Effects of Chemotherapy on Pathologic and Biologic Characteristics of Locally Advanced Breast Cancer

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In 42 patients with locally advanced breast cancer treated with neoadjuvant chemotherapy followed by surgery and radiation therapy, the effects of chemotherapy on tumor architecture, morphometric nuclear and nucleolar characteristics, DNA ploidy, proliferation index measured by mitotic activity index, expression of differentiation antigens, and microvessel density were studied. Pretreatment biopsy specimens were available to compare with mastectomy specimens for 24 patients, and subclavicular biopsy specimens taken before chemotherapy were available for 9 patients. In the remaining patients, fine-needle aspiration was performed before chemotherapy, and morphologic and biologic features of the tumors could be studied only after chemotherapy. In 23 patients, only microscopic tumor or no tumor was left after chemotherapy, and in these patients we observed a characteristic pattern of relatively cellular fibrous tissue with lymphocytic infiltrate, iron-loaded macrophages, and, when present, scattered foci of tumor cells in between. We found a reduction in mitotic activity index and in global microvessel density over all the tumors as a group. There was, however, no consistent pattern of changes in nuclear and nucleolar morphometric characteristics, DNA ploidy, and expression of differentiation antigens, and no pathologic or biologic features were predictive for response to chemotherapy. (Key words: Breast cancer, locally advanced; Neoadjuvant chemotherapy; Tumor biology; Tumor morphology) Am J Clin Pathol 1997;107:211–218.

Neoadjuvant chemotherapy is increasingly being used in the treatment of inoperable breast cancer[1] and as primary treatment in less advanced stages of disease to facilitate breast conservation.[2] Patients with such disease can serve as an in vivo model for study of the effects of chemotherapy on tumor morphologic and biologic characteristics of breast carcinoma. Although a few studies have been performed, some on cell kinetic changes after chemotherapy[3–5] and some on cellular or histologic changes[6–9] or changes in oncogene expression,[9] the effects of chemotherapy on tumor architecture and cell biologic characteristics have not been studied extensively. Serial monitoring may provide insight into biologic features of tumors during chemotherapy, mechanisms of action of chemotherapy, and pathologic and biologic characteristics correlated with response.

We evaluated the effects of neoadjuvant chemotherapy on tumor architecture, morphometric nuclear and nucleolar features, DNA ploidy, cellular proliferative activity, expression of differentiation antigens, and microvessel density in 42 patients with locally advanced breast cancer.

MATERIALS AND METHODS

Between 1990 and 1995, 42 patients with stage IIIA and stage IIIB breast cancer were enrolled in a study in which they received neoadjuvant chemotherapy followed by surgery and radiation therapy.[10] Chemotherapy consisted of moderately high doses of doxorubicin (90 mg/m^2) and cyclophosphamide (1,000 mg/m^2) on day 1, followed by granulocyte-macrophage colony-stimulating factor (GM-CSF; 250 µg/m^2 subcutaneously or intravenously) on days 2 through 11.[11] In the second and fourth cycles, the dosages were reduced in all patients. This treatment protocol was established in an earlier dose-seeking study.[12] Cycles were repeated every 3 weeks. Initially it was the intention to give four to six treatment cycles, but as the study progressed, six cycles were given whenever possible. All patients responded well enough to the chemotherapy to allow subsequent

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mastectomy (Madden operation) with axillary lymph node dissection, followed by radiation therapy (4,005 cGy in 15 fractions to the thoracic wall and axilla).

In 24 patients, the diagnostic procedure was an incisional biopsy. For 9 patients referred from other hospitals, subclavicular biopsied tissue was the only available specimen. In 9 patients, only preoperative fine-needle aspiration for cytologic analysis was performed to confirm the clinical diagnosis; for these patients only mastectomy tissue after chemotherapy was available for pathologic examination and cell biologic assessment. Written informed consent was obtained from all patients, and the protocol was approved by the ethical and scientific review committees of the University Hospital Vrije Universiteit Amsterdam.

