

Changes in Microvessel Density As Assessed by CD34 Antibodies after Primary Chemotherapy in Human Breast Cancer¹

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ABSTRACT

Background: Several papers have shown that quantitation of tumor angiogenesis in primary breast cancer by counting blood vessels gives an independent assessment of prognosis. The impact of chemotherapy ± endocrine therapy on the extent of angiogenesis is unknown.

Methods: Matched pair histological tumor samples were obtained before and after primary chemotherapy from 120 breast cancer patients recruited in the same institution. The first 55 cases received cyclophosphamide, methotrexate, and 5-fluorouracil ± Tamoxifen, whereas the subsequent 65 were submitted to single agent epirubicin. Patients underwent an incisional biopsy at diagnosis and definitive surgery on completion of three or four chemotherapy cycles. Microvessel density (MVD) was performed after staining with the CD34 monoclonal antibody.

Results: MVD slightly decreased after chemotherapy [median 51.26 mm² (range 2.33–163.1) and 44.27 mm² (2.33–121.16; $P < 0.001$); this small reduction neither correlated with tumor response nor with changes in Ki67 expression. MVD at baseline significantly correlated with MVD assessed at definitive surgery (Spearman $r = 0.70$, $P < 0.001$). In multivariate analysis, c-erbB2 status showed an independent role in predicting the reduction in MVD that just failed to attain the statistical significance ($P = 0.08$), whereas baseline parameters,

such as T, N, steroid hormone receptor, bcl-2, p53, c-erbB2, and Ki67 expression, did not enter the model.

Conclusions: Primary chemotherapy is able to modestly reduce the MVD in breast tumors. This small change is not biologically important, because the baseline neoangiogenesis status is not substantially changed. The change in microvessel count after chemotherapy could be potentially influenced by the c-erbB2 status.

INTRODUCTION

The role of angiogenesis in the understanding of tumor biology is a subject of growing interest. It has been demonstrated experimentally that tumor cell proliferation and growth, as well as metastatic spread, is preceded and favored by the formation of new blood vessel (1). Consequently, angiogenic determination could provide complementary information to that obtained from the tumor biological profile and could thus be used for prognostic assessment and as a therapeutic target in human tumors.

Data from experimental and clinical studies indicate that BC is an angiogenesis-dependent tumor (2). It has been suggested that the intensity of angiogenesis may be inversely correlated with the time of survival of patients with invasive BC (3–11), although not all studies have found this association (12–16). These discrepancies may be the result of the various methodological aspects, such as methods for staining and counting vessels and patient selection.

Very little information is available regarding the effects of traditional cancer therapies (endocrine and chemotherapy) on normal and pathological angiogenesis.

In murine models, TAM³ has been shown to inhibit angiogenesis either in ER-positive BCs (MCF-7; Refs. 17 and 18) or in ER-negative tumors (19). The antiangiogenic effect of TAM has been confirmed in humans in a recent study involving a small number of matched pair BC specimens obtained before therapy by core biopsy and afterward at definitive surgery (20). When considering cytotoxic drugs, paclitaxel has been shown to inhibit angiogenesis *in vivo* (21), whereas doxorubicin and mitomycin C were shown to have an enhanced suppression of tumor growth when combined with neoangiogenesis inhibitors (22).

The administration of primary chemotherapy in BC patients permits *in vivo* assessment of treatment-induced changes in tumor biology. The analysis of histological BC samples from patients randomized to a trial of preoperative *versus* adjuvant chemo-endocrine therapy showed a median score of microvessels significantly lower in the preoperative treatment group with

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³ The abbreviations used are: TAM, Tamoxifen; CR, complete response; ER, estrogen receptor; MVD, microvessel density; CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; SD, stable disease; BC, breast cancer.

respect to the adjuvant treated group (23). These data indirectly suggest that primary chemotherapy causes reduction in MVD.

In the present study, we evaluated the variation in the MVD by CD34 immunostaining before and after chemotherapy in a matched series of BC patients with operable disease diagnosed and followed up in the same institution.

