Vascular Integrin $\alpha_\beta_3$: A New Prognostic Indicator in Breast Cancer

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ABSTRACT

Blood vessel density is a prognostic indicator of multiple tumor types. Recently, it has been established that tumor-associated blood vessels express elevated levels of integrin $\alpha_\beta_3$. In fact, there is evidence that integrin $\alpha_\beta_3$ identifies the most proliferative endothelial cells within human breast carcinomas. Therefore, we evaluated breast cancer tissue in terms of both blood vessel density and $\alpha_\beta_3$ expression. We found that the antibody LM609 to integrin $\alpha_\beta_3$ preferentially stains the blood vessels of small caliber. Furthermore, comparative studies between LM609 and anti-CD31 antibodies on normal breast indicate that very low and weak expression of integrin $\alpha_\beta_3$ was found on vessels within normal tissue, whereas CD31 antigen was expressed in almost all vasculature. Indeed, expression of integrin $\alpha_\beta_3$ was significantly higher in tumors of patients with metastasis than in those without metastasis. In a series of 197 consecutive patients with invasive breast cancer and long follow-up, vascular expression of integrin $\alpha_\beta_3$ in tumor vascular “hot spots” was found to be the most significant prognostic factor predictive of relapse-free survival in both node-negative and node-positive patients. These findings support the contention that angiogenesis plays a critical role in breast cancer progression and suggest that integrin $\alpha_\beta_3$ is an endothelial cell marker with significant prognostic value and potential usefulness as a target for specific antiangiogenic therapy.

INTRODUCTION

The formation and growth of new blood vessels from preexisting ones, i.e., angiogenesis, is a pivotal biological process in mammalian organisms. Angiogenesis is necessary in a variety of physiological processes, such as embryonic development, chronic inflammation, and wound repair, in which it is highly regulated (reviewed in Refs. 1 and 2). Abnormal angiogenesis is involved in the pathogenesis of several diseases, such as chronic inflammatory disorders and degenerative ocular diseases, and it is necessary for tumor growth and progression (reviewed in Ref. 1). Tumor-induced angiogenesis is initiated by tumor and stromal cell release of angiogenic peptides and/or by down-regulation of endogenous angiogenesis inhibitors (reviewed in Ref. 3) and involves modifications of the ECM.

Several studies suggest that endothelial cell migration and invasion through ECM are regulated by cellular adhesion receptors (reviewed in Refs. 4 and 5). Previous studies (6–8) demonstrate that integrin $\alpha_\beta_3$ plays a key role in angiogenesis induced by a variety of stimuli in vivo. Integrin $\alpha_\beta_3$ promotes vascular endothelial cell survival in vivo (9). In fact, during angiogenesis, proliferative endothelial cells become apoptotic in response to antagonists of integrin $\alpha_\beta_3$, which also causes regression of vascularization and tumor growth by a mechanism involving p53, the cell cycle inhibitor p21$^{WAF/CIP1}$, and alterations of the bcl-2/bax ratio (5, 9).

Initial studies (10) examined the functional role of integrin $\alpha_\beta_3$ in human breast cancer and provided evidence that this integrin is highly expressed on angiogenic vessels associated with invasive breast cancer and that systemic administration of LM609 antibody directed to integrin $\alpha_\beta_3$ is capable of disrupting intratumoral neovessels, thereby inhibiting tumor formation in a severe combined immunodeficient mouse/human chimeric model.

We undertook these studies to further define the expression of integrin $\alpha_\beta_3$ in human breast cancer. LM609 antibody preferentially immunostains the intratumoral microvessels of smaller caliber. This staining pattern was selective for invasive cancer and specific for tumor-induced neovascularization. In contrast, integrin $\alpha_\beta_3$ expression was not readily detected in nonangiogenic benign breast tissue. The degree of VECs stained by LM609 antibody was significantly higher in tumors of patients with metastasis, and it was moderately associated with...
endothelial cell but not tumor cell proliferative rate. In addition, the LM609 antibody defined the vascular "hot spot" of each cancer in a consecutive series of 197 breast cancer patients and proved to be a significant predictor of clinical outcome of the patients, both before and after adjustment for the conventional prognostic indicators.

MATERIALS AND METHODS

**Patient Population.** The first preliminary study was undertaken on 46 patients with invasive breast carcinoma who underwent surgery between 1990 and 1991 at the Vicenza General Hospital. This series was made up of pairs of cases who had disease recurrence (24 patients) and cases who developed metastasis (22 patients), randomly selected from a larger series of consecutive patients who underwent surgery during the same period and who had available follow-up data. The main characteristics of the patients were as follows. The median age was 59 years (range, 29–87 years). Sixteen patients were pre- or perimenopausal, and 26 were postmenopausal patients. Histological types were ductal invasive (38 cases) and lobular or other invasive types (8 cases). Tumor sizes were pT1 (24 cases), pT2 (17 cases) and pT3 in 5 cases. There was axillary node involvement (N+) in 28 cases, whereas 18 cases were N−. Histological grades (Bloom and Richardson) were G1 (11 cases), G2 (15 cases), and G3 (20 cases). Sixteen patients did not receive adjuvant therapy, whereas 10 were treated with adjuvant chemotherapy and 20 were treated with adjuvant tamoxifen. The median follow-up of the series exceeded 5 years. In addition, 15 samples of tissue from subjects with normal breast were studied for comparison.

A second cohort of 197 consecutive patients with operable invasive breast carcinoma who underwent surgery between 1987 and 1991 at the same institution and who had no evidence of distant metastatic disease at diagnosis were studied for prognostic purposes. Patients were followed until the date of their death, the date they were last known to be alive, or the end of the follow-up period, whichever came first. The criteria of administration of adjuvant treatments to node-positive patients were described previously (11).

**Tissue Evaluation.** Tumors were classified adopting the 1989 pathological staging system of the American Joint Committee on Cancer (1992) regarding primary tumor size and axillary lymph node involvement. Tumors were assigned a histological grade from I (low) to III (high), according to the Bloom and Richardson grading scheme.

**Antibodies and Immunohistochemical Studies.** Antibodies used for the study included: previously characterized affinity-purified anti-integrin αvβ3, LM609 (6, 10); monoclonal anti-CD31 clone JC/70A (purchased from DAKO, Glostrup, Denmark); anti-CD105 monoclonal E-9 (kindly supplied by Prof. Shant Kumar, University of Manchester, Manchester, United Kingdom); and monoclonal anti-Ki-67 (purchased from DAKO). The dilutions used were: 1:1000; 1:400; 1:50, and 1:50, respectively.

All the immunohistochemical determinations were performed on representative samples snap-frozen in liquid nitrogen and stored at −80°C until sectioning. Cryostat sections, 4–6 μm thick, were fixed in cold acetone at 4°C for 10 min, air-dried, and incubated at room temperature with the above antibodies for 1 h. After being washed with PBS, bound antibodies were visualized using antimouse biotinylated antibody, the streptavidin-peroxidase complex (DAKO), and 3,3′-diaminobenzidine. For the detection of the nuclear antigen Ki-67, as a marker of cell proliferation, and of the membrane epitope E-9, the latter being a marker of endothelial cells, a double immunostaining technique was adopted, as reported elsewhere (12).

E-9-positive endothelial cells stained red, and proliferating cell nuclei stained dark brown. We performed a systematic comparison in the same fields of the samples tested between the panendothelial marker anti-CD31, as an internal positive control, and LM609 to test the sensitivity and specificity of the latter antibody for microvessels. For negative controls, in all instances, we used nonspecific IgG as the primary antibody.

**Criteria of Evaluation.** To quantitate and compare expression of integrin αvβ3 and CD31 antigen, microvessels expressing either antigen were counted in five fields for each sample within a ×100 field that stained positive for both integrin αvβ3 and CD31. The primary field was chosen from areas that combined a large number of total microvessels based on the CD31-positive staining, and then the additional four fields were derived by moving in four separate directions from that of the original field.