Specimen Preparation

Fresh surgical specimens were cut in slices approximately 0.5 cm thick, tumor dimension was measured, and the material was fixed in neutral 4% buffered formaldehyde. Representative tumor samples were embedded in paraffin. If no apparent macroscopic tumor was present after chemotherapy, fibrotic areas in the region where the tumor was located preoperatively were sampled extensively. Slices 4 μm thick were cut and stained with hematoxylin-eosin for histologic analysis, mitotic activity counting, morphometry, and immunohistochemical analysis. For DNA cytometry, cell suspensions were prepared from 50-μm–thick slices of the representative paraffin block of the primary tumor according to standard procedures[13] and stained with 4'-6-diamino-2-phenylindole (DAPI) (Partec; Enkhuizen, The Netherlands), taking 4-μm "sandwich" slices before and after the thick slices to examine them for presence of invasive tumor. All assessments were performed by two investigators (A.H.H. and P.J.V.D.) blinded to clinical outcome.

Histopathologic Analysis

For the prechemotherapy breast biopsy and mastectomy specimens, tumor type, presence of in situ carcinoma, benign breast disorders, and angioinvasion were recorded. The presence of lymphocytic infiltration, stroma between tumor cells, necrosis, and macrophages was scored semiquantitatively: −, absent; +, present; and ++, abundant. After chemotherapy, stromal cellularity was compared with prechemotherapy cellularity to detect active stroma proliferation, and was noted as −, no increase; +,

slightly increased; or ++, evidently increased. In the axillary dissection specimens, we counted the number of lymph nodes containing tumor and registered changes that suggested previous presence of tumor metastasis (eg, nodular fibrotic areas and iron-loaded macrophages). For the prechemotherapy subclavicular biopsy specimens, only tumor type was recorded.

Mitotic Activity Counting and Morphometry

On hematoxylin-eosin–stained slides, the most poorly differentiated and most proliferative areas were selected, avoiding areas with in situ carcinoma, necrosis, and many nonmalignant cells were not examined. A measurement field approximately 0.5×0.5 cm was marked for counting of mitotic cells and nuclear and nucleolar morphometric measurements. Mitotic cells were counted at 400× magnification using a 40× objective (field diameter, 450 μm) in 10 consecutive high-power fields, starting at the spot within the measurement field with the highest density of mitotic cells. The total number of mitotic cells counted in these 10 fields was considered the mitotic activity index (MAI).[14] In case of less than 10 fields of vision, the number of mitotic cells counted was extrapolated to 10 fields to obtain the MAI. This is an established reproducible procedure.[15] Nuclear and nucleolar morphometry were performed using an interactive digitizing video overlay system (QPRODIT; Leica, Cambridge, England) at a final magnification of approximately ×3,000. One-hundred nuclei (or the maximum number of nuclei available) were selected according to a systematic random sampling method,[16] and their contours and nucleoli were traced. For each case, the mean of the nuclear and nucleolar areas was calculated.

DNA Flow Cytometry

DNA flow cytometry was performed with a mercury lamp–based flow cytometer (PAS II; Partec, Münster, Germany) within 3 hours after DAPI staining. The first peak in the DNA histogram was assumed to represent DNA-diploid cells. DNA diploidy was defined by the presence of only one cell cycle in the DNA histogram, and DNA nondiploidy by the presence of more than one cell cycle.

