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Advances in Brief

Association of Macrophage Infiltration with Angiogenesis and Prognosis in Invasive Breast Carcinoma[1]

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Abstract

Angiogenesis is a key process in tumor growth and metastasis and is a major independent prognostic factor in breast cancer. A range of cytokines stimulate the tumor neovasculature, and tumor-associated macrophages have been shown recently to produce several important angiogenic factors.

We have quantified macrophage infiltration using Chalkley count morphometry in a series of invasive breast carcinomas to investigate the relationship between tumor-associated macrophage infiltration and tumor angiogenesis, and prognosis. There was a significant positive correlation between high vascular grade and increased macrophage index ($P = 0.03$), and a strong relationship was observed between increased macrophage counts and reduced relapse-free survival ($P = 0.006$) and reduced overall survival ($P = 0.004$) as an independent prognostic variable. These data indicate a role for macrophages in angiogenesis and prognosis in breast cancer and that this cell type may represent an important target for immunoinhibitory therapy in breast cancer.

Introduction

Angiogenesis is important in tumor growth and metastasis. Recent studies have shown microvessel density in tumors to be a major prognostic factor in various forms of cancer, including invasive carcinoma of the breast. Moreover, high microvessel density gives independent prognostic information for both relapse-free and overall survival (1).

Angiogenesis is a multistep process involving the formation of new capillaries from an existing vascular network with remodeling of the extracellular matrix, endothelial cell migration and proliferation, capillary differentiation, and anastomosis. The neovasculature within tumors is highly proliferative compared to normal vessels, and several factors are known to stimulate angiogenesis, including VEGF,[3] bFGF, EGF/transforming growth factor α, TNF-α, platelet-derived endothelial cell growth factor/thymidine phosphorylase, hepatocyte growth factor/scatter factor, and insulin-like growth factor I (2).

In invasive breast carcinomas, the neoplastic cell population is often outnumbered by such stromal cells as TAMs, which can comprise more than 50% of the total tumor mass (3). It is thought that monocytes in the peripheral circulation are recruited to the tumor site by the release of the chemotactic cytokines, monocyte chemotactic protein-1 (4), CSF-1 (5), granulocyte macrophage CSF (6), and VEGF (7) by such tumors. Once recruited, monocytes differentiate to become TAMs and are modified in the tumor microenvironment to secrete several growth factors, such as EGF (8), TNF-α (9), VEGF, and bFGF (10).

There are drugs that inhibit macrophage function, such as Linomide, which is antiangiogenic *in vivo* but not *in vitro* (11). This effect is mediated by its ability to inhibit both the entry of macrophages into tumors and their secretion of TNFα (12). Furthermore, a recent study has reported that prolonged immunosuppression reduced the incidence of breast cancer in a large cohort of patients, suggesting that breast cancer is in some way promoted by immune mechanisms (13).

Collectively, these findings have led to increased interest in the role of TAMs in the regulation of tumor development and angiogenesis and prompted us to evaluate the relationship between TAM infiltration and tumor angiogenesis in a series of invasive breast carcinomas. We also correlated macrophage infiltration with overall and relapse-free survival for the same set of patients. To study the relationship between angiogenesis and macrophage infiltration, a variation of the Chalkley point-counting method for assessment of vascular density (14) was employed in a similar fashion to assess the macrophage density (MϕI) for each tumor.

Materials and Methods

Immunohistochemistry. A consecutive series of 101 breast carcinomas was assessed for vascular grade and MϕI. The endothelial cell marker CD31 and the commonly used macrophage marker CD68 were stained immunohistochemically using the mouse monoclonal antibodies JC70a (DAKO) and KP1 (DAKO) on 5-μm sections of routinely processed paraffin-embedded biopsies to identify blood vessels and macrophages, respectively. The alkaline phosphatase antialkaline phosphatase method was used to amplify the primary antibody signal, and the stain was developed using a new fuchsin substrate kit (DAKO), yielding an insoluble red reaction product. A hematoxylin nuclear counterstain was also applied.