Tumors were heterogeneous in their microvessel density, but the areas of most intense neovascularization were identified by scanning the tumor sections at low power (×100), and then the areas of invasive carcinoma with the highest number of distinct microvessels stained with the above endothelial markers were recorded. Once the single area of highest neovascularization (vascular hot spot) was identified for each tumor, individual microvessels were counted on a ×200 field (i.e., ×20 objective lens and ×10 ocular lens; 0.738 mm² per field) as reported previously (10–13).

Because the most prominent integrin αvβ3 staining was colocalized in the most highly vascularized area by CD31 staining, integrin αvβ3 expression was quantitated by counting LM609-positive microvessels in the same areas in which the highest expression of CD31 was assessed. Three independent observers (P. C. B., E. B., and G. R.) evaluated vascular vessel counts.

The methodology of identifying endothelial cells and counting cycling cells has been described previously (14). Five adjacent fields were analyzed at ×400, each field crossing the entire tumor tissue available on the slide. In every field, the cycling endothelial or tumor cells were counted (P. B. V.). The mean of the fractions of cycling cells of the five fields was used for evaluation. A minimum of 450 and a maximum of 2300 endothelial cells (median, 970 cells), and a minimum of 750 and a maximum 3250 tumor cells (median, 1780 cells) were counted per section, respectively.

**Statistical Methods.** In the preliminary study, the hypothesis that integrin αvβ3 was identically distributed in the three subgroups of patients was evaluated using the nonparametric KW test (15). A multiple comparison procedure was subsequently adopted that was suitable for protection from type I statistical error. The association between the number of microvessels stained by LM609 with the other continuous variables was evaluated by Spearman’s rank correlation coefficient.
The Kolmogorov-Smirnov test was used to compare the distributions of microvessel counts in the groups of patients identified by the modalities of the discrete variables.

For the prognostic study on 197 patients, RFS and OS were calculated considering the time elapsed between the date of surgery and the date of the first recurrence (local or distant) and the date of death for all causes, respectively. Univariate and multivariate analyses (the latter only for RFS) were performed using Cox's regression models. Because integrin α3β1 expression and CD31 vascular count were measured on a continuous scale, their prognostic relationships were studied by a flexible restricted cubic spline approach (16). Conventional categories were adopted for the other prognostic variables. The null hypotheses β=0 was tested by Wald statistic and the additional prognostic contribution of each variable, when the other variables were considered, was tested by LRT.

Due to the high correlation found between integrin α3β1 expression and CD31 counts, two alternative multivariate Cox regression models, including either integrin α3β1 expression or CD31 vascular counts, were performed. The other variables retained clinically relevant were also considered in the multivariate models to adjust the prognostic effect of the two above-mentioned variables.

To describe the prognostic trend for integrin α3β1 expression, the hazard values for three equally spaced values of absolute microvessel counts (50, 70, and 90 counts) were estimated by the full multivariate Cox regression models. In each model two hazard ratios (70 versus 50 and 90 versus 70) and the corresponding 95% confidence limits were calculated. In the presence of linear trends, we would expect equal values of the HRs for equal increments of integrin α3β1 expression. In addition, the estimated RFS curves obtained from the full Cox

Fig. 1  Comparison of the pattern of staining of the microvessels obtained by Mab LM609 or Mab anti-CD31. Tissue sections were processed as described in "Materials and Methods." A, invasive ductal breast carcinoma, poorly differentiated (G3). Shown are three vessels of large caliber stained by anti-CD31 antibody (×100, red arrow) within the tumor stroma. This picture is shown only to highlight the different pattern of staining of the two antibodies regarding the caliber of the vessels. This particular field was not considered for the evaluation of microvessel count. B, the same tumor described above. In the same field (×100), the same three vessels of larger caliber are not stained by LM609 antibody (red arrow). As a positive internal control, the picture presents a longitudinal microvessel positively stained by the LM609 antibody (green arrow) within the tumor stroma. C, the picture shows, at high resolution (×400), a typical endothelial cell positively stained by both LM609. No background is observed (red arrow). D, the same picture stained using the anti-CD31 antibody, for comparison (×400; red arrow).
regression models were provided corresponding to the three above-considered integrin α3β1 expression values in the patients with N− or N+ tumors separately.