Immunohistochemical Analysis

For relevant blocks, immunohistochemical staining with mouse antibodies (Table 1) was carried out on paraffin-embedded material obtained before and after

TABLE 1. MONOCLONAL ANTIBODY USED FOR IMMUNOHISTOCHEMICAL STAINING

Antibody	Target	Source	Dilution
JC70	CD31	Dako, Glostrup, Denmark	1:40
AE1-3	Cytokeratin	Boehringer Mannheim, Germany	1:100
CAM5.2	Cytokeratin 8	Becton-Dickinson, San Jose, Calif	1:5
EMA	Epithelial membrane antigen	Dako	1:50
NSE	Neuron-specific enolase	Dako	1:50
CEA	Carcinogen embryonic antigen	Netherlands Cancer Institute, Amsterdam	1:10
Vimentin	Mesenchymal cells	Clone V9	1:4,000

chemotherapy. Staining for CD31 (JC70) was performed only on prechemotherapy breast biopsy tissue, not on subclavicular biopsy samples. The avidin-biotin immunoperoxidase method (Vectastain; Vector Laboratories, Burlingame, Calif) was used. Endogenous biotin was blocked with streptavidin (0.1% in phosphate-buffered saline solution [PBS]) and D-biotin (0.01% in PBS) (Sigma, St Louis, Mo). For vimentine and CD31 staining, antigen retrieval by microwave thermocycling, three times for 5 minutes in 0.01 mol/L of buffered citrate at pH 6.0, was performed.[17] Slides were incubated at room temperature for 1 hour or overnight at 4°C (CD31). PBS was used for washing steps. Samples were scored positive for epithelial membrane antigen (EMA), AE1-3, CAM5.2, neuron-specific enolase (NSE), carcinogen embryonic antigen (CEA), and vimentin if most cells expressed these antigens. Estrogen receptor staining was performed on frozen sections according to the manufacturer's protocol (Abbott Diagnostics, Chicago, Ill). The histoscore was applied,[18] and when the score was greater than 100, the estrogen receptor was considered positive.

Microvessel Counting

In representative sections, microvessel density (MVD) was assessed by (1) systematically counting microvessels over the entire tumor area at ×400 magnification using a ×40 objective (global MVD), and (2) subjectively counting microvessels in the tumor area (consisting of four fields) having the highest MVD at low magnification (hot-spot MVD).[19,20] All microvessel counts were converted to square millimeters.

Statistical Analysis

For statistical analysis, grouping was performed using logical classes for the discrete variables. For continuous variables, the cutoff was the median value. To assess correlations, confusion matrices were computed and tested for significance with the X^2 test. Preoperative and postoperative data for the continuous variables were compared using the paired t-test. P values $<.05$ were regarded as significant. All tests were performed with the Biomedical Package (BMDP; Statistical Solutions, Cork, Ireland).

RESULTS

Patient characteristics are given in Table 2. Of the 21 patients with stage IIIB disease, 11 had inflammatory breast cancer.

Table 3 shows the pathologic response to chemotherapy. Six patients responded completely, with no malignant cells left after extensive sampling. In 17 patients, only microscopic foci of tumor cells were present; 3 of these had only a few tumor cells in one lymph node. Six patients had diffuse microscopic carcinoma; that is, no macroscopic tumor was visible, but at histologic examination diffuse infiltration of tumor cells in all segments of the breast was noted. Thirteen patients had macroscopic disease. In patients with only microscopic tumor or without any tumor left, we observed a characteristic pattern of relatively cellular fibrous tissue (Fig 1, A), with reactive lymphocytic infiltration, iron-loaded macropages (see

TABLE 2. PATIENT CHARACTERISTICS BEFORE TREATMENT

Number of patients	42
Age (y)	
Mean	47
Range	26–63
Clinical stage	
IIIA	21
IIIB	21
Inflammatory breast cancer	11
Tumor diameter (cm)	
Mean	9
Range	5–15
Axillary lymph node involvement (clinical)	35

TABLE 3. PATHOLOGIC RESPONSE TO CHEMOTHERAPY

	No. of Patients
No residual tumor	6
Minimal microscopic tumor*	17
Diffuse microscopic tumor	6
Macroscopic tumor	13
Axillary lymph nodes	
Negative	18
1–3 positive	8
4–10 positive	15
>10 positive	1
Top positive	8

*Three patients had only few tumor cells in one lymph node.