To evaluate the spatial distribution of macrophage hot spots and their relationships to areas of angiogenesis, a subset of 46 cases was double stained for CD68 and Von Willebrand factor (formerly factor VIII-related antigen, an endothelial cell marker). Double staining of Von Willebrand factor and CD68 was achieved using a peroxidase-labeled EPOS rabbit polyclonal antibody (identifying blood vessels; DAKO) with the mouse monoclonal antibody KP1 (identifying macrophages). The peroxidase-labeled anti-Von Willebrand factor antibody was applied first; this is a single step method, and 3, 3′-diaminobenzidine (DAKO) was used as the chromogen, yielding an insoluble brown reaction product. This was followed by the monoclonal antibody KP1 utilizing the standard alkaline phosphatase antialkaline phosphatase method and new fuchsin substrate as the chromogen. This produced single sections with blood vessels stained brown and macrophages stained red, and a hematoxylin nuclear counterstain was also applied.

ER and EGFR. Estrogen receptor analysis was performed using the method described by the European Organization for Research and Treatment of Cancer. Breast Cancer Co-operative group (15). Tumors with cytoplasmic estrogen receptor levels of more than 5 fmol/mg of protein were considered positive. The EGFR level was determined using a ligand binding method described previously (16). Tumors with an EGFR level of greater than 20 fmol/mg of protein were considered positive.

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[3] The abbreviations used are: VEGF, vascular endothelial cell growth factor; bFGF, basic fibroblast growth factor; TNFα, tumor necrosis factor α; CI, confidence interval; TAM, tumor-associated macrophage; MϕI, macrophage index; CSF, colony-stimulating factor; ER, estrogen receptor; EGF, epidermal growth factor; EGFR, EGF receptor; CVC, Chalkley vessel count.

Vascular and Macrophage Index. Microvessel density was assessed in the three most vascular areas or "hot spots" following a brief scan of the entire section at low power. For each area, and eyepiece-mounted 25-point Chalkley array graticule was used to assess the vascular grade using a ×25 objective lens with a ×10 eyepiece as described by Fox *et al.* (14). The mean of the three Chalkley counts was used for the subsequent statistical analysis. To assess the MϕI, the same Chalkley point-counting method was employed to assess the three areas of densest macrophage infiltration. Similar to the method employed for vascular enumeration, the Chalkley point array was rotated so that the maximum number of points coincided with stained macrophages, and a count was taken. The vascular and macrophage hot spots are chosen rather than random fields, because these areas are thought to be the most biologically important regions.

The double-stained sections were assessed for vascular index and MϕI simultaneously by first scanning the section at low power to find the three most highly vascularized areas. Each area was then assessed for vascularity using the Chalkley point array and then immediately assessed for MϕI using the same method. The section was then scanned again to determine the three densest areas of macrophage infiltration; each area was then assessed for MϕI and then assessed immediately for vascularity.

Statistical Analysis. Spearman rank and pairwise correlation were used to investigate relationships between continuous patient and tumor variables, and a χ^2 test for categorical variables. Survival curves were plotted using the method of Kaplan and Meier, and the log rank test and Cox univariate duration model were used to evaluate differences between life tables. A Cox multivariate proportional hazard model was used to investigate the overall effect of patient and tumor variables on survival. The analysis was performed using Stata release 3.1 (Stata Corp., College Station, Texas).

Results

Correlation of MϕI with Angiogenesis. Data from the series of breast tumors examined was divided by tertiles into three categories corresponding to low, medium, and high vascular grade. We have reported previously that the upper third group by vascular grade has a worsened prognosis when compared to the middle and lower groups (14). When comparing the mean MϕI for the three vascular groups, it was found that the mean MϕI for the high-vascular grade group was significantly higher than that found for the combined lower and middle-vascular grade groups ($P = 0.03$; Fig. 1*a*). This demonstrates a positive correlation between high vascular grade and increased MϕI.

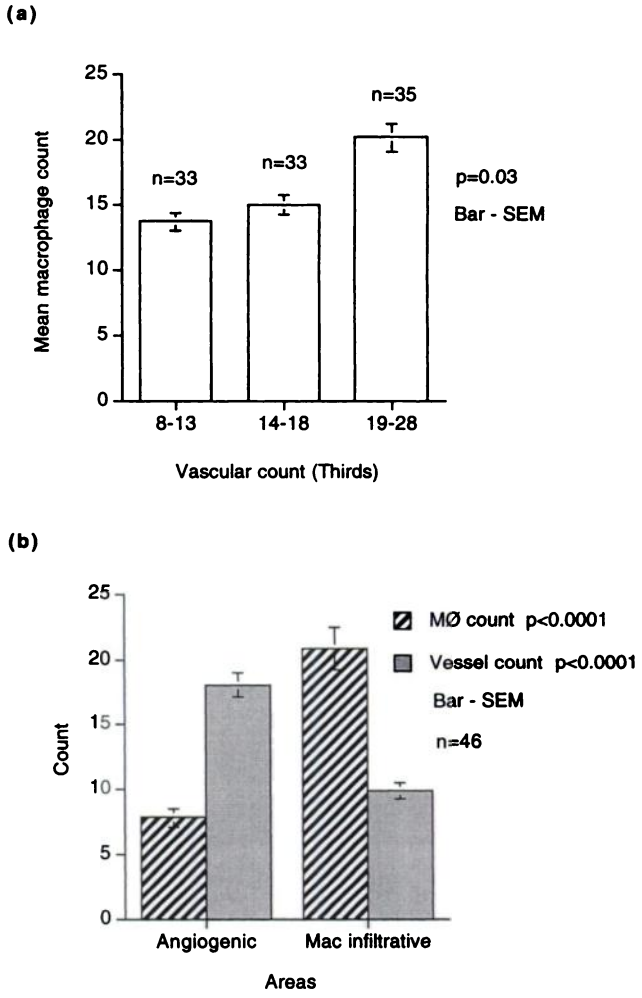


Fig. 1. Macrophage infiltration and angiogenesis. *a*, comparison of macrophage infiltration with degree of vascularization (split into thirds). *b*, distribution of macrophages in relation to areas of angiogenesis.

Spatial Relationships between Areas of Angiogenesis and Macrophage Activity. The three most dense areas of macrophage content were enumerated simultaneously for macrophage and vascular density; this was followed by the three most dense areas of vascular activity being evaluated in the same manner. The overall results are presented in Fig. 1*b*, where it is shown that there is an inverse relationship between macrophage density and vascular density. That is, the highly angiogenic areas were poorly populated with macrophages when compared to the more macrophage-dense fields, which were similarly poorly vascularized. Macrophage density in low-angiogenesis areas was higher than in high angiogenesis areas ($P < 0.0001$). Angiogenesis was higher in low macrophage areas than high-macrophage areas ($p < 0.0001$). This inverse relationship was maintained when highly vascularized and poorly vascularized tumors were examined separately.

Correlation of MϕI with Relapse-free and Overall Survival. In this series of cases, a total of 91 patients were evaluable for survival. Taking MϕI as a continuous variable in a univariate Cox proportional hazard model, increasing MϕI was a significant indicator of worsened prognosis for both relapse-free and overall survival [(for relapse-free survival, $P = 0.01$, hazard ratio = 1.03 (CI, 1.00–1.06), for overall survival, $P = 0.05$, hazard ratio = 1.03 (CI 1.00–1.07)]. Two cut points for MϕI were chosen corresponding to the median (12) and the upper third (18). At the median cut point, 41 cases were in the low (<12) category, with 50 cases falling into the high (≥12) category, whereas, using the upper tertile cut point, 60 cases fell into the low (<18) category, with 31 cases being in the high (≥18) category.

