mice per condition.

*Immunohistochemical and immunofluorescence analysis.* Tumors resected from mice were washed twice with sterile PBS and either embedded in OCT media for snap freezing or paraffin embedded for histological analysis. 3-μm sections of frozen tissue were stained with either mAb LM609, P2B1, or in combination with polyclonal antibody 016P. Two color immunofluorescence staining was performed as previously described (16). Briefly, tissues were allowed to incubate with murine mAbs LM609 or P2B1 together with rabbit polyclonal antibody 016P for 2 h at room temperature. Tissue sections were washed three times in PBS and incubated with both goat anti-rabbit and goat anti-mouse antibodies as described previously (16). Two color staining was detected with secondary antibodies labeled with either FITC or rhodamine for immunofluorescence and immunoperoxidase or alkaline phosphatase for immunohistochemical analysis. Histological analysis of paraffin sections were stained with hematoxylin and eosin as previously described (20). Tissue sections were photographed with an Olympus BX60 compound microscope at a magnification of 200.

*Quantification of angiogenesis.* Tumors and surrounding tissue was embedded, snap frozen in liquid nitrogen, and sectioned. 3-μm sections were stained for the presence of human angiogenic blood vessels with both mAbs LM609 (anti-αvβ3) and P2B1 (anti-human CD31). The number of tumor-associated human blood vessels were determined by counting immunoreactive vessels from at least five sites showing significant vascularization examined by high power (×200) microscopy as described previously (5–7), with the use of an Olympus BX60 compound microscope fitted with epifluorescence. Blood vessel counts were made by three independent observers in a double blind fashion.

*Statistical analysis.* Statistical analysis was performed with a Stat Works Program for Macintosh computers, Cricket Software Inc., Philadelphia, PA. Data was analyzed for statistical significance with Students *t* test.

## Results

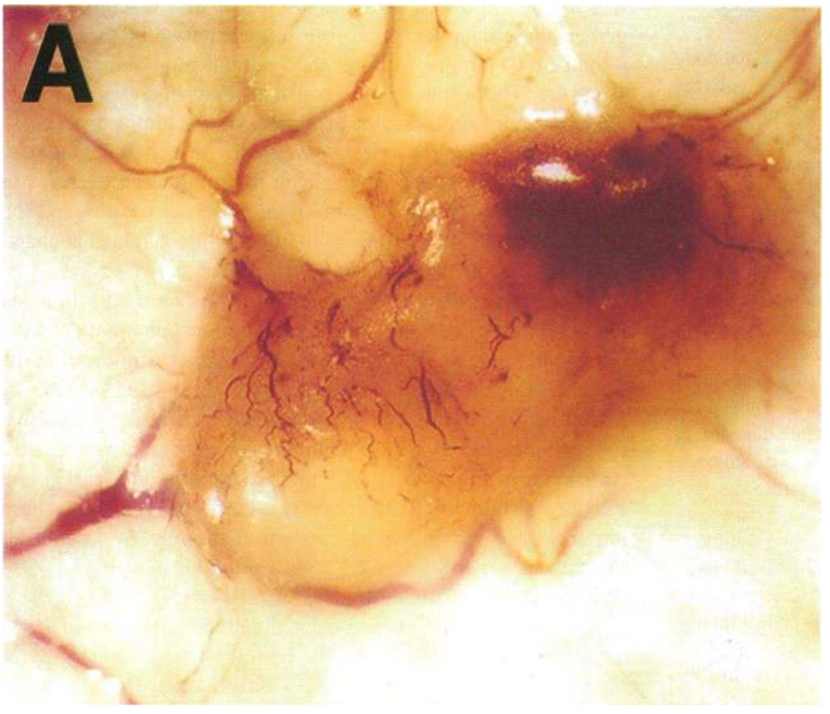
*Integrin αvβ3 is a marker of blood vessels associated with human breast tumor biopsies.* To examine the expression of integrin αvβ3 in human tumor-associated vasculature, biopsies from normal human breast, fibrocystic disease, or breast carcinoma were examined. Malignant human breast carcinoma biopsy and adjacent normal skin overlaying this biopsy were co-stained with mAb LM609 (anti-αvβ3) and polyclonal antifactor VIII antibody. As shown in Fig. 1 *A*, factor VIII (red), a known marker of blood vessels was expressed in vessels of normal breast tissue while little, if any, αvβ3 integrin (brown) was expressed in these same vessels. In contrast, integrin αvβ3 was highly expressed in vessels associated with malignant human breast carcinoma (Fig. 1 *B*). To further evaluate the vascular expression of integrin αvβ3 in various human breast tissue lesions and cancer, representative biopsies were stained with mAb LM609. As demonstrated in Fig. 2, *A–C*, minimal levels of αvβ3 were expressed in either fibrocystic, normal, or benign lobes of human breast tissue, respectively. However, in all malignant breast carcinomas tested (Fig. 2, *D–F*), αvβ3 integrin was highly expressed in the tumor-associated vasculature. Together, these data document four representative biopsies out of a total of 10 tumors with matched normal biopsy tissue (Figs. 1 and 2). These findings suggest that integrin αvβ3 may be a clinically relevant indicator of human breast tumor disease.