Fig 1, B), and, when present, scattered foci of tumor cells in between. The characteristic pattern of changes in lymph nodes with metastases consisted of nodular hyaline fibrotic areas and, when present, scattered foci of tumor cells in between (Fig 2, A), and occasionally with iron-loaded macrophages (see Fig 2, B).
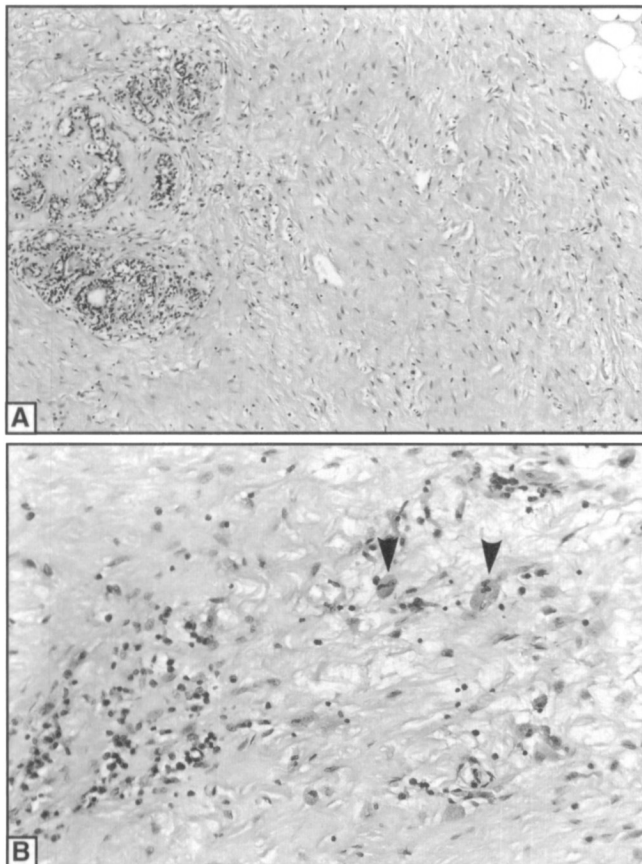
In 33 patients, we were able to assess tumor type both before and after chemotherapy (Table 4). In 25 patients, comparison before and after chemotherapy did not show a change in tumor type. In one patient with a mixed ductal-mucinous carcinoma before chemotherapy, only the mucinous component was left after chemotherapy. The preoperative presence of in situ carcinoma in 4 patients remained unchanged after chemotherapy. In another 4 patients, in situ carcinoma was present in the mastectomy specimen but was not seen in the preoperative biopsied tissue. Benign breast disorders were observed more often after chemotherapy (see Table 4).

Morphometric and DNA cytometric changes are shown in Table 5. DNA index tended to be lower after chemotherapy, but was not significant. Mean nuclear and nucleolar surface area were not significantly different after chemotherapy.

For mitotic activity index, sequential results were available in 25 patients. Proliferation index decreased significantly ($P=.02$) after chemotherapy. This was

FIG 1. Reactive changes in the breast after chemotherapy. A, Cellular fibrous tissue (hematoxylin-eosin [H&E], magnification ×330). B, Lymphocytic infiltrate and iron-loaded macrophages (arrowheads) (H&E, magnification ×330).
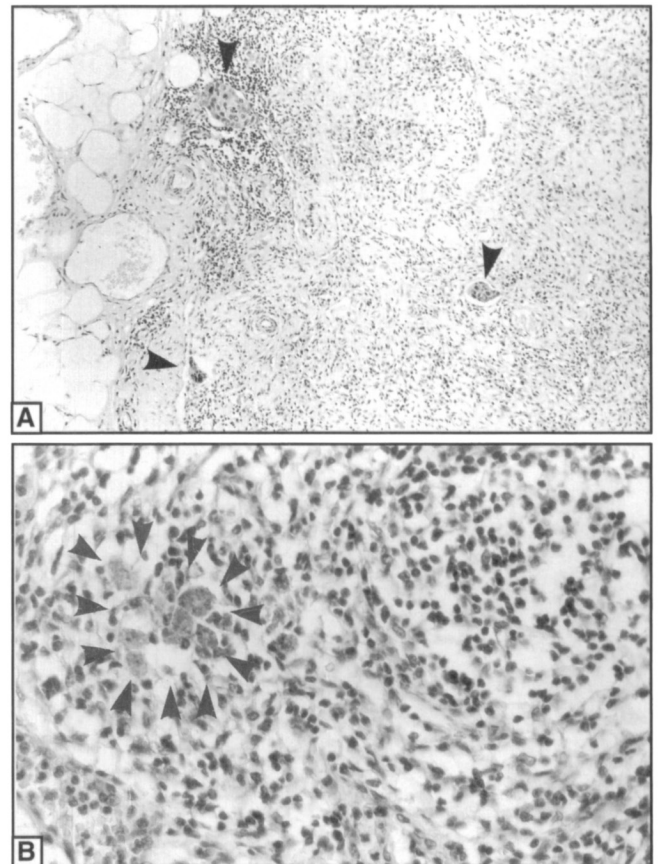

FIG 2. Reactive changes in lymph nodes after chemotherapy. A, Hyaline fibrotic areas with scattered foci of tumor cells (arrowheads) (hematoxylin-eosin [H&E], magnification ×330). B, Iron-loaded macrophages (arrowheads) (H&E, magnification ×330).

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TABLE 4. COMPARISON OF TUMOR TYPE BEFORE AND AFTER CHEMOTHERAPY

Histologic Findings*	Before Chemotherapy	After Chemotherapy
Type		
Ductal	27	27
Lobular	3	4
Medullary	1	0
Papillary-mucinous	1	1
Ductal-mucinous	1	0
Mucinous	0	1
Total	33	33
In situ carcinoma present	4	8
Benign breast disorders present	3	14

*Cases with only preoperative cytologic findings not listed.

TABLE 5. EFFECT OF CHEMOTHERAPY ON MORPHOMETRIC CHARACTERISTICS AND DNA PLOIDY

	Before Chemotherapy (mean)	After Chemotherapy (mean)	P
Nuclear surface area	60.2	61.3	.8
Nucleolar surface area	3.6	4.1	.3
DNA index	1.4	1.2	.06

recorded in patients with only microscopic disease left and in patients with macroscopic disease remaining after chemotherapy. Mitotic activity index increased in only five patients, and was unchanged in two.

Expression of keratin markers (CAM5.2 and AE 1/3) and other cellular differentiation markers (EMA, CEA, NSE, vimentine, and estrogen receptor) did not significantly change after chemotherapy (Table 6).

Chemotherapy did not influence angioinvasion, amount of infiltration, or necrosis consistently, but in most cases the number of macrophages increased (Table 7). In 21 of 24 tumors (88%), cellular stroma was present between tumor cells before chemotherapy, and no increase was noted after chemotherapy.

Microvessel density counted with the hotspot method showed a median of 74/mm² (range, 18–161 mm²) before chemotherapy and 70/mm² (range, 19–168/mm²) after chemotherapy. In the 21 patients in whom samples before and after chemotherapy

TABLE 6. EXPRESSION OF DIFFERENTIATION MARKERS BEFORE AND AFTER CHEMOTHERAPY

Antigen	Before Chemotherapy		After Chemotherapy	
	Positive	Negative	Positive	Negative
Cytokeratin 8	28	2	33	0
Cytokeratin	29	0	33	0
Endothelial membrane antigen	28	1	31	2
Vimentin	5	25	7	26
Carcinogen embryonic antigen	13	17	13	20
Neuron-specific antigen	10	21	10	23
Estrogen receptor	14	25	10	23

were studied, microvessel density decreased in 11 patients and increased in 10. Global microvessel counts, however, showed a different pattern: median density before chemotherapy was 54/mm² (range, 25–101/mm²), and after chemotherapy was 49/mm² (range, 17–93 mm²), which was a significant decrease (*P*=.02). In 14 patients, global microvessel density decreased, and increased slightly (3%–20%) in 5. In the patients with complete pathologic response, we counted microvessels in the area where the tumor had been. Both counting methods revealed a decrease in 2 patients and no change in the others.

Patients with only microscopic disease or without any tumor after chemotherapy responded well to chemotherapy. We tried to identify whether any pretreatment characteristics were predictive of a good pathologic response, but found that none of the studied pathologic and cell biologic characteristics were.

DISCUSSION

We describe our observations on the effects of chemotherapy on pathologic and biologic features in a group of 42 patients with locally advanced breast carcinoma. This chemotherapy regimen induced marked reduction of tumor in 23 patients; in 17 patients only microscopic disease was left, and in 6 patients tumor cells were totally eradicated.

There was no change in tumor type after chemotherapy. However, in one patient with mixed ductal-mucinous carcinoma, only the mucinous component remained after chemotherapy. Perhaps the extensive amount of extracellular mucin makes the tumor cells relatively inaccessible to chemotherapy. Some patients had in situ carcinoma after chemotherapy that was

TABLE 7. EFFECT OF CHEMOTHERAPY ON HISTOLOGIC FEATURES

| | No. of Cases Studied Before and After CT | Present Before Chemotherapy | | After Chemotherapy | | | | | |
| | | | | Increased | | Decreased | | Unchanged | |
		n	%	n	%	n	%	n	%
Angioinvasion	24	7	29	5	21	6	25	13	54
Macrophages	24	1	4	18	75	0	—	6	25
Infiltration	24	11	45	13	54	3	13	8	33
Necrosis	24	1	4	6	25	0	—	18	75

CT = chemotherapy.

not observed in the preoperative biopsy specimen. This is probably due to biopsy sampling error. The same may also apply to the increase in benign breast disorders after chemotherapy, because it is not likely that chemotherapy induces these disorders. Every patient with in situ carcinoma or benign breast abnormalities (eg, adenosis, apocrine metaplasia, and mammary duct ectasia) before chemotherapy also demonstrated these features after chemotherapy. These preinvasive lesions therefore do not seem to be sensitive to chemotherapy.

A few studies have noted the cellular changes of breast carcinomas in biopsy or fine-needle aspiration specimens obtained after chemotherapy.[6–9] Changes described include enlarged nuclei, nuclear vacuolization, and foamy cytoplasm. Some studies observe these changes more often after an almost complete response.[6,7] We also observed enlargement of nuclei and nucleoli after chemotherapy in 15 patients, but in 9 patients nuclear and nucleolar surface area was reduced. Overall, no change consistently reached statistical significance, and no correlation between pathologic response and cellular changes was noted.

Cellular differentiation markers such as keratin markers (CAM5.2 and AE1/3), EMA, vimentin, CEA, NSE, and estrogen receptor did not change after chemotherapy (see Table 7). There is therefore no chemotherapy-induced differentiation or dedifferentiation with regard to these markers.

The MAI decreased significantly after chemotherapy. In a minority of patients, the proliferating compartment increased. All patients with only microscopic disease left showed decreased proliferation. It seems that chemotherapy indeed acts, at least in part, by killing cycling cells. However, in some patients a subclone of cells survives, with increased proliferative capacity. If we assume this reflects the status of non-eradicated metastatic cells, increased proliferation may indicate poor prognosis. Several other studies have

reported on the kinetic changes after chemotherapy.[3–5,9] Two did not show a consistent decrease,[4,9] one showed a decrease only in responding patients,[5] and one showed a decrease only in patients with a high pretreatment proliferation fraction.[3] We found no significant relation with pretreatment proliferation and pathologic response but a nearly significant relation between posttreatment proliferation and pathologic response to chemotherapy ($P=.07$). Three studies showed positive correlation between pretreatment proliferation and response,[4,9,21] and another demonstrated negative correlation between posttreatment proliferation and response.[3] Differences between these study findings can probably be explained in part by the different techniques used to assess proliferation index.