The predictive capability of the models was investigated by the Harrell c statistic (17). This statistic assumes values ranging from 0.5 to 1.00. If the model has no predictive capability, it is expected to be 0.5, and it approaches 1.00 in the case of high prognostic capability.

Because adjuvant treatments were not allocated in a random fashion, the univariate analyses were performed separately for the following groups of patients: N−, not treated; N+, treated with adjuvant chemotherapy; and N+, treated with hormone therapy. The multivariate analyses were performed separately for N− and N+ patients. We also performed an analysis on all the series. However, we advise that the results of the latter analysis be interpreted with caution because the nodal status effect can be confused with the treatment effect. Consequently, it is influenced by the criteria adopted to allocate the patients to each adjuvant treatment (11).

RESULTS

Expression of Integrin α3β1 and CD31 Antigen in Breast Disease. To establish the total tumor-associated blood vessel count in both invasive cancer of the breast and normal breast tissue, cryostat sections of 46 invasive cancers and 15 normal breast tissues were stained with the panendothelial cell marker anti-CD31 antibody. In all tumors (Fig. 1A) and normal tissue studied, CD31 antigen was shown to be consistently associated with VECs, independently of their caliber. In adjacent sections of the same tissue frozen block, we also detected integrin α3β1 antigen. However, expression of integrin α3β1 was restricted to VECs lining tumor-associated microvessels and was preferentially expressed on the vessels of minor caliber.
**Fig. 3** Double immunostaining with the antibodies E-9 and Ki-67 showing an invasive ductal carcinoma of the breast with high tumor cell proliferation rate (brown nuclei) and low proliferating endothelial cells (brown nuclei and red vessel). Arrow, a typical proliferating endothelial cell.

**Fig. 4** The degree of expression of integrin αβ3 (LM609 antibody) on blood endothelial cells in invasive breast cancer is moderately associated with high rate of proliferating endothelial cells as assessed by double staining techniques using E-9 and Ki-67 antibodies (n = 46). (Fig. 1B). LM609 is highly specific for tumor-associated endothelium, and it does not cross-react with unrelated structures or normal vessels (Fig. 1C). Among the 46 invasive cancers, the median number of microvessels immunostained by LM609 at the hot spot was 67 per field (range, 18–151), whereas little or no αβ3 was observed in vessels of normal breast tissue. As shown in Fig. 2, an invasive ductal breast carcinoma is represented by both an area of hot spot and non-hot spot stained with either anti CD31 or LM609. LM609 detects primarily small caliber hot spot vessels (Fig. 2B).

**Correlation of LM609-stained Microvessels with Endothelial Cell Proliferation Rate.** To further characterize the αβ3-positive vessels at the hot spots, we double-stained the tumor sections with Mab E-9, directed to the proliferation marker CD105, and Ki-67 antibody (Fig. 3). There was an association between these markers as suggested by the Spearman’s rank correlation ρ, value of 0.51 (P = 0.001; Fig. 4).

Conversely, integrin αβ3 expression on VECs was not correlated with tumor size (KS test = 0.26; P = 0.33); axillary nodal status (KS = 0.29; P = 0.24); histological grading (KS = 0.19; P = 0.72) or tumor cell proliferative rate (Ki-67 labeling index; Spearman’s ρ = 0.15).

**Integrin αβ3 and Metastasis.** To establish whether αβ3 expression on VECs was associated with tumor malignancy, we examined LM609 staining within cryostat sections from patients who developed metastasis (n = 22) and compared it to staining in sections from patients that showed no disease recurrence (n = 24). The median number of LM609-stained...
**Prognostic Value of Integrin α3β3 Expression.** To evaluate the prognostic value of α3β3 in breast cancer biopsies, we examined a series of 197 consecutive patients with invasive breast cancer for the presence of VEC-associated α3β3. The median follow-up of these patients was 86 months for both RFS and OS (range, 1–112 months and 5–122 months, respectively). The main clinicopathological characteristics of the cohort studied, stratified by axillary lymph node status and adjuvant therapy, are listed in Table 1.