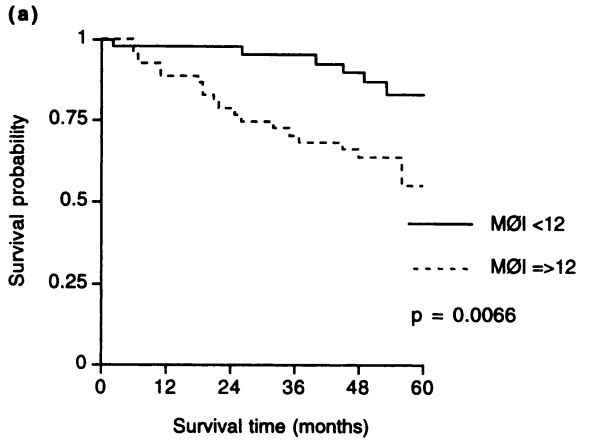
Comparing MϕI to relapse-free survival, at the median cut point a higher relapse rate occurred in the high-MϕI category (hazard ratio, = 3.2, $P = 0.007$; Fig. 2*a*). Likewise, using the upper third cut point, there was an increased relapse rate in the high-MϕI group (hazard ratio = 2.73, $P = 0.008$).

When examining the effect of MϕI on overall survival, a similar effect was observed with higher death rates at above the median (hazard ratio = 11.19, $P = 0.0036$; Fig. 2*b*) and in the upper third MϕI class (hazard ratio = 3.4, $P = 0.0233$).

Examining node-negative tumors separately, using the median cut point for MϕI, it was found that MϕI was a significant predictor of poorer outcome for relapse-free survival in this group ($P = 0.013$).

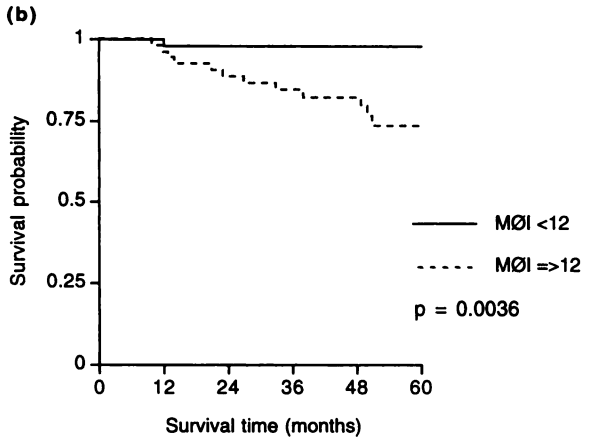
When angiogenesis was examined for its effects on survival, it was found that cases that were highly angiogenic had a worse overall and relapse-free survival rate than cases in the low-angiogenesis group (for overall survival, $P = 0.02$; for relapse-free survival, $P = 0.009$).

Multivariate Survival Analysis. Because it was shown that the cases with high MϕI above the median have a worse prognosis, multivariate analysis was carried out with other factors.



	Patients	Relapses	Predicted
MØI <12	41	6	12.93
MØI =>12	50	20	13.07

Hazard ratio = 3.32, 95% CI = 1.33,8.27, Chi squared = 7.39, p = 0.0066



	Patients	Deaths	Predicted
MØI <12	41	1	6.25
MØI =>12	50	12	6.75

Hazard ratio = 11.19, 95% CI = 1.45,8.62, Chi squared = 8.48, p = 0.0036

Fig. 2. Macrophage infiltration and survival. *a*, relapse-free survival at median MϕI cut point. *b*, overall survival at median MϕI cut point.

MϕI was significant as an independent prognostic indicator for both relapse-free and overall survival ($P = 0.038$ and $P = 0.037$, respectively), with high MϕI indicating worse prognosis (Table 1). When macrophages are taken into account, angiogenesis was not significant, although it was the most significant of the other factors. A separate multivariate analysis excluding MϕI showed CVC to be independently prognostic for relapse-free survival ($P = 0.018$), but in this group, CVC failed marginally to achieve significance for overall survival ($P = 0.081$), although it was the most significant of all the other factors, with the exception of node status.

Interaction of MϕI with Angiogenesis and Effects on Survival. Because both vascular grade and MϕI were found to prognostically significant, and there was a positive correlation between the two factors, we assessed the interactions between MϕI and angiogenesis and their effects on relapse-free and overall survival. The median MϕI cut point was used in these analyses.

