

Development of Tumor-infiltrating Lymphocytes in Breast Cancer after Neoadjuvant Paclitaxel Chemotherapy¹

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ABSTRACT

Purpose: Neoadjuvant chemotherapy for breast cancer creates new possibilities for the analysis of biological factors in the tumor and/or host, which may play a role in the response to treatment. In this study we analyzed whether changes in local antitumor immunity take place after neoadjuvant paclitaxel therapy and if they correlate with response to treatment.

Experimental Design: Neoadjuvant chemotherapy (paclitaxel, 200 mg/m² q2w, 4 treatments) was followed by definitive surgical management. Histological sections from the pre- and post-treatment surgical specimens of 25 patients were analyzed for the extent of lymphocytic infