[CANCER RESEARCH 59, 856–861, February 15, 1999]

# Breast Carcinoma: Vascular Density Determined Using CD105 Antibody Correlates with Tumor Prognosis[1]

**Shant Kumar,[2] Ashur Ghellal, Cheng Li, Ged Byrne, Najib Haboubi, Ji Min Wang, and Nigel Bundred**

*Department of Pathological Sciences, The University of Manchester, Manchester M13 9PT, United Kingdom [S. K., A. G., C. L.]; Christie Hospital, Manchester, M204BX United Kingdom [S. K., C. L.]; Department of Surgery, Withington Hospital, M208LR Manchester, United Kingdom [A. G., G. B, N. H., N. B.]; and Pharmingen, San Diego, California 2121-1111 [J. M. W.]*

## ABSTRACT

**Angiogenesis is essential for tumor growth and metastasis. There are conflicting reports as to whether microvessel density (IMD) in breast cancers is associated with prognosis. This could be due to the use of different antibodies to endothelial cell markers, variation in tissue pretreatment protocols, and nonstandardized counting methods. We have assessed the IMD in 106 breast carcinomas using a pan-endothelial marker, CD34, and a recently described mAb to CD105, which preferentially reacts with endothelial cell in angiogenic tissues. IMD values (separated as above or below median) for CD105 expression showed a statistically significant correlation with overall ($P = 0.0029$) and disease-free survival ($P = 0.0362$). In contrast, blood vessel counts using a pan-endothelial marker CD34 did not correlate with overall or disease-free survival ($P = 0.2912$ and $P = 0.3153$, respectively). When IMD values were subdivided into quartiles and assessed for their prognostic values, there was a statistically significant difference in the overall survival across CD105, but not CD34, values ($P = 0.0017$ and $P = 0.7997$, respectively) and also disease-free survival ($P = 0.0431$ and $P = 0.5066$, respectively). Further analysis of IMD values demonstrated that there were no deaths in the lowest quartile for CD105 and it differed from the other three quartiles. However, examination of clinical details of patients in the lowest quartile failed to reveal clustering of patients known to be associated with low-risk factors. Multivariate analysis confirmed that IMD values using CD105 were an independent prognostic factor. These results suggest that the ability to quantitatively distinguish between tumor neovascularization and preexisting vessels may be important in the assessment of tumor angiogenesis, but requires confirmation in a greater number of patients with a longer follow-up.**

## INTRODUCTION

Angiogenesis, the formation of new blood vessels from a preexisting vascular bed, is a complex multistep process involving extracellular matrix remodeling, EC[3] migration and proliferation, microvessel differentiation, and anastomosis. These processes are controlled by angiogenic factors that regulate one or more of these key events. Angiogenesis is essential for tumor growth and metastasis. The degree of vascularization (*i.e.*, IMD) has the potential to be used as a prognostic marker (1, 2). The extent of neovascularization in invasive breast carcinomas is an independent predictor of metastasis either to axillary lymph nodes, distant sites, or both (2–4). Subsequently, several researchers have demonstrated that high IMD is an independent prognostic factor in many other histological types of human tumors, whereas some investigators have failed to find any such correlation (reviewed in Refs. 2 and 5). The reason for this discrepancy is not known. IMD in tumors has been assessed by staining tissues with pan-endothelial antibody against antigens such as CD34, CD31 and von Willebrand factor. ECs are highly heterogeneous, therefore, it begs the question whether a pan-endothelial marker is an ideal reagent to evaluate IMD in a tumor. Previously, we and others have reported that anti-CD105 antibodies, unlike pan-endothelial markers, preferentially bind to "activated" ECs *in vitro* and in tissues participating in angiogenesis (6–11). For instance, in irradiated human umbilical vein endothelial cells, CD105 was highly up-regulated compared with normal unirradiated cells (10). Furthermore, after immunostaining of tissue sections representing various histological types of human tumor including breast cancer, CD105 was found to be strongly expressed in ECs of tumor blood vessels, but was either undetectable or only weakly present in blood vessels of most normal tissues (7–9). CD105 was present in large amounts in biopsies from psoriatic skin, rheumatoid joint and healing wound, CD105 was present in abundant quantities compared with their normal counterparts (11, 12).[4] Krupinski *et al.* (13) showed that whereas little or no CD105 was observed in ECs of normal brain, blood vessels in the penumbra of infarcts in stroke patients stained intensely with mAb to CD105 (13). Recently, a mAb to CD105, conjugated to a toxin, was reported to inhibit growth of human breast tumor xenografts in SCID mice (6). In an unpublished immunoscintigraphy study, we observed that $^{131}$I-labeled mAb to CD105 localized in some patient's intracranial tumors.

Our hypothesis is that the use of CD105 antibody should reduce the incidence of false-positive staining of normal blood vessels entrapped within a tumor and those located within the close vicinity of a cancerous mass. Therefore, it is likely to be a better reagent in the visualization and quantification of IMD in a tumor. In this study, we have compared the IMD in breast cancers using a mAb to the pan-endothelial marker CD34 and a mAb to CD105, which is more specific for ECs of blood vessels in tissues undergoing angiogenesis.