*Growth of human breast carcinoma in the SCID mouse/human chimera model.* To evaluate the role of angiogenesis in the proliferation of human breast carcinoma, we examined the growth of human breast tumors in full-thickness human neonatal foreskin transplanted onto SCID mice. As shown in Fig. 3 *A*, αvβ3-negative MCF-7PB cells injected intradermally into the human skin formed well-defined solid tumors containing numerous blood vessels infiltrating the central mass of these tumors. To determine the origin of these tumor-associated blood vessels, frozen sections of this tissue were stained with antibodies known to react specifically with human but not murine blood vessels. As shown in Fig. 3 *B* and *C*, mAbs LM609 (anti-αvβ3) and P2B1 (anti-human CD31) reacted with blood vessels in these tumor sections as indicated by the red staining. As a control, these tissues were costained with a polyclonal antibody 016P directed to human or mouse factor VIII (green). When these images were merged a direct colocalization (yellow) of integrin αvβ3 or CD31 with factor VIII was observed among many of the vessels detected. These data clearly demonstrate the presence of human angiogenic blood vessels within these human breast tumors.

*Role of vascular integrin αvβ3 in human breast tumor growth and angiogenesis.* To assess the effects of mAb LM609 on human breast tumor growth, tumor-bearing mice were injected via the tail vein with mAb LM609 or control antibody W6/32 directed to human MHC class I molecule. Mice were treated twice weekly for a total of 3 wk. Resulting tumors were measured with calipers to determine volume and then resected for wet weight determination. As indicated in Fig. 4, *A* and *B*, systemic administration of mAb LM609 resulted in a 5- and 10-fold reduction of tumor weight and volume, respectively, as compared to controls ($P = 0.002$). In fact, 8 of the 12 mice treated with mAb LM609 showed no evidence of tumor growth while 8 of 11 mice treated with control mAb W6/32 developed relatively large tumors. Animals injected with PBS alone showed similar tumor growth to those treated with mAb W6/32 (data not shown).

*mAb LM609 directed to vascular integrin αvβ3 blocks breast tumor-induced human angiogenesis.* Morphological analysis of the LM609-treated tumors indicated a significant reduction in tumor-associated blood vessels. As shown in Fig. 5 *A*, tumors from mice treated with control mAb W6/32 showed numerous blood vessels infiltrating the tumors. However, a reduced number of vessels were associated with tumors from LM609-treated animals. These results suggest that mAb LM609 significantly blocks angiogenesis as well as breast tumor growth in the microenvironment of the human skin.