High integrin α3β3 expression on intratumoral microvessels was found to be a strong prognostic indicator of shorter RFS and OS on univariate analysis in all the subgroups of patients, including: N− patients (LRTRFS = 42.80, P < 0.0001; and LRTOS = 17.60, P = 0.00015); N+ patients treated with adjuvant chemotherapy (LRTRFS = 9.46, P = 0.0088; and LRTOS = 8.82, P = 0.012) and N+ patients treated with adjuvant hormone therapy (LRTRFS = 20.80, P < 0.0001; and LRTOS = 10.20, P = 0.0059). The graphic representations of the relationship between the degree of integrin α3β3 expression on VECs, as a continuous variable, with the logarithm of the hazard ratio for RFS and OS, are shown in Fig. 6. On univariate analysis, also microvessel counts by anti-CD31 antibody staining was a highly significant prognostic factor in all the subgroups (N−, LRTRFS = 47.10, P < 0.0001; and LRTOS = 19.50, P < 0.0001; N+ treated with chemotherapy, LRTRFS = 8.48, P = 0.014, and LRTOS = 10.50, P = 0.05; N+ treated with hormone therapy, LRTRFS = 19.50, P < 0.0001, and Research.
The correlation between nodal status and adjuvant therapy. The hormone therapy was adopted, taking into account the structural, combined variable with modalities N-tumor size, all were not found to be of prognostic significance.

In the subgroup of N+ patients the model included: menopausal status, tumor size, histological grading, and the combined variable nodal status/adjuvant therapy and integrin αβ3 expression. This latter variable, again, was the strongest prognostic factor (LRT = 70.53, P < 0.0001) being the predictive capability of the model c = 0.81.

The alternative Cox regression models with CD31 counts were performed with the same strategies used for integrin αβ3. The results obtained were similar and, as reported in Table 3, CD31 was the strongest prognostic factor in all the subgroups of patients. The predictive capability of the models including CD31 is similar to those obtained for integrin αβ3 expression (c = 0.89, c = 0.72, and c = 0.80, respectively, for N−, N+, and overall series). The multivariate model performed in the overall series excluding the markers of angiogenesis (i.e., integrin αβ3 or CD31) has a poorer predictive capability (c = 0.68), thus confirming the prognostic relevance of assessment of microvessel counts in breast cancer (19, 20).

To provide a graphic representation of the prognostic impact of integrin αβ3, the RFS curves for three absolute values of microvessels stained with LM609 were estimated by the multivariate Cox regression models performed separately on N− and N+ patients (Fig. 7). A patient with a N− tumor and an absolute microvessel count of 70 had a 7 times higher recurrence risk compared to one with an absolute value of 50; however, comparing the absolute values of 90 versus 70, the HR was smaller (HR = 2.50). A similar trend was observed for N+ patients.

**DISCUSSION**

**Integrin αβ3 Expression Is Specific for Tumor-induced Angiogenesis.** Determination of angiogenic activity may have important clinical application in the management of patients with breast cancer as a new prognostic tool and target for novel anticancer therapeutic strategies based on angiosuppressive treatments (18).

Previous studies have used panendothelial markers, including factor VIII-related antigen, CD31, CD34, and so on, to identify microvessels in biopsies of human cancers and found that the patients with highly vascularized tumors had significantly poorer outcome than those with low vessel counts (reviewed in Refs. 18 and 19). However, the panendothelial markers used in the above studies do not target tumor-induced neovascularization because they stain all vessels, inclusive those of normal tissues, where they react with both vascular and lymphatic endothelium (20). In this report, several lines of evidence suggest that integrin αβ3, on blood vessels serves as a marker of neovascularization in breast cancer tissue. First, by comparing the staining pattern obtained using either LM609 or anti-CD31 antibodies, we found that the former antibody identifies an absolute lower number of intratumoral microvessels at the hot spot (Table 1). This is because LM609 antibody preferentially stains the small-caliber blood vessels, consistent with the notion that angiogenesis is restricted to the growth of small venules and capillaries (1). Second, elevated expression of integrin αβ3 on intratumoral vasculature corresponded to high vascular endothelial cell proliferation rate as detected by costainings with E-9 and Ki-67 antibodies. It was claimed that the E-9