## MATERIALS AND METHODS

**Patients.** One hundred six tissues from primary invasive breast cancers detected at the South Manchester University Hospitals were used for this study. All patients underwent axillary node clearance combined with either mastectomy or wide local excision with radiotherapy of their primary tumor and have been followed up for a median period of 5 years (range, 3–9). Histopathological prognostic parameters of each tumor were assessed by a single experienced pathologist using H&E-stained preparations. Serial sections of snap frozen tissues were used to assess angiogenesis. Axillary lymph nodes were dissected from the axillary clearance specimens, and the total number of nodes found together with the number of involved nodes were recorded. Any patient who had axillary nodal involvement received adjuvant therapy, which was usually tamoxifen (20 mg/day) and/or chemotherapy. Disease-free and overall survival were calculated as the period from surgery until the date of first recurrence or death, respectively.

**Immunohistochemical Localization of CD34 and CD105.** Cryostat sections (5 $\mu$m) were air-dried and fixed in cold acetone for 1 min. Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide in deionized water for 10 min. Nonspecific binding of the antibodies was blocked with 1%

Received 8/10/98; accepted 12/16/98.

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

[1] S. K. is in receipt of a grant from the Christie Hospital endowment funds and C. L. is a Wellcome Trust Fellow.

[2] To whom requests for reprints should be addressed, at Department of Pathological Sciences, Medical School, Manchester University, Manchester M13 9PT, United Kingdom. Phone: 0161-275-5298; Fax: 0161-275-5289.

[3] The abbreviations used are: EC, endothelial cell; IMD, intratumoral microvascular density; ER, oestrogen receptor; PR, progesterone receptor; mAb, monoclonal antibody.

[4] Unpublished data.

Table 1 *Details of IMD (means and medians) using mAbs to CD34 and CD105 and categorical variables*

| | IMD values for | | | | | | |
|---|---|---|---|---|---|---|---|
| | CD34 | | | | CD105 | | |
| Parameter | N | Mean | Median | $P^a$ | Mean | Median | *P* |
| Age (years) | | | | 0.827 | | | 0.913 |
| <50 | 29 | 2.71 | 2.75 | | 3.48 | 3.60 | |
| ≥50–57.4 | 26 | 3.02 | 2.87 | | 3.71 | 3.62 | |
| ≥57.5–62.9 | 28 | 2.69 | 2.71 | | 3.67 | 3.60 | |
| ≥63 | 23 | 2.87 | 2.71 | | 3.38 | 3.75 | |
| Menopause | | | | 0.535 | | | 0.625 |
| Pre | 26 | 2.38 | 2.62 | | 3.40 | 3.55 | |
| Peri | 6 | 3.29 | 3.37 | | 3.52 | 3.40 | |
| Post | 74 | 2.83 | 2.75 | | 3.64 | 3.75 | |
| M stage | | | | 0.615 | | | 0.920 |
| M0 | 101 | 2.81 | 2.75 | | 3.55 | 3.70 | |
| M1 | 5 | 2.93 | 3.00 | | 3.69 | 3.25 | |
| ER | | | | 0.832 | | | 0.303 |
| Negative | 27 | 2.74 | 2.75 | | 3.71 | 3.75 | |
| Positive | 40 | 2.79 | 2.75 | | 3.44 | 3.50 | |
| Progesterone receptor | | | | 0.551 | | | 0.894 |
| Negative | 30 | 2.77 | 2.71 | | 3.57 | 3.75 | |
| Positive | 37 | 2.76 | 2.75 | | 3.53 | 3.60 | |
| Histology | | | | 0.807 | | | 0.589 |
| Ductal | 95 | 2.801 | 2.75 | | 3.53 | 3.60 | |
| Lobular | 11 | 3.14 | 3.00 | | 3.47 | 3.50 | |
| Grade | | | | 0.298 | | | 0.340 |
| Well differentiated | 25 | 2.59 | 2.50 | | 3.47 | 3.60 | |
| Moderate differentiated | 36 | 2.80 | 2.75 | | 3.31 | 3.37 | |
| Poorly differentiated | 44 | 2.97 | 3.00 | | 3.82 | 3.75 | |
| Lymph node | | | | 0.738 | | | 0.787 |
| Not involved | 49 | 2.83 | 2.75 | | 3.67 | 3.70 | |
| Involved | 50 | 2.84 | 2.75 | | 3.53 | 3.67 | |
| Tumor size | | | | 0.015[b] | | | 0.333 |
| <2.5 cm | 29 | 3.00 | 2.875 | | 3.62 | 3.85 | |
| 2.5–3 cm | 6 | 3.15 | 3.125 | | 3.725 | 4.20 | |
| >3 cm | 70 | 2.71 | 2.50 | | 3.52 | 3.55 | |

[a] *P*s estimated by Mann-Whitney test, except tumor size and age, which are obtained by Kruskal-Wallace test.
[b] Statistically significant.

(v/v) normal goat serum in Tris buffered saline (0.01 M, TBS) for 10 min. Serial sections were incubated with mAb E-9 (CD105; 1:1000; see Ref. 8) or CD-34 (1:100; QBEND-10; Serotec Ltd., Oxford, United Kingdom) for 1 h at room temperature. The slides were washed with TBS and incubated with biotinylated secondary antibody (1:100; DAKO, Golstrup, Denmark) in 1% (v/v) goat serum in TBS for 30 min at room temperature, followed by washing with TBS for 2–3 min. The streptavidin biotin complex (1:100 in TBS; ABC complex; DAKO) was applied for 30 min at room temperature and washed with TBS. The slides were treated with 0.02% diaminobenzidine (Sigma) and hydrogen peroxide (0.3%) in deionized water, then counterstained with Mayers hematoxylin, dehydrated, and mounted with DPX (a mixture of disterene, plasticizer and xylene; BDH, Leicester, United Kingdom). A section wherein the primary or secondary antibody had been omitted was used as a negative control in every case.

**Quantification of Microvessels.** IMD as visualized by staining for CD34 and CD105 was quantified by light microscopy without knowledge of patient details. The most vascular areas (*i.e.*, the so-called hot-spots) in a tumor were located at low magnification, and vessels were counted using a Chalkley point eye piece graticule at ×400 magnification (2). Any brown-staining EC or group of cells in contact with a spot in a grid was counted as an individual vessel. The mean of four Chalkley counts for each tumor was calculated and used in statistical analysis.

**Statistical Analysis.** Statistical analysis was performed by using Mann-Whitney and Kruskal-Wallace tests of central location, Spearman's correlation and survival analysis using Cox regression, and Kaplan-Meier graphs with the log rank test. CD34 and CD105 were treated as continuous (ratio) variables, although they were divided into quartiles for the purpose of survival analysis with Kaplan-Meier graphs. Their association with age and tumor size was measured with Spearman's correlation coefficient. Means and medians of CD34 and CD105 were tabulated for categories of categorical variables such as stage. Differences between the medians were tested using the Mann-Whitney or Kruskal-Wallace tests. Kaplan-Meier graphs and log rank tests were used to examine the prognostic value of IMD data for overall and disease-free survival. Multivariate models (Cox proportional hazards) were then used to ascertain their prognostic value relative to standard prognostic factors, such as stage or nodal status. Greenwood's confidence intervals identified how survival differed between the quartiles.

## RESULTS

Of the 106 patients, 89 patients are alive (81 without relapse and 8 with relapsed disease) and 17 patients have died (Tables 1 and 2). Of those who died, all but one died of breast cancer during the follow-up

Table 2 *The relationship between quartiles for the IMD and overall and disease-free survival*

| | Quartiles for CD34 | | | | | Quartiles for CD105 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ≤2.4 | ≤3.0 | ≤3.7 | >3.7 | *P* | ≤2.8 | ≤3.7 | ≤4.4 | >4.4 | $P^a$ |
| Number of patients | 33 | 41 | 15 | 17 | | 22 | 32 | 36 | 16 | |
| Overall survival | | | | | 0.7997 | | | | | 0.0017 |
| Disease-free survival | | | | | 0.5066 | | | | | 0.0431 |
| Alive without relapse | 26 | 31 | 12 | 12 | | 18 | 26 | 28 | 9 | |
| Alive with relapse | 1 | 6 | 0 | 1 | | 4 | 3 | 1 | 0 | |
| Died with relapse | 6 | 4 | 2 | 4 | | 0 | 3 | 7 | 6 | |
| Died of other causes | 0 | 0 | 1 | 0 | | 0 | 0 | 0 | 1 | |

[a] *P* using log rank test. Patients in the first quartile did best both with regard to overall and disease-free survival.

Table 3 *Some clinical details of breast-cancer patients who have died of disease or relapsed*

| S. No | Survival in days | | Outcome | Microvascular density using | | Other parameters | | |
|---|---|---|---|---|---|---|---|---|
| | Overall | Disease-free | | CD105 | CD34 | Nodes[b] | Grade | T stage |
| 1 | 630 | 482 | A[a] | 3.6 | 2.25 | 1 | 3 | 2 |
| 2 | 956 | 593 | D | 4.20 | 3.33 | 1 | 2 | 2 |
| 3 | 482 | 257 | A | 4.00 | 2.50 | 1 | 3 | 3 |
| 4 | 2108 | 431 | A | 5.82 | 2.00 | 0 | 2 | 1 |
| 5 | 533 | 453 | D | 2.90 | 1.66 | 1 | 2 | 3 |
| 6 | 3487 | 1256 | A | 3.00 | 2.25 | 0 | 2 | 2 |
| 7 | 2890 | 590 | D | 4.40 | 3.00 | 0 | 3 | 2 |
| 8 | 734 | 532 | D | 4.30 | 3.00 | 1 | 2 | 2 |
| 9 | 1410 | 627 | D | 3.90 | 3.75 | 1 | 3 | 1 |
| 10 | 658 | 510 | D | 5.50 | 3.00 | 1 | 3 | 2 |
| 11 | 441 | 441 | D | 3.10 | 2.33 | 1 | 2 | 3 |
| 12 | 2519 | 1050 | A | 2.00 | 2.66 | 1 | 2 | 3 |
| 13 | 961 | 961 | D | 5.10 | 3.33 | 1 | 3 | 3 |
| 14 | 2171 | 1098 | D | 5.10 | 2.00 | 1 | 3 | 2 |
| 15 | 1405 | 1180 | D | 5.80 | 1.66 | 1 | 2 | 2 |
| 16 | 557 | 557 | D | 4.60 | 3.66 | 1 | 3 | 1 |
| 17 | 1146 | 1000 | D | 4.30 | 2.33 | 1 | 2 | 2 |
| 18 | 442 | 163 | A | 3.25 | 3.00 | 1 | 3 | 3 |
| 19 | 304 | 272 | D | 3.00 | 3.75 | 1 | 3 | 2 |
| 20 | 357 | 271 | D | 6.90 | 3.75 | 0 | 3 | 1 |
| 21 | 545 | 462 | A | 3.25 | 3.00 | 0 | 3 | 3 |
| 22 | 641 | 579 | A | 3.30 | 5.50 | 0 | 3 | 1 |
| 23 | 377 | 377 | D | 4.00 | 3.75 | 1 | 2 | 3 |
| 24 | 369 | 324 | D | 4.00 | 2.25 | 1 | 2 | 2 |

[a] A, alive with relapse; D, dead.
[b] Lymph-node status: 0, involved; 1, not involved.

period. The median age at diagnosis was 57 years (range, 32–79), and the mean was 56.37 (SE ± 1.61). Some clinical details of patients and their IMD values obtained using mAbs to CD34 and CD105 together with actual elapsed time from diagnosis to relapse or death are presented in Table 3. The staining of ECs was intense, specific and easy to visualize for both CD105 and CD34 (Fig. 1). There was no statistically significant correlation between IMD values obtained using anti-CD105 and anti-CD34 antibodies ($r = 0.103$ and $P = 0.287$; Spearman's correlation test).

The mean and medians of IMD values assessed by CD105 and CD34 staining were tabulated for the following categorical variables: menopause status, age and tumor size, M stage, ER and PR status, histology, and grade and nodal involvement (Table 1). The medians were tested for equality between the categories using the Mann-Whitney and Kruskal-Wallace tests. Only tumor size (*i.e.*, the smaller the tumor, the higher was the IMD value) was statistically significantly correlated with IMD value using the anti-CD34 antibody.

Of the 106 tumors stained with anti-CD105 antibody, 54 had microvessel counts below the median (3.7; range, 0.40–7.00) and 52 microvessel counts were above the median. IMD values obtained using anti-CD34 showed that 74 tumors were below the median (3; range, 1.5–5.75) and 32 tumors were above the median. The IMD values (separated as above or below median) for CD105 significantly correlated with overall ($P = 0.0029$; Fig. 2*A*) and disease-free survival ($P = 0.0362$; Fig. 2*C*). In contrast, IMD values for CD34 showed no correlation with overall ($P = 0.2912$; Fig. 2*E*) or disease-free survival ($P = 0.3153$; Fig. 2*G*).

The IMD values obtained by CD105 staining were subdivided into quartiles: ≤2.8, ≤3.7, ≤4.4, and >4.4 (Fig. 2*B*). The quartiles for CD34 were: ≤ 2.4, ≤ 3.0, ≤ 3.7, and >3.7 (Fig. 2*F*). The log rank test revealed statistically significant differences in overall survival between the quartile values for CD105 ($P = 0.0017$), but the differences were not significant for CD34 ($P = 0.7997$). With regard to disease-free survival, IMD values obtained using CD105 were significant ($P = 0.0431$; Fig. 2*D*), whereas those for CD34 were not statistically significant ($P = 0.5066$, Fig. 2*H*).