Dividing the series of cases into high and low vascular grade, it was found that for relapse-free survival, MϕI was able to split the low-vascular grade group into two, with high-MϕI cases having a worsened prognosis when compared to the low-MϕI cases ($P = 0.023$; Fig. 3). A similar finding was observed when examining overall survival, with MϕI again splitting the low-vascular grade group ($P = 0.0017$). However, in the high-vascular grade group, MϕI was unable to split the series into two more prognostically significant subgroups in either relapse-free ($P = 0.71$) or overall survival ($P = 0.72$).

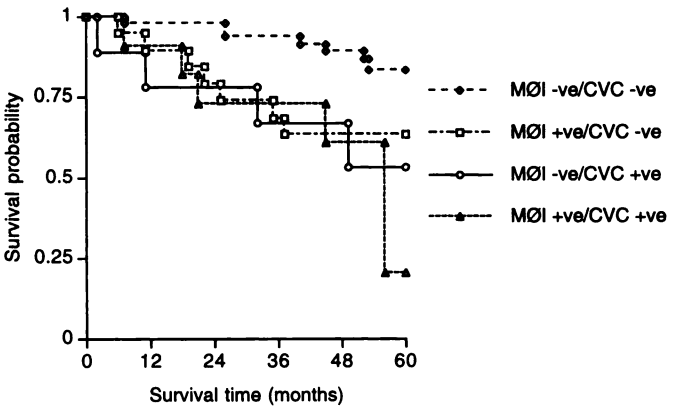
Dividing the series by high and low MϕI, it was found that vascular grade was able to further stratify the low-MϕI group into two prognostically different subgroups for both relapse-free ($P = 0.022$; Fig. 3) and overall survival ($P = 0.0005$), with the high vascular grade category having the worse prognosis. This was not observed when vascular grade was used to stratify the high-MϕI group of cases ($P = 0.43$ for relapse-free survival; $P = 0.84$ for overall survival).

Comparison of MϕI with Other Prognostic Variables. MϕI was compared to other patient and tumor variables, including age, tumor size, nodal status, grade, ER, and EGFR. Split by median, using χ^2 tests at cut point 12 for MϕI, no significant associations were evident.

Discussion

We have shown that there is a significant and positive correlation between angiogenesis and macrophages in breast tumors, with highly vascular tumors having higher numbers of macrophages. This observation could be due either to the presence of more vessels, allowing the infiltration of more macrophages, or the proangiogenic activity of macrophages inducing the proliferation, migration, and differentiation of endothelial cells.

Although the former explanation cannot be excluded, the latter appears to be more likely in light of recent evidence showing that macrophages play an important role in promoting angiogenesis by secreting proangiogenic cytokines and/or enzymes that degrade the extracellular matrix (10). Also in favor of the latter is the observation that the areas of greatest macrophage density or macrophage hot spots



CVC split by MØI		MØI split by CVC	
CVC + / MØI +	p = 0.71	MØI + / CVC +	p = 0.43
CVC + / MØI -		MØI + / CVC -	
CVC - / MØI +	p = 0.023	MØI - / CVC +	p = 0.022
CVC - / MØI -		MØI - / CVC -	

Fig. 3. Effects of interaction between macrophage infiltration and angiogenesis on survival. *−ve*, negative; *+ve*, positive.

Table 1 *Multivariate Cox proportional hazard analysis*

Prognostic indicator	Cut point	Overall survival			Relapse-free survival		
		Hazard	95% CI	*P* value	Hazard	95% CI	*P* value
MϕI	12	9.43	1.15–77.3	0.037	2.79	1.06–7.33	0.038
CVC	7	3.57	0.87–14.5	0.076	2.19	0.89–5.41	0.089
Age	50	1.60	0.29–8.93	0.593	1.18	0.42–3.33	0.758
ER	10	0.72	0.19–2.68	0.623	0.68	0.27–1.70	0.405
EGFR	20	0.66	0.17–2.64	0.558	0.68	0.26–1.80	0.443
Nodes	0	6.09	1.58–23.5	0.009	1.91	0.78–4.64	0.155
Size	2	1.09	0.28–4.30	0.900	2.48	0.82–7.48	0.108

are situated away from the vascular hot spots, implying active migration rather than accumulation in areas around blood vessels. This type of distribution has also been noted by Hildenbrand *et al.*, who also observed a positive relationship between high macrophage grade and increased expression of urokinase-plasminogen activator in breast cancer (17).