![Graph](https://example.com/graph.png)
Table 2  Multivariate analysis on relapse-free survival
Model includes integrin α₃β₁ expression.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Node-negative patients</th>
<th>Node-positive patients</th>
<th>All patients</th>
</tr>
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<tbody>
<tr>
<td>Microvessel counts with integrin α₃β₁ expression</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Linear component</td>
<td>11.10 0.0009</td>
<td>7.27 0.0070</td>
<td>17.80 &lt;0.0001</td>
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<tr>
<td>Nonlinear component</td>
<td>7.46 0.0063</td>
<td>2.52 0.1100</td>
<td>8.90 0.0029</td>
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<td>Menopausal status, post vs. pre/peri</td>
<td>7.77 0.0053</td>
<td>2.58 0.1000</td>
<td>5.26 0.0218</td>
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<td>Tumor size, pT₂₃ vs. pT₁</td>
<td>3.34 0.0670</td>
<td>1.24 0.2660</td>
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<td>Histological grading, G₁ vs. G₂</td>
<td>2.60 0.1070</td>
<td>5.19 0.0227</td>
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<tr>
<td>No. of involved axillary nodes, ≥3 vs. &lt;3</td>
<td></td>
<td>1.11 0.2900</td>
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<tr>
<td>Adjuvant therapy, hormone vs. chemotherapy</td>
<td></td>
<td>0.08 0.7830</td>
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</tr>
<tr>
<td>N+ CMF vs. N-</td>
<td></td>
<td>0.84 0.3590</td>
<td></td>
</tr>
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</table>

"One degree of freedom.

Table 3  Multivariate analysis on relapse-free survival
Alternative model including CD31 counts.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Node-negative patients</th>
<th>Node-positive patients</th>
<th>All patients</th>
</tr>
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<tbody>
<tr>
<td>Microvessel counts by anti-CD31</td>
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<td></td>
</tr>
<tr>
<td>Linear component</td>
<td>11.20 0.0009</td>
<td>5.93 0.0149</td>
<td>16.90 &lt;0.0001</td>
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<tr>
<td>Nonlinear component</td>
<td>7.09 0.0078</td>
<td>1.61 0.2050</td>
<td>8.60 0.0034</td>
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<td>Menopausal status, post vs. pre/peri</td>
<td>1.52 0.2180</td>
<td>2.53 0.1110</td>
<td>2.71 0.0995</td>
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<td>Tumor size, pT₂₃ vs. pT₁</td>
<td>1.62 0.2030</td>
<td>0.62 0.4320</td>
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<tr>
<td>Histological grading, G₁ vs. G₂</td>
<td>0.85 0.3580</td>
<td>4.20 0.0405</td>
<td></td>
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<tr>
<td>No. of involved axillary nodes, ≥3 vs. &lt;3</td>
<td></td>
<td>1.03 0.3100</td>
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<td>Adjuvant therapy, hormone vs. chemotherapy</td>
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<td>0.005 0.9450</td>
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<td>N+ CMF vs. N-</td>
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<td>0.09 0.7690</td>
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<td>N+ TAM vs. N-</td>
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<td>0.12 0.7280</td>
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"One degree of freedom.

antibody, specific against the CD105 antigen, was selectively expressed on “activated” intratumoral vessels (21). Therefore, the correlation found between the expression of integrin α₃β₁, CD105, and proliferative endothelial cell nuclei suggests that integrin α₃β₁ is a marker of angiogenic blood vessels. Finally, the comparative studies between LM609 and anti-CD31 antibodies on normal breast tissue indicate that very low and weak expression of integrin α₃β₁ was found on quiescent normal vessels, whereas CD31 antigen was expressed in almost all vasculature, regardless of their size. We found that the number of VECs within tumoral stroma was significantly higher than that of normal tissue regarding integrin α₃β₁ expression, but not CD31 antigen (data not shown). These findings confirm and extend the results previously reported by Brooks et al. (10) demonstrating the presence of α₃β₁ in a small series of human breast cancers.