DNA ploidy tended to be lower after chemotherapy, but this did not reach statistical significance, and there was no relation between DNA ploidy and pathologic response to chemotherapy. In previous reports, the relationship between DNA ploidy and response to chemotherapy varied. Two studies reported a better response in aneuploid tumors,[4,7] whereas another reported a better response in diploid tumors.[22]

In addition to cellular changes, the stromal components of these tumors before and after chemotherapy were studied because it is becoming clearer that the stroma is important in tumor biology.[23] In several patients, we observed clusters of residual tumor cells scattered in cellular fibrous areas after chemotherapy. Earlier it had been reported that fibrous capsules can shield tumors from immune mechanisms.[24] We hypothesized that chemotherapy induces a stromal reaction that could form a physical barrier to subsequent chemotherapy and prohibit an ongoing response. However, we did not observe an increase in stromal cellularity, suggesting there is no induction of stroma proliferation, nor did the amount of stroma before chemotherapy correlate with pathologic response. These results are preliminary, however,

because we used only a semiquantitative estimation of the amount of stroma and did not study all the components of stroma in detail (eg, different types of collagen). This aspect needs further study and is currently under investigation. Necrosis was seen only in 25% of patients after chemotherapy. Probably in the other patients necrotic cells had been already cleared by macrophages, which in most patients were increased after chemotherapy.

Another important aspect of the stroma that recently has been studied extensively is tumor neovascularization. There is mounting evidence that angiogenesis is relevant in the biologic aggressiveness of breast cancer. In the multistep process of malignancy, the onset of angiogenesis behaves independent of the other pathways of tumor progression.[25,26] The switch from the avascular to the vascular phase is generally accompanied by primary tumor growth and progression.[27] Several studies using immunohistochemical methods have shown a significant relationship between metastasis or worse survival and high microvessel density counted with the hotspot method.[18,28–32] Until now, angiogenesis has mainly been studied in early stage I or II breast cancer. Hotspot microvessel counting, a method generally used by most investigators, did not show a significant change after chemotherapy. We also counted microvessels in entire tumor sections to investigate whether there was a global change in vessel number, and with this method a significant decrease after chemotherapy was observed. This may indicate that chemotherapy can inhibit proliferation of endothelial cells, leading to less vascularized tumors. This has been described in an in vivo model.[33] Alternatively, GM-CSF may be stimulatory; some studies have shown this cytokine can induce proliferation and migration of endothelial cells.[34,35] In some patients there was, indeed, increased vessel density after chemotherapy. The precise role of chemotherapy and growth factors in endothelial cell growth and growth inhibition needs further investigation. With both counting methods, there was no correlation between microvessel density and pathologic response. This might suggest that degree of vascularization (ie, accessibility of tumor cells to therapy) does not have a major effect on chemotherapy. However, this should be interpreted with caution, because microvessel counts do not give information about functional capacity of blood vessels.

In conclusion, in patients with no tumor or only microscopic tumor left after chemotherapy, the typical morphologic pattern consists of fibrous tissue, lymphocytic infiltrate, and iron-loaded macrophages, and lymph nodes with nodular hyaline fibrotic areas, sometimes with iron-loaded macrophages. Tumor cells when still present are scattered in between. Chemotherapy does not induce a consistent pattern of changes with regard to DNA ploidy, nuclear and nucleolar morphometric features, and cellular differentiation markers. There was, however, a significant decrease in cell proliferation and in global amount of microvessels. Further study of the effects of chemotherapy and growth factors on angiogenesis and stromal reactions, and the relation between these factors and response to chemotherapy is warranted.

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