The factors that induce macrophage hot spots are not clear but may include necrosis and hypoxia. Angiogenesis occurs in vascular hot spots (14), which are biologically important because they provide a route through which tumor cells can metastasize. Tumor cells in the nonvascular regions experience hypoxic stress and/or undergo cell death. Macrophages may then migrate into these areas, chemotactically following a gradient of substances released by stressed cells such as lactate, low oxygen, and/or VEGF released by the hypoxic tumor cells themselves. Once in these hypoxic areas, they may become angiogenically active, and it has been shown previously that experimental hypoxia can induce the release of bFGF by monocytic cells *in vitro* (18). Upon induction or completion of angiogenesis in that area, macrophages may then move away or undergo apoptosis.

Focal macrophage infiltration also appears to be an important prognostic factor in invasive carcinoma of the breast, being predictive of a worsened outcome and of reduced relapse-free and overall survival when MϕI is high. Indeed, from these data it would seem that MϕI is a more powerful predictor of survival than nodal status, again suggesting that macrophages may have a direct role in disease progression. Other studies have found associations between higher levels of macrophage infiltration and markers of poor prognosis such as Ki-67 (17), and Pupa *et al.* (19) have described a positive correlation between macrophage infiltration and *c-erb*B2 as well as high grade in invasive breast carcinoma. However, in a large series of 1207 cases, they found that the presence of a general lymphoplasmacytic infiltrate in these poor prognosis groups did improve outcome. Also, it was seen in a subset of 72 cases of *c-erb*B2-positive cases that the lymphoplasmacytic infiltrate had a high proportion of macrophages, although the group for which the macrophage proportions were known was not analyzed for survival, and the macrophages were not quantified.

A minority of low-angiogenic tumors in this study were seen to have high macrophage counts and *vice versa*. This shows that macrophages are not necessarily a major angiogenic influence in all highly vascular tumors, and it will be of interest to assess angiogenic factors expressed in these two types of tumors. Also, tumors with high MϕIs and low angiogenesis may not yet have developed increased vascularization, because this type of study represents only a single point in the evolution of a tumor. Tumors that have both low vascular grade and low MϕI may represent an early stage in the induction of angiogenesis and may have been destined to progress to highly vascularized tumors with a greater macrophage density at a later stage.

Both angiogenesis and MϕI were seen to be prognostic factors, and these appear to be related. When the survival data for vascular grade and MϕI are combined, it is observed that high vascular grade can predict a worsened outcome in the low MϕI subclass of cases (but is unable to split the high-MϕI subclass into two prognostically different groups). Also, MϕI can split the low-vascular grade group into two prognostically different groups. This suggests that if either factor is high, the pathway toward metastasis and poorer outcome is favored; if either is low, then the other factor generates a poor prognosis pathway. If both factors are high, prognosis is not significantly worsened than when either is high, possibly because they act via a similar final pathway.

Taken together, our data indicate that TAMs may play a central role in tumor angiogenesis and tumor progression, and that assessment of MϕI could be used clinically to predict outcome in breast cancer patients. It is also apparent that the TAM population of breast carcinomas could be an effective target for future antiangiogenic therapies. Recent findings suggest that this could be achieved using interleukin 10 and Linomide. In mice, transplantable tumors transfected with interleukin 10 show lower levels of macrophage infiltration than their nontransfected counterparts (20), whereas treatment of tumor-bearing rats with Linomide markedly reduced the tumor infiltration and TNFα secretion of macrophages as well as the degree of tumor neovascularization (12). On the basis of the data presented here, breast cancer is a key tumor type for assessing these new therapies.

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