To quantify the antiangiogenic effects of systemically administered mAb LM609, frozen sections of control and experimental-treated tumors were stained with anti-CD31 antibody. As shown in Fig. 5 *B*, numerous human tumor-associated blood vessels were present in tissues from mice treated with control antibody W6/32. In contrast, a marked reduction in angiogenic human vessels was evident in LM609 treated tumors (Fig. 5 *C*). In fact, LM609 treatment resulted in an approximate three- to fourfold reduction in human blood vessels as detected by reactivity with anti-human CD31 antibody (Fig. 5 *D*). These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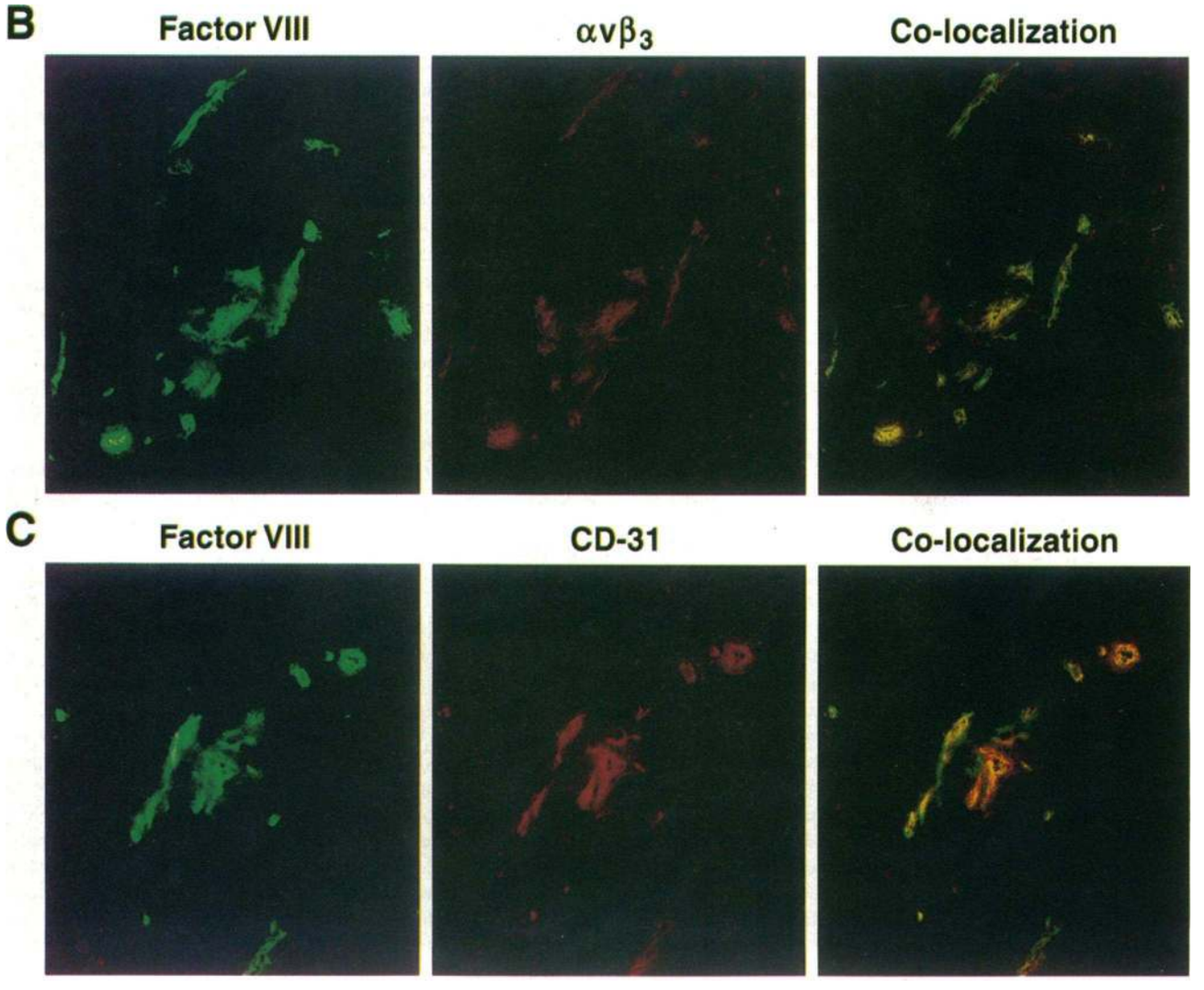
B
Factor VIII
αvβ3
Co-localization
C
Factor VIII
CD-31
Co-localization