Analysis of data for CD105 using Greenwood's confidence inter-

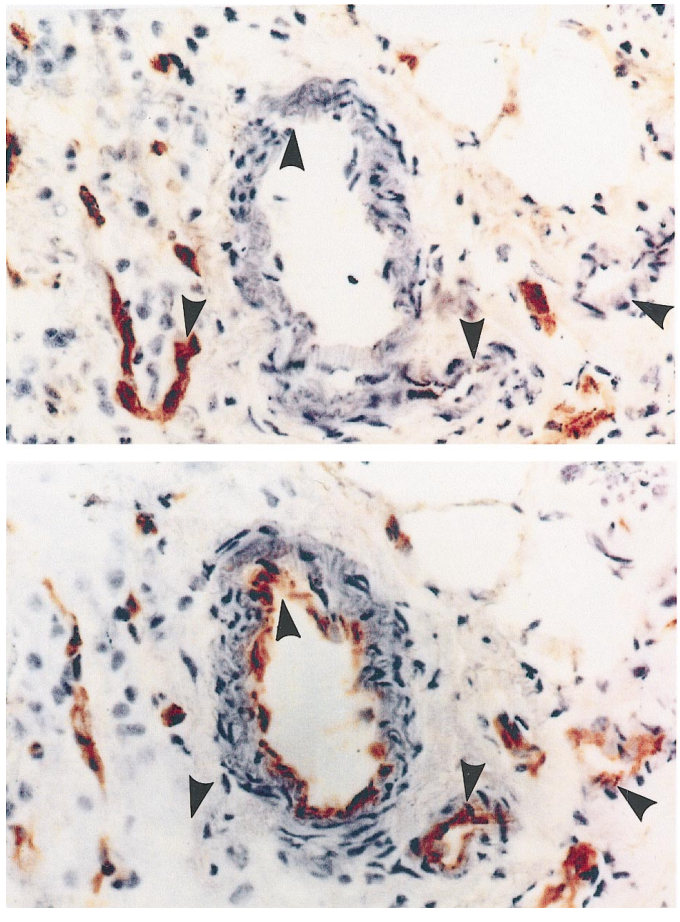

Fig. 1. The indirect immunoperoxidase technique was used to demonstrate reactivities of mAbs against CD105 (*top*) and CD34 (*bottom*) in serial sections of breast carcinomas. Although the intensity of staining was similar in both tissue sections, the heterogeneity of blood vessels visualized by the two antibodies is obvious and has been indicated by *arrowheads*.

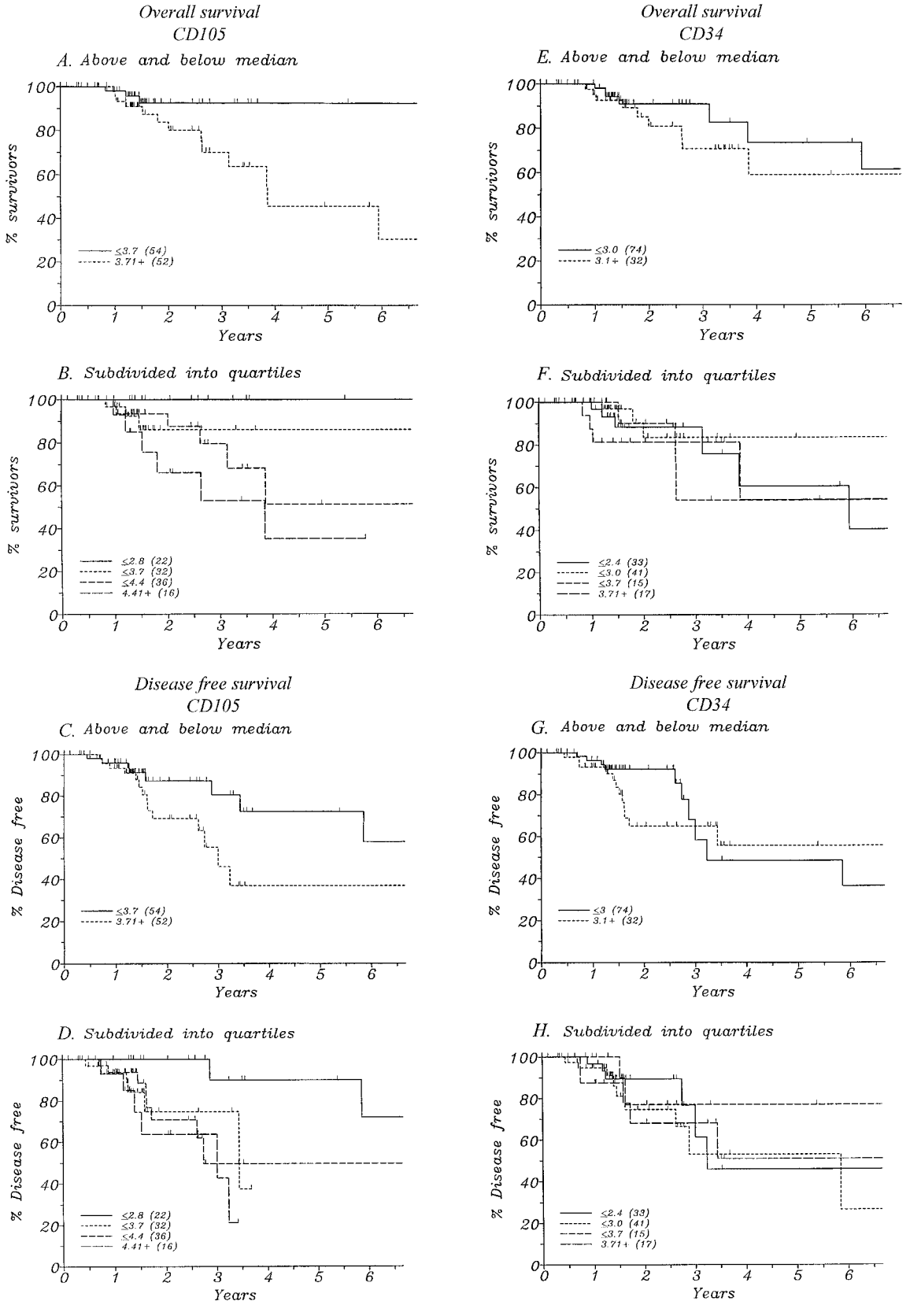


Fig. 2. Life tables showing overall and disease-free survival for 106 patients when the IMD values were obtained using an anti-CD34 or anti-CD105 antibody. The values were subdivided (i) into subgroups above and below the median and (ii) into quartiles. *A*, IMD values obtained using anti-CD105 antibody showed there was a correlation with overall survival when divided according to the median (median, 3.7; $P = 0.0029$) and when divided into quartiles ($P = 0.0017$; *B*). This indicates that patients with a high IMD have a greater risk of death. *C* and *D*, IMD values indicated that there was also significant correlation with disease-free survival when the IMD values for CD105 were divided according to whether they were above and below the median ($P = 0.0362$; *C*) and quartiles ($P = 0.0431$; *D*). This, again, confirms that patients with high IMD values have a greater risk of recurrence of disease and death. *E* and *F*, IMD values using anti-CD34 antibody showed there was no correlation with overall survival when divided according to the median (median, 3.0; $P = 0.2912$; *E*) or into quartiles ($P = 0.7997$; *F*). *G* and *H*, IMD values using anti-CD34 antibody showing there was no correlation with disease-free survival when divided according to above and below median ($P = 0.3153$; *G*) or into quartiles ($P = 0.5066$; *H*).

Table 4 *Cox proportional hazard model for overall and disease-free survival*

Univariate statistics use the score statistics, which are equivalent to the log rank test for categorical variables. Combined statistics (multivariate) use the likelihood ratio statistics. Using the Cox proportional hazard model, CD105 proved to be most dominant factor when compared with other possible prognostic factors. Here in a multivariate analysis significance obtained using CD105 data is independently significant irrespective of whichever variable is added to the model (middle column in both tables).

| | Univariate analysis | Multivariate analysis—significance of: | |
|---|---|---|---|
| | Significance of variable | CD105 | Variable |
| Overall survival | | | |
| CD105 | 0.001 | | |
| Age | 0.629 | <0.0001 | 0.783 |
| Menopausal status | 0.590 | 0.0001 | 0.523 |
| Grade | 0.084 | 0.0001 | 0.215 |
| Nodal involvement | 0.201 | 0.0002 | 0.0093 |
| Tumor size | 0.509 | <0.0001 | 0.431 |
| ER | 0.0279 | 0.014 | 0.010 |
| PR | 0.433 | 0.019 | 0.198 |
| Disease-free survival | | | |
| CD105 | 0.0033 | | |
| Age | 0.135 | 0.0011 | 0.048 |
| Menopausal status | 0.363 | 0.0012 | 0.197 |
| Grade | 0.037 | 0.0007 | 0.060 |
| Nodal involvement | 0.119 | 0.0024 | 0.242 |
| Tumor size | 0.272 | 0.0008 | 0.498 |
| ER | 0.0211 | 0.032 | 0.0066 |
| PR | 0.348 | 0.028 | 0.236 |

vals revealed that, for both overall and disease-free survival, the lowest quartile differed from other quartiles. There were no deaths in the lowest quartile. However further examination of clinical details of the patients in the lowest quartile failed to identify any particular factor that may have led to the observed differences.

From Table 3, it can be seen that median IMD values obtained using mAbs to CD105 and CD34 for patients who have either died of disease ($n = 16$) or relapsed ($n = 8$) were 4.0 and 3.0, whereas the corresponding values for the remaining patients who are well and alive ($n = 81$) were a little lower *viz.*3.5 and 2.75, respectively.

CD105 and a selection of possible prognostic variables were tested for overall survival and disease-free survival using the Cox proportional hazard models (Table 4). For overall survival, only CD105 and ER were highly significant at the 5% level as individual prognostic variables. Only CD105, ER, and grade were significant prognostic variables at the 5% level for disease-free survival (Table 4). Combined statistics (multivariate analysis) showed that CD105 was a statistically significant independent prognostic factor for both overall and disease-free survival ($P < 0.001$). Briefly, in multivariate models the prognostic significance of a variable depends on the prognostic significance of IMD values obtained using mAb to CD105 and *vice versa*. For instance, CD105 is independently significant (*i.e.,* it is significant whatever variable is added to the model; *Column 2* in Table 4). In contrast, nodal involvement and age with regard to overall survival and disease-free survival, respectively, are not independent variables (*i.e.,* their significance depends on CD105). Univariate *P*s are from score statistics, and multivariate *P*s are from likelihood ratio statistics.

## DISCUSSION

The most important finding to emerge from this study is that the mAb to CD105, which is more specifically reactive with ECs of blood vessels in tissues undergoing angiogenesis, proved to be an ideal reagent to quantify microvessels in breast cancers. In contrast, conventional pan-endothelial antibody CD34 was an unreliable marker for visualizing tumor-associated ECs. Furthermore, IMD values obtained using CD34 and CD105 did not show any significant correlation with each other.

Folkman (14, 15) has demonstrated the importance of angiogenesis in the development and metastasis of tumors, but the first quantitative evidence that angiogenesis in human tumors could predict the probability of metastasis was reported for melanoma (16). This study demonstrated a clear demarcation between tumors with little angiogenesis, which did not metastasize, and tumors in which an increase in angiogenesis correlated with a rising rate of metastasis. In the majority of more recent studies, tumor angiogenesis and prognosis have been shown to correlate in a variety of tumors such as of breast, prostate, colon, lung, and brain (2). The implication of these data are that assessment of tumor angiogenesis may prove valuable in the selection of patients for antiangiogenic therapy. However, some reports have failed to find any correlation between tumor prognosis and IMD (reviewed in Refs. 2 and 5) and, curiously, others have found that high IMD was associated with better prognosis (17). The most probable reasons for this discrepancy are the variability in the reactivity of EC antibodies, differences in tissue pretreatment procedures and blood vessel counting methods. The endothelium of tumor and normal tissues is heterogeneous and thus pan-EC antibodies may not stain all tumor blood vessels to the same degree. For instance, antibodies to CD34 and von Willebrand factor, another widely used pan-endothelial marker, generally react well with ECs in large blood vessels, but their expression is diminished or absent from some microvessels in normal and many tumor tissues (8). For these reasons, CD34 antibody may not be an ideal reagent to visualize tumor-associated blood vessels. Furthermore, pan-EC antibodies, including CD34, are not entirely specific for ECs (8, 18). There are also significant differences in the staining of ECs by antibodies from different suppliers, and the quality of staining is grossly affected by different pretreatment methods of tissues (19). In contrast to the pan-endothelial antibody CD34, anti-CD105 specifically reacts with ECs of all angiogenic tissues, including tumors, but only weakly or not at all with those of most normal tissues (6–8, 12, 13). The use of this antibody would have the advantage that normal blood vessels entrapped within a tumor will not be stained by CD105 antibody and, thereby, the probability of false-positive staining will be diminished. Thus, the properties of CD105, as validated by this study, offer a unique opportunity to more accurately assess tumor angiogenesis. This probably explains the observation that CD34 staining correlated with tumor size, but CD105 staining did not. Generally, the smaller the tumor, the higher was the IMD value with CD34. It suggests that compared with large tumors, smaller tumors contain a higher proportion of entrapped normal blood vessels from the host that would be stained by a pan-endothelial marker *viz.*mAb to CD34 but not by CD105, which is more selective in its reactivity. Interestingly, Thompson *et al.* (20), who investigated early tumor vascularization in mouse mammary adenocarcinoma, found that with increasing tumor size, the relative volume of vessels within transplanted tumors initially rapidly increased, then reached a plateau, corresponding to ~1.5% of tumor volume: a 400% increase in vascular density compared with the surrounding host tissue. A key conclusion from their morphometric study was that tumors acquired much of their vasculature by vessel incorporation from the host tissues.

Apart from the differences in immunological reactivity in ECs, as demonstrated by the use of EC antibodies, three others factors need consideration. First, within a tumor, not all blood vessels are functional at all times (21). Whether the two EC antibodies we have used differentially stain functional *versus* nonfunctional blood vessels is not known. Second, in normal organs, IMD in formalin-fixed and paraffin-embedded sections is many fold lower than can be demonstrated by vascular casts (22). The proportion of microvessels that

were not stained by the antibodies to ECs used by ourselves or others is not known. Third, IMD data does not take into account the possible occurrence of lymphatic vessels in a tumor because at present there is no antibody that will specifically stain lymphatic endothelium. However, this is unlikely to be a potential source of discrepancy because tumors do not possess lymph vessels (14).

In summary, IMD, when assessed using a mAb to CD105, is an independent predictor of prognosis in patients with breast cancer, but this association is not found using the pan-endothelial marker CD34. Thus, the ability to quantitatively distinguish between tumor neovascularization and preexisting vessels may be important in the assessment of tumor angiogenesis. We would like to add a cautionary note that the patient population studied by us, although unselected, does not seem typical of breast cancer. For instance, lymph node status, a well established prognostic factor in breast cancer, failed to show up as a significant independent variable. It was only significant ($P < 0.009$) for patient survival when considered in conjunction with IMD obtained using mAb to CD105. Furthermore, a high proportion of patients who were alive with relapse had low IMD values, after a longer follow-up period, and might be expected to do poorly. This would alter the statistical analysis. Therefore, further confirmatory study is warranted on a larger cohort of patients with a longer follow-up period.