The primary aim of this study was to investigate the effect of primary chemotherapy on tumor MVD. The secondary aims were to: (a) correlate changes in MVD with tumor response and reduction in proliferative activity; and (b) search for baseline predictors of MVD variation.

PATIENTS AND METHODS

Patients. The study was carried out on 157 consecutive patients recruited in our institution from August 1990 to January 1997 with an operable breast tumor or locally advanced disease (T2–4N0–1M0). The patients had been enrolled in two consecutive Phase II studies that aimed to evaluate the activity of the CMF regimen administered in association with TAM in cases with ER+ tumors and that of the single agent epirubicin. None of the patients had objective skin inflammation or edema. On first presentation, an incision biopsy was performed on each patient, and a small wedge of tissue (0.5–0.8 cm) was removed. Initial staging comprised clinical examination, bilateral mammography, echography, chest X-ray, liver echography, or computed tomography scan, bone scintigraphy. All patients gave informed consent to the diagnostic procedures and the proposed treatment.

Treatment. Chemotherapy was started within 1 or 2 days from diagnosis. The first consecutive 76 patients received the CMF chemotherapy regimen, which was given on days 1 and 8 every 28 days (24). The dose of cyclophosphamide and 5-fluorouracil was 600 mg/m² body surface area, and the dose of methotrexate was 40 mg/m². The subsequent 81 patients received epirubicin 60 mg/m² on days 1 and 2 every 21 days (25). The first consecutive 45 patients, with ER+ BC at first biopsy, received additional TAM treatment (30 mg/daily) in association with the CMF treatment. TAM was administered after obtaining the results of the receptor status, ~20 days from the first biopsy, and continued up until surgery. Each month, the size of the primary tumor and the size of the axillary lymph nodes, when appreciable, were measured carefully with a caliper by the same clinician. Response was assessed by the clinical measurement of the changes in the product of the two largest diameters recorded in two successive evaluations. According to the WHO criteria (26) tumor progression was defined as an increase of ≥25% in tumor size, SD as an increase of <25% or a reduction of <50%, partial response as a tumor shrinkage >50%, and CR as the complete disappearance of all clinical signs of disease.

Surgery was planned after full clinical reassessment. Quadrantectomy or modified radical mastectomy was performed when indicated in association with full axillary dissection. All patients subjected to quadrantectomy underwent irradiation of the residual breast (60 Gy delivered over 6 weeks).

Histopathologic Grade and Immunohistochemistry. The degree of malignancy was assessed according to the Elston and Ellis grading system, which classifies tumors into grade I (well differentiated), grade II (moderately differentiated), and grade III (poorly differentiated; Ref. 27).

The immunohistochemical methodology used in this study is fully described elsewhere (25). Briefly, an antigen retrieval step was performed by heating a tissue section in a citrate buffer. The primary antibodies applied were: CD34/QB-END [mouse monoclonal (Novocastra Lab, Newcastle upon Tyne, United Kingdom), dilution 1:25, overnight incubation at 4°C], ER [mouse monoclonal 6F11 (Novocastra Lab), dilution 1:50, 1-h incubation at room temperature], PgR [mouse monoclonal 1A6 (Novocastra Lab), dilution 1:20, 1-h incubation at room temperature], Ki67 [mouse monoclonal Mib-1 (DAKO, Glostrup, Denmark), dilution 1:30, 1-h incubation at room temperature], p53 [mouse monoclonal D07 (Novocastra Lab), dilution 1:100, 1-h incubation at room temperature], bcl-2 [mouse monoclonal 124 (DAKO), dilution 1:40, overnight at 4°C], and c-erbB2 [mouse monoclonal CB11 (Novocastra Lab), overnight at 4°C].

Biotinylated horse antimouse IgG and avidin-biotin-peroxidase complex were applied as a staining method (Vectastatin ABC kit; Vector Laboratories, Inc., Burlingame, CA). A solution containing hydrogen peroxide (0.06% volume for volume) and diaminobenzidine 4 HCl (3,3'-diaminobenzidine; 0.05 volume for volume) was used as chromogen.