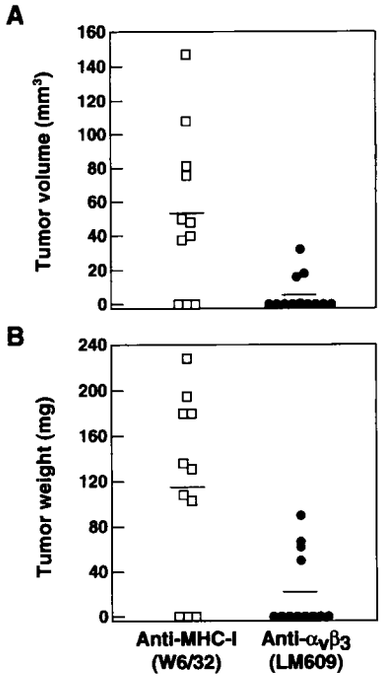


*Figure 4.* Antagonist of integrin $\alpha v\beta 3$ blocks human breast carcinoma growth in human skin. Tumor bearing mice were treated systemically (250 $\mu$g/50 $\mu$l) with mAbs LM609 (anti-$\alpha v\beta 3$) or control W6/32 (anti-MHC class I), twice weekly for a total of 3 wk by tail vein injection. Tumors were resected and tumor weights and volume determined. (*A*) Tumor volume, (*B*) Tumor weight. Bars indicates the mean of each data set.

data demonstrate that systemic administration of mAb LM609 inhibits human breast tumor–induced angiogenesis and this is likely due to an effect on human blood vessels, since these vascular cells are the only cells that react with mAb LM609 in these tissues (Fig. 3).

*Blocking human breast tumor angiogenesis results in a less invasive tumor phenotype.* Systemic administration of mAb LM609 prevented the growth of breast tumors in 8 of the 12 animals (Fig. 4). Tumors from the four mice that did develop measurable lesions were further examined histologically. As indicated in Fig. 6 *C*, the small tumors remaining after mAb LM609 treatment had very few viable blood vessels (*arrows*) and a well defined tumor margin (*arrow heads*). Control-treated tumors however, (Fig. 6 *A* and *B*), showed numerous blood vessels and a highly invasive tumor margin. In fact, in these tumors numerous blood vessels contained malignant tumor cells that had actually invaded through the blood vessel walls (Fig. 6 *B*). No vessels containing malignant tumor cells were detected in tumors from LM609-treated mice, suggesting a less invasive tumor phenotype.

## Discussion

Numerous studies have provided suggestive evidence that angiogenesis is of critical importance for the growth and metastasis of many solid tumors including breast carcinomas (21–23). In fact, breast cancer patients whose tumors had high vessel counts had a poor clinical prognosis while patients with low vessels counts generally had minimal if any detectable metastatic disease (5–8).

We recently showed that integrin $\alpha v\beta 3$ is highly expressed on angiogenic blood vessels in human granulation tissue and in the chick embryo (15, 16). In this report we provide evidence that integrin $\alpha v\beta 3$ is highly expressed on malignant breast tumor vasculature, while little if any is expressed on vessels from normal, fibrocystic, or benign breast tissue lesions. This selective vascular expression of integrin $\alpha v\beta 3$, combined with studies indicating angiogenesis as a reliable indicator of breast cancer status, suggests $\alpha v\beta 3$ may prove to be a prognostic marker of malignant human breast tumors.