## REFERENCES


1. Fidler, I. J., and Ellis, L. M. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell, *79:* 185–188, 1994.
2. Fox, S. B., Gatter, K. C., and Harris, A. Tumor angiogenesis. J. Pathol., *179:* 232–237, 1996.
3. Gasparini, G., Pozza, F., and Harris, A. L. Evaluating the potential usefulness of new prognostic and predictive indicators in node-negative breast cancer patients. J. Natl. Cancer Inst., *9:* 1206–1219, 1996.
4. Weidner, N. Factor VIII related antigen. J. Natl. Cancer Inst., *85:* 674–675, 1993.
5. Page, D., and Jensen, R. Angiogenesis in human breast carcinoma; what is the question? Hum. Pathol., *26:* 1173–1174, 1995.
6. Seon, B. K., Matsuno, F., Haruta, Y., Kondo, M., and Bareas, M. Long-lasting complete inhibition of human solid tumors in SCID mice by targeting endothelial cells of tumor vasculature with antihuman endoglin immunotoxin. Clin. Cancer Res., *3:* 1031–1044, 1997.
7. Derbyshire, E., and Thrope, P. Targeting the tumour endothelium using specific antibodies. *In:* R. Bicknell, C. Lewis, and N. Ferrara (eds.), Tumour Angiogenesis, pp. 343–356. Oxford: Oxford University Press, 1997.
8. Wang, J. M., Kumar, S., Pye, D., Haboubi, N., and Nakib, L. Breast carcinoma; comparative study of tumor vasculature using two endothelial-cell markers. J. Natl. Cancer Inst., *86:* 386–388, 1994.
9. Wang, J. M., Kumar, S., Pye, D. A., van Agthoven, Krupinski, J., and Hunter, R. D. A monoclonal antibody detects heterogeneity in vascular endothelium of tumors and normal tissues. Int. J. Cancer, *54:* 363–370, 1993.
10. Wang, J. M., Kumar, S., van Agthoven, A., Kumar, P., Pye, D., and Hunter, R. D. Irradiation induces up-regulation of E9 protein (CD105) in human vascular endothelial cells. Int. J. Cancer, *62:* 791–796, 1995.
11. Westphal, J. M., Willems, H. W., Schalkwijk, C. J. M., Ruiter, D. J., and DeWaal, R. M. W. Characteristics and possible function of endoglin. A TGF-$\beta$ binding protein. Behring. Inst. Mitt., *92:* 15–22, 1993.
12. Kumar, S., Wang, J. M., and Bernabeu, C. CD 105 and angiogenesis. J. Pathol., *178:* 363–366, 1996.
13. Krupinski, J., Kaluza, J., Kumar, P., Kumar, S., and Wang, J. M. Role of angiogenesis in patients with cerebral ischemic stroke. Stroke, *25:* 1794–1798, 1994.
14. Folkman, J. Angiogenesis and tumor growth. N. Engl. J. Med., *334:* 921, 1996.
15. Folkman, J. Fighting cancer by attacking its blood supply. Sci. Am., *275:* 116–120, 1996.
16. Srivastava, A., Laidler, P., Davies, R., Horgan, K., and Hughes, L. E. The prognostic significance of tumor vascularity in intermediate-thickness (0.76–4.0 mm thick) skin melanoma. A quantitative histological study. Am. J. Pathol., *133:* 419–423, 1988.
17. Lindmark, G., Gerdin, B., Sundberg, C., Paplan, L., Bergstrom, R., and Gimelius, B. Prognostic significance of the microvascular count in colorectal cancer. J. Clin. Oncol., *14:* 461–466, 1996.
18. Martin, L., Green, B., Renshaw, C., Lowe, D., Rudland, P., Leinster, S. J., and Winstanley, J. Examining the technique of angiogenesis assessment in invasive breast cancer. Br. J. Cancer, *76:* 1046–1054, 1997.
19. Abdalla, S., Haboubi, N., and Kumar, S. Quantification of microvessel density in human tumors; the effects of various pre-treatment methods and endothelial antibodies. Int. J. Oncol., *9:* 923–926, 1996.
20. Thompson, W. D., Shiach, K. J., Fraser, R. A., McIntosh, L. C., and Simpson, J. G. Tumors require their vasculature by vessel incorporation not vessel ingrowth. J. Pathol., *151:* 323–332, 1987.
21. Jain, R. Vascular and interstitial physiology of tumours: role in cancer detection and treatment. *In:* R. Bicknell, C. Lewis, and N. Ferrara (eds.), Tumour Angiogenesis, pp.343–356. Oxford: Oxford University Press, 1997.
22. Narayan, K., Withers, R., and Garcia, C. Guinea pig spinal cord as a model for the study of late radiation injury and repair. Int. J. Oncol., *4:* 809–814, 1994.