Immunohistochemical Scoring. All samples had a negative control slide (no primary antibody) of an adjacent section to assess the degree of nonspecific staining. Positive controls included breast carcinomas known to exhibit high levels of each marker.

All staining was scored by counting the number of positive stained cells and expressed as a percentage of the total tumor cells (≥1000) counted across five to ten representative fields of the section using a standard light microscope equipped with a 10 × 10 square graticule. Nuclear staining of tumor cells was evaluated for Ki67 and p53, whereas cytoplasm staining was considered for bcl2, and cell membrane staining was considered for c-erbB2. Reproducibility of counting was assessed by a second investigator rescoring 10 slides.

Vascularity was defined as described previously (5, 7) as the number of vessels per field counted in the area of highest vascular density. Single endothelial cells, endothelial cell clusters, and microvessels in the tumors, clearly separated from adjacent microvessels, were counted. Peritumoral vascularity and vascularity in areas of necrosis were not scored. Branching structures were counted as single vessels. Individual stained microvessels were point counted at ×400 magnification using a square grid graticule. This corresponded to a field size of 0.43 mm². Five fields per tumor section were counted in the areas that appeared to contain the greatest number of microvessels. The average of counts in these fields was considered in the analysis. All figures in the text are quoted per mm².

The relative intensity of ER and PgR staining was assessed in a semiquantitative fashion as described previously by McCarty *et al.* (28), incorporating both the intensity and distribution of specific staining. A value (HSCORE) was derived from the sum of the percentages of positive-stained epithelial cells multiplied by the weighted intensity of staining. Specimens were deemed receptor positive if the HSCORE was >100 (29). For the other biological parameters, a cutoff of ≥5% positive cells was introduced to discriminate p53-positive and p53-negative primary malignancies, as reported previously (30), and a cutoff of ≥10% of stained cells was considered for c-erbB2 positivity. No cutoffs were introduced for bcl2 expression. The

immunohistochemical evaluation at definitive surgery was performed by the same pathologists who remained blinded to the disease response and the score assessed at first biopsy.

Statistical Analysis. Nonparametric statistical methods (Wilcoxon test for paired data, Mann-Whitney *U* test for unpaired data, and Spearman rho for simple correlation analysis) were used, when indicated, in the primary analyses of the data. Multiple group comparison for MVD at baseline was performed by ANOVA. To take into account a possible confounding effect of baseline MVD, ANCOVA was performed instead of ANOVA for multiple group comparison when considering the reduction in CD34 expression and CD34 staining at residual tumors. Multivariate analysis was performed by multiple linear regression. Statistical analysis was performed on an IBM-compatible personal computer using the Statistica for Windows (31) software package.

RESULTS

A group (120) patients of 157 (55 receiving CMF ± TAM and 65 epirubicin) was evaluable for microvessel count assessment before and after treatment and was considered in the present analysis. The characteristics of patients included in the study are shown in Table 1. MVD was not available in 4 patients at the end of treatment because they attained a pathological CR. Among the remaining 33 cases, 21 were discarded because of insufficient tumor samples, and 12 cases were not located. The characteristic of those patients did not differ from those included in the study.

MVD did not show any relationship with tumor volume and lymph node status, as well as with baseline p53, bcl2, c-erbB2, Ki67, and steroid hormone receptor expression (Table 2). After treatment (median three cycles, range two to six), 25 patients attained a clinical CR (20.7%), 58 a clinical partial response (48.3%), and 35 an SD (29.1%), whereas only 2 progressed (1.7%).

As reported in Table 3, primary chemotherapy was able to slightly but significantly reduce the microvessel count either in overall cases or in the subgroup submitted to epirubicin but not in the subset receiving CMF + TAM. The treatment-induced reduction in microvessel staining notwithstanding, a significant correlation was found between microvessel count obtained at baseline and that obtained after treatment (Spearman $r = 0.70$, $P < 0.001$; Fig. 1).

Baseline MVD did not vary stratifying patients as destined to obtain a clinical response (either complete or partial), SD, or progressive disease. Changes in microvessel count neither correlated with the clinical response (Fig. 2) nor with the changes in Ki67 expression (Spearman $r = 0.01$, $P = n.s.$).

A number of baseline prognostic parameters, such as T, N, menopause, histological grading, Ki67, p53, bcl-2, ER, PgR, and c-erbB2 expression, was evaluated in multivariate analysis to assess their role in predicting the microvessel count reduction. None of them showed a significant independent association (Table 4). A trend of inverse relationship was found for c-erbB2 expression and CMF ± TAM treatment.

DISCUSSION

Assessment of changes in neoangiogenesis before and after primary chemotherapy, together with tumor response, may allow for early prediction of relapse and survival of BC patients.

Table 1 Patient characteristics

No.	120
Age	
Median	56
Range	26–71
Menopausal status	
Premenopause	41 (34.2%)
Postmenopause	79 (65.8%)
TNM ^a	
T ₂	87 (72.5%)
T ₃	23 (19.2%)
T ₄	10 (8.3%)
N ₀	63 (52.5%)
N ₁	57 (47.5%)
Grade	
II	43 (35.8%)
III	77 (64.2%)
Receptor status	
ER ⁺ ^b	91 (75.8%)
ER [−]	29 (24.2%)
PgR ⁺ ^b	57 (47.5%)
PgR [−]	63 (52.5%)
Ki67	
≤10%	39 (32.5%)
11–29%	64 (53.3%)
≥30%	17 (14.2%)
c-erbB2	
Positive	26 (23.0%)
Negative	87 (77.0%)
Missing	7
P53	
Positive	39 (32.5%)
Negative	81 (67.5%)
Bcl-2	
Positive	91 (77.1%)
Negative	27 (22.9%)
Missing	2
CD34	
Median	51.26
Range	2.33–163.1

^a TNM, Tumor-Node-Metastasis.

^b ER+ (HSCORE > 100); PgR+ (HSCORE > 100).

To our knowledge, only one study has reported changes in neoangiogenesis with chemotherapy (23). This is the first study involving matched pair specimens. This has been made possible by the systematic employment of incisional biopsy instead of fine needle aspiration in the diagnosis of BC.

The present study shows that cytotoxic treatment, administered at conventional dose schedule, led to a small decrease in the MVD, confirming previous unmatched pair data (23). The modest change in MVD observed in the present study does not appear biologically relevant (although significant) and could be perhaps ascribed simply to intratumoral variation. MVD at baseline significantly correlated with MVD observed at the end of treatment, so that patients with elevated intratumoral vascularization at baseline tended to maintain their microvessel status after chemotherapy. Because tumor spread to distant sites is dependent on access to vasculature, these data suggest that cytotoxic treatment is not able to substantially alter the metastatic potential of the tumor. The schedule adopted (i.v. push injection instead of continuous infusion; Ref. 32) and the relative short time of treatment duration could partially account for the

Table 2 CD34 expression at baseline according to biological variables

Variables	Mean	SE	SD	Median	Range	P
T ₂	60.88	3.79	35.12	53.59	16.31–163.1	
T _{3–4}	53.59	5.31	30.51	48.93	2.33–130.48	0.37 ^a
N ₀	56.34	3.81	29.97	51.26	18.64–163.1	
N ₁	61.60	5.01	37.86	53.59	2.33–163.1	0.07 ^a
G2	57.82	5.63	36.93	51.26	16.3–163.1	
G3	59.45	3.71	32.36	53.59	2.33–163.1	0.32 ^a
ER+	60.09	3.69	35.00	53.53	2.33–163.1	
ER–	55.04	5.69	30.63	46.60	2.33–130.48	0.43 ^a
PgR+	59.33	4.70	35.16	51.26	2.33–163.1	
PgR–	58.43	4.17	33.09	53.59	2.33–163.1	0.64 ^a
Ki67						
≤10%	51.74	5.13	32.06	44.27	2.33–153.78	
11–29%	60.79	4.36	34.86	53.59	2.33–163.1	
≥30%	68.44	8.31	33.26	61.74	2.33–130.48	0.20 ^b
c-erbB2+	62.73	7.59	38.70	48.93	2.33–163.1	
c-erbB2–	55.68	3.40	31.53	51.26	2.33–163.1	0.17 ^a
p53+	56.22	4.95	30.92	51.26	2.33–163.1	
p53–	60.14	3.96	35.43	53.59	2.33–163.1	0.36 ^a
bcl-2+	58.74	3.76	35.66	51.26	2.33–163.1	
bcl-2–	60.15	5.53	28.73	55.92	2.33–130.48	0.21 ^a