Previous work in our laboratory has indicated that integrin $\alpha v\beta 3$ plays a functional role in angiogenesis in the chick embryo (15, 16). To extend this work we examined a more clinically relevant model of tumor growth and angiogenesis, which has been successfully used to examine human melanoma growth (17, 18). Human breast tumors established from MCF-7PB cells were allowed to grow in full thickness human skin transplanted onto SCID mice. Systemic administration of mAb LM609 directed to the integrin $\alpha v\beta 3$, significantly reduced human angiogenesis and the resulting growth of a human breast carcinoma. In addition, we also observed similar results when examining growth of an $\alpha v\beta 3$ negative human melanoma cell line (M21L) in this model (data not shown). Taken together, these results suggest that mAb LM609 and perhaps other $\alpha v\beta 3$ antagonists may impact the angiogenic response and growth of various human tumors. In the case of breast carcinoma, mAb LM609 caused a three- to fourfold reduction in the number of human tumor-associated blood vessels as compared to antibody or buffer controls. This reduction in human angiogenesis was directly linked to the reduced tumor growth in this model. However, the variable response to LM609 treatment may be due, in part, to the fact that some murine vessels invade these human skin grafts over time. Thus, our results are likely to be an under estimate since mAb LM609 does not react with murine $\alpha v\beta 3$, and would not be expected to impact murine blood vessels feeding these tumors.

Interestingly, not only did mAb LM609 block human angiogenesis and tumor growth, but it also significantly altered the invasive behavior of these tumors. Histological analysis of the tumor tissue remaining after mAb LM609 treatment revealed only small confined lesions with well defined borders. In contrast, control-treated tumors had irregular and highly invasive tumor margins. In fact, control-treated tumors showed numerous blood vessels containing malignant tumor cells that had invaded through the vessel walls. These characteristics are consistent with a highly invasive and metastatic tumor (24, 25). Con-

*Figure 3.* Human angiogenesis and breast carcinoma growth in the SCID mouse/human chimeric model. $\alpha v\beta 3$ negative human breast carcinoma cells ($3 \times 10^6$) were injected intradermally within the human skin transplanted onto SCID mice as described in Materials and Methods. Tumors were allowed to grow for 3 wk until a palpable mass was detected on all animals. (*A*) Human breast tumor growing within the human skin showing associated blood vessels. Photos were taken at a magnification of 10. To determine the origin of the blood vessels, tumors were embedded in OCT, snap frozen, and 3-$\mu$m sections were cut for immunofluorescence analysis. (*B*) Cryostat sections of human breast tumors from SCID mice stained with antifactor VIII polyclonal antibody 016P (green), anti-$\alpha v\beta 3$ mAb LM609 (red), colocalization of factor VIII and $\alpha v\beta 3$ (yellow). (*C*) Cryostat sections of human breast tumors stained with antifactor VIII polyclonal antibody 016P (green), anti-CD31 mAb P2B1 (red), colocalization of factor VIII, and human specific CD31 (yellow). Photographs were taken at a magnification of 200.

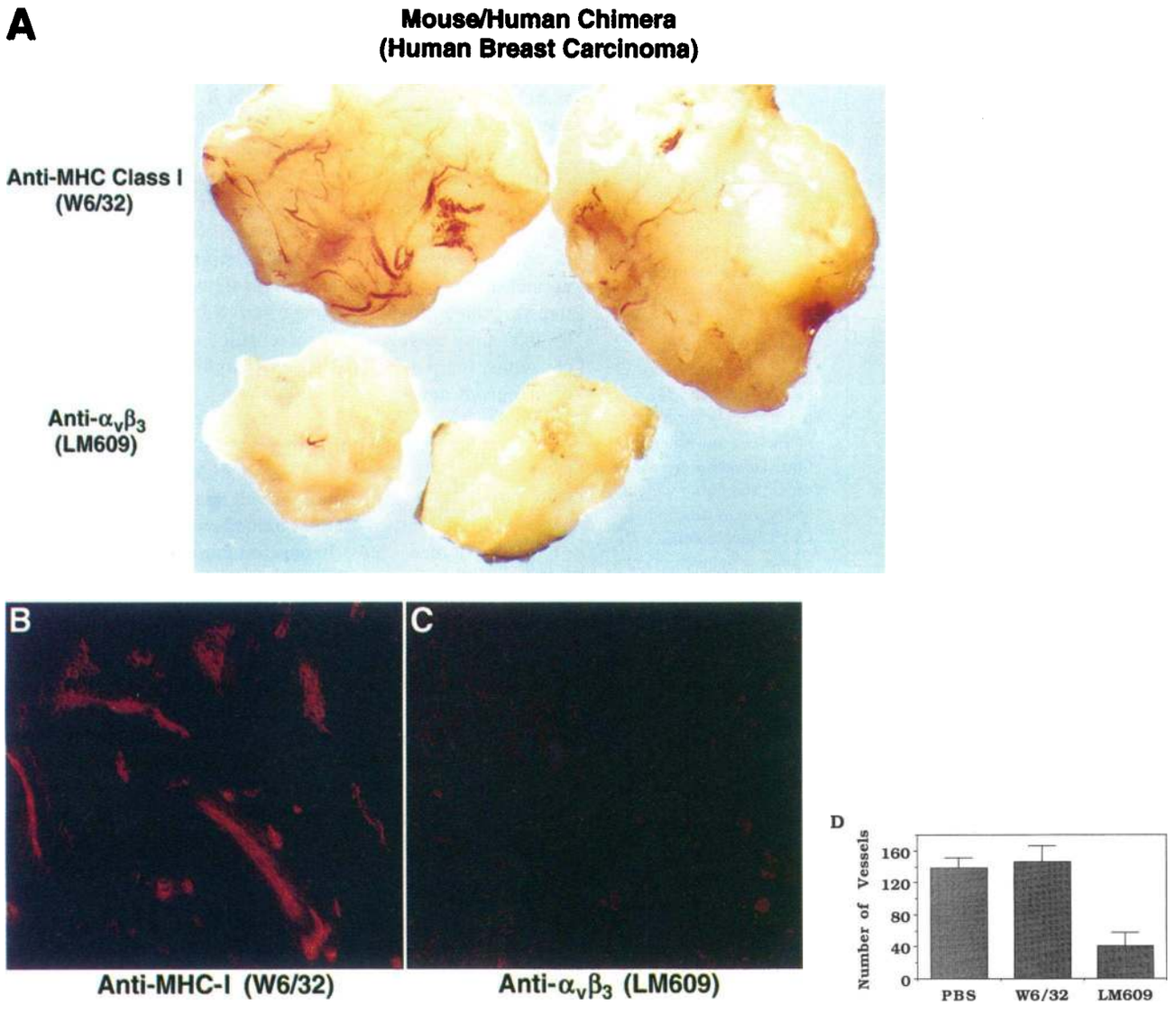


*Figure 5.* Antagonists of integrin $\alpha v\beta 3$ block human angiogenesis. (*A*) Tumors removed from the human skin grafts were examined morphologically with a stereo microscope and photographed at a magnification of 10. Cryostat sections of tumors treated with mAb W6/32 (*B*) or LM609 (*C*), were stained by indirect immunofluorescence. Positive immunoreactivity is indicated by staining with mAb P2B1 (anti-human CD31) in red. Tissue sections were photographed at a magnification of 200. (*D*) Quantification of human tumor-associated blood vessels from five randomly selected high power (×200) microscopic fields. Blood vessel counts were determined by three independent observers in a double blind fashion. Bars indicate means±SE.

versely, no blood vessel infiltration was observed in tumors from LM609-treated mice.

Previous studies have suggested that angiogenesis contributes to the invasive and metastatic spread of tumor cells (1, 2, 5, 10). Furthermore, angiogenic blood vessels have been characterized by an increased secretion of various proteases including members of the serine and matrix metalloproteinase families (26–30). Our results (Fig. 6) may be explained by increased proteolytic activity from vascular-derived proteases which could significantly alter the extracellular matrix in the immediate vicinity of the tumor. This proteolyzed matrix may facilitate the invasive and metastatic potential of these human breast carcinomas. Alternatively, tumor cell invasiveness may be influenced by growth factors and chemokines present in the serum that diffuse into the tumor and surrounding stroma from leaky angiogenic tumor-associated vessels. These factors may in turn promote tumor cell proliferation and motility. Thus, by limiting angiogenesis, tumor cells may be deprived of factors that contribute to their malignant phenotype.

In this report, we provide several lines of evidence that antagonists of integrin $\alpha v\beta 3$ may offer an effective treatment for human breast cancer and tumor angiogenesis. First, integrin $\alpha v\beta 3$ is highly expressed on the vasculature of invasive and malignant human breast tumors, whereas this receptor is minimally expressed on quiescent vessels in normal breast tissue. Second, systemic administration of mAb LM609, directed to integrin $\alpha v\beta 3$ blocks human angiogenesis and breast tumor growth in the microenvironment of the human skin. Third, this treatment has no apparent effect on normal human tissue since these human skin grafts appeared unaffected by LM609 treatment as indicated by normal dermal architecture and histology. Finally, systemic administration of mAb LM609 appeared to

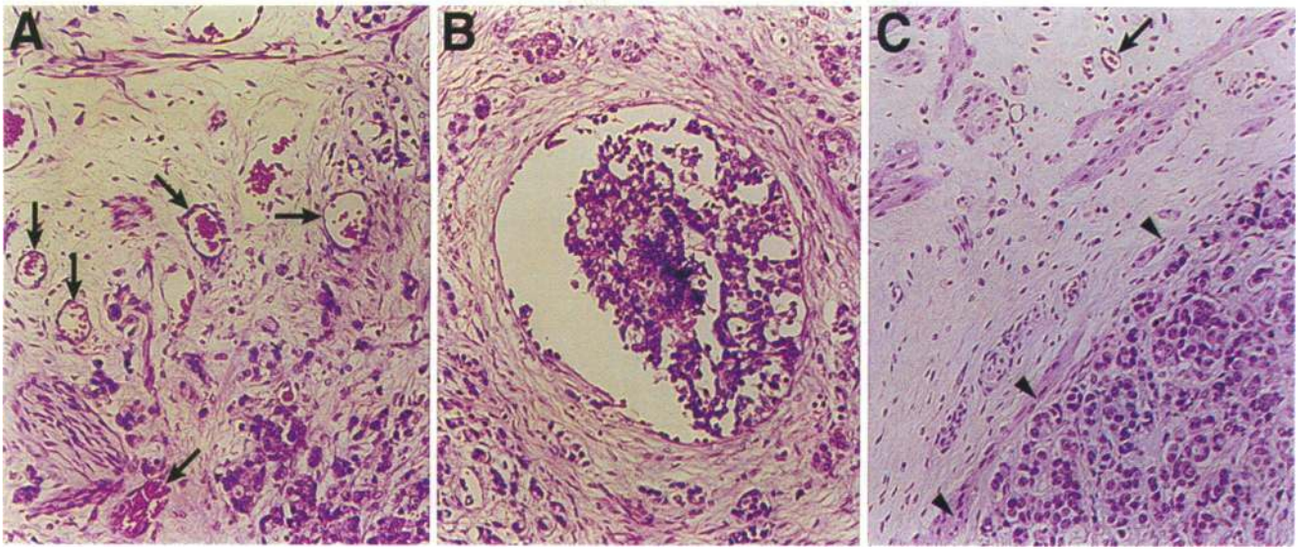


*Figure 6.* Blocking human angiogenesis alters the invasive phenotype of human breast carcinomas. Paraffin-embedded sections of control (*W6/32*) or experimental (*LM609*)-treated tumors were stained with hematoxylin and eosin for histological analysis (*A*) mAb W6/32-treated tumor showing irregular and invasive tumor margins and numerous blood vessels (*arrows*). (*B*) W6/32-treated tumor showing an example of a blood vessel containing malignant tumors cells that have invaded through the vessel wall. (*C*) mAb LM609-treated tumor showing well defined borders (*arrow heads*) and few blood vessels (*arrows*). Tissue sections were photographed at a magnification of 200.

reduce the invasive behavior of these human breast carcinomas. Together, these findings implicate integrin $\alpha v\beta 3$ as a marker of blood vessel infiltration in human breast cancer and suggests that antagonists of integrin $\alpha v\beta 3$ may provide a powerful new antiangiogenic approach for the treatment of human breast cancer.

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