Vascular Integrin α₃β₁ Expression and Metastasis.

To verify whether the levels of expression of integrin α₃β₁ are predictive of metastasis, we compared the absolute highest vascular counts with LM609 staining in a subgroup of primary tumors without metastasis and in those of patients who developed metastasis. We found that the breast cancers that showed evidence of metastasis had a significantly higher VEC integrin α₃β₁ expression than did those without metastasis (Fig. 5). These results may be explained by experimental evidence that elevated levels of integrin α₃β₁ are stimulated by production of angiogenic peptides, such as basic fibroblast growth factor, and cytokines, such as tumor necrosis factor-α (9). Alternatively, activated endothelial cells produce various proteases that could alter the structure of ECM and, thereby, facilitate tumor cells invasiveness and metastasis (10).

Vascular Integrin α₃β₁ Expression, Prognosis, and Potential Therapeutic Implications.

Expression of integrin α₃β₁ was found to be a strong prognostic factor in a consecutive series of breast cancer patients after surgery with long-term follow-up. Of particular relevance, in all the patient subgroups studied, tumors containing elevated microvessel counts with LM609 staining had a significantly poorer prognosis relative to those with low expression of integrin α₃β₁. Multivariate analyses, including the conventional prognostic indicators, suggest...
that integrin \( \alpha_3\beta_1 \) expression on tumoral vasculature was an independent prognostic indicator of recurrence in the patients with node-negative as well as in those with node-positive disease treated with adjuvant therapy.

The degree of integrin \( \alpha_3\beta_1 \) expression on VECs discriminated between different prognostic groups when the impact of this variable was adjusted for the other covariates included in the multivariate model (Fig. 7). Integrin \( \alpha_3\beta_1 \) expression on intratumoral vasculature had, in this series, a prognostic value similar to that of the panendothelial marker CD31, as suggested by the alternative model of multivariate analysis reported in Table 3. Furthermore, the prognostic value of integrin \( \alpha_3\beta_1 \) compares favorably with the results of previous studies, which assessed the prognostic significance of microvessels count determined using various panendothelial markers in biopsies from breast cancer (11, 13, 22, 23). Finally, the multivariate model, which did not include a marker of angiogenesis, had a poorer predictive value as compared to those of the models reported in Tables 2 and 3 (as assessed using the Harrell \( c \) statistic). These findings further support the clinical relevance of angiogenesis as a marker of malignant breast cancer. In this context, LM609 antibody would be considered a more specific and sensitive marker of angiogenesis. However, this can be further tested in prospective studies.

The results of this study coupled with the previous reports (10) that systemic administration of LM609 antibody blocked angiogenesis and breast tumor growth, suggest that antagonists of integrin \( \alpha_3\beta_1 \) may provide an antiangiogenic approach worthy of serious consideration to improve treatment of human breast cancer. In fact, a humanized form of LM609 (Vitaxin) has recently completed Phase I clinical trials in patients with advanced stage cancer. This antibody showed no toxic side effects and appeared to provide clinical benefit in some of the patients (24). Furthermore, recent evidence has been reported suggesting that integrin \( \alpha_3\beta_1 \) is a target molecule for disruption of tumor vasculature induced by administration of tumor necrosis factor and IFN-\( \gamma \) (25). Because inhibition of angiogenesis is presently considered one of the more promising novel therapeutic approaches for cancer patients, the development of markers of angiogenic activity is an integral part of proper clinical trial designs in inhibitors of angiogenesis (26). In conclusion, it seems likely that the use of the LM609 antibody to determine integrin \( \alpha_3\beta_1 \) vascular expression, which is detectable by a single and reliable immunohistochemical assay, may become part of breast cancer evaluation as a prognostic marker. Furthermore, LM609 staining may be a useful surrogate marker predictive of the efficacy of systemic administration of antagonists of integrin \( \alpha_3\beta_1 \) or of other inhibitors of angiogenesis capable of blocking endothelial cell growth (reviewed in Ref. 26).

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