^a Mann-Whitney *U* test.^b ANOVA.

Table 3 CD34 expression before and after primary chemotherapy according to the treatment administered

CD34 (No. patients)	Before chemotherapy (Pretreatment biopsy)	After chemotherapy (Definitive surgery)	P ^a
Overall patients (120)			
Median	51.26	44.27	
Range	2.33 ÷ 163.1	2.33 ÷ 121.16	
Mean	58.86	51.32	
SE	3.11	2.27	
SD	33.93	23.99	0.009
CMF + Tamoxifen (55)			
Median	41.94	37.28	
Range	2.33 ÷ 153.78	2.33 ÷ 121.16	
Mean	49.73	44.18	
SE	4.14	3.20	
SD	30.71	22.84	n.s.
Epirubicin (65)			
Median	59.41	48.93	
Range	2.33 ÷ 163.10	2.33 ÷ 121.16	
Mean	66.69	57.29	
SE	4.35	3.00	
SD	34.83	23.45	0.02

^a Wilcoxon test for paired data.

results obtained. The reduction in tumor vascularization did not correlate with tumor response, suggesting that the antiangiogenic activity of chemotherapy, if any, does not contribute to the tumor shrinkage.

Dividing patients according to the treatment administered, reduction in MVD seemed to be confined in the subgroup receiving epirubicin. Caution, however, should be adopted in interpreting these data coming from a nonrandomized comparison.

The absence of relationship between tumor response and reduction in neoangiogenesis is in contrast with a previous study, by Marson *et al.* (20), showing a greater reduction in BC angiogenesis in tumors that have responded to primary TAM. The two studies, however, are not comparable. Different meth-

odologies were used to assess the microvessel staining (Factor VIII *versus* CD34); in addition, TAM was administered for 6 months in the Marson study, whereas the treatment duration in our series was shorter (3–4 months). Finally, it is impossible in our study to ascertain the contribution that TAM may have had in reducing the microvessel count as the drug was never administered alone.

We have demonstrated previously in the same series that the proliferation activity, as assessed by immunohistochemical Ki67 expression, was also affected significantly by primary chemotherapy, whereas steroid hormone receptor, oncogene, and tumor suppressor gene expression did not substantially change (33). In the present study, reduction in Ki67 did not correlate with changes in microvessel count, suggesting that the

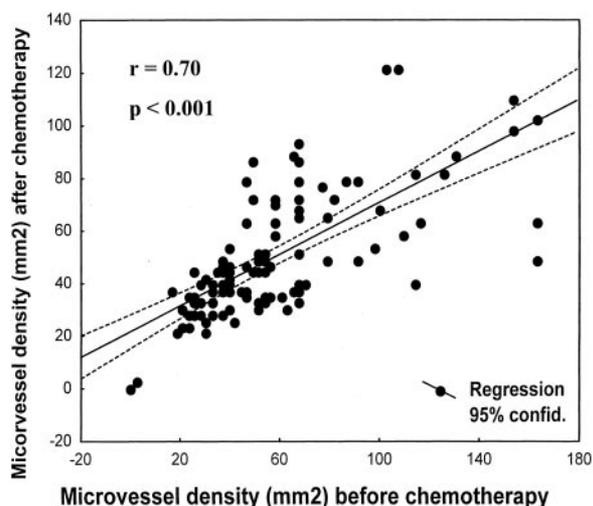


Fig. 1 Correlation between MVD assessed before and after primary chemotherapy.

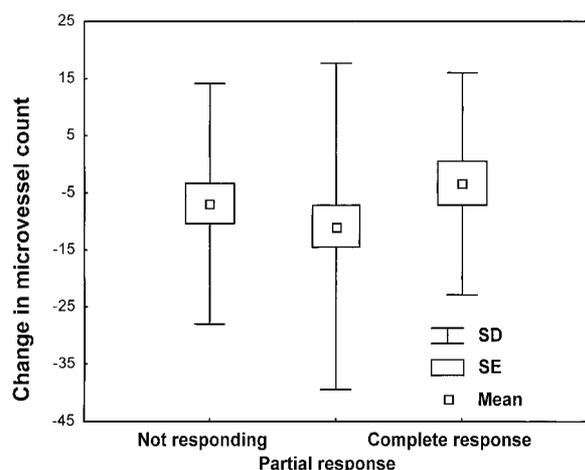


Fig. 2 Distribution of changes in MVD according to the clinical response. Data are: mean; \pm SE; \pm SD.

antiproliferative properties of chemotherapy does not affect the endothelial cells of intratumor neofomed vessels.

Our study also explored the relationship between intratumoral neovascolarization and chemo-responsiveness, and the results showed a lack of correlation between baseline microvessel count and subsequent clinical response (either complete or partial) to the therapy. These data are consistent with those of one study involving a group of patients with locally advanced disease submitted to doxorubicin (34).

A number of studies has correlated the neoangiogenesis status with biological prognostic parameters, such as p53 (35, 36), bcl2 (37), Ki67 (38), and c-erbB2 (4, 39), but contradictory data have been provided. In the present series, the microvessel count did not correlate with clinical and biological parameters, such as T, N, grading, and steroid hormone, p53, bcl2, Ki67, and c-erbB2 expression. Only a relatively small number of cases has been assessed, so that we cannot exclude that some relationships

Table 4 Independent variables associated with microvessel density reduction according to a multivariate regression analysis^a

Variables	B ^b	SE-B	P
Lymph node	-0.60	2.46	0.81
ER	-0.55	2.24	0.81
Tumor size	1.13	2.40	0.64
p53	-1.42	2.39	0.55
Menopause	-1.89	2.21	0.39
PgR	2.43	2.26	0.28
Histology grade	2.40	2.32	0.30
Ki67	-2.36	1.78	0.19
bcl-2	-4.14	2.53	0.10
c-erbB2	-4.39	2.54	0.08
Treatment ^c	-3.68	2.10	0.08

^a Backward stepwise regression analysis.

^b B, estimated regression coefficient; SE-B, SE of B; P, the significant level of the Wald statistics.

^c CMF \pm Tamoxifen vs. epirubicin.

could attain the statistical significance if evaluated in a greater number of cases.

Noteworthy, when these biological and clinical parameters were considered together in a multivariate analysis to assess their independent role in predicting the treatment-associated reduction of the MVD, none attained the statistical significance, but a trend in favor of an inverse relationship was observed with the c-erbB2 status. The stimulatory role of c-erbB2 of angiogenesis in cancer is well acknowledged, although the signaling pathway linking HER2 with angiogenesis remains less well understood (40). C-erbB2 overexpression, that is not modified by chemotherapy (25), could maintain this stimulatory effect during treatment, thus counteracting the general tendency of MVD reduction. Our data, if confirmed in a greater number of cases, could offer an additional mechanism of treatment resistance linked to the c-erbB2 expression.

To conclude, this study shows that conventional chemotherapy in BC patients is able to induce a small reduction that has no biological significance, as it does not substantially affect the baseline neoangiogenesis status. The association of conventional cytotoxic drugs with specific antiangiogenic agents should be studied in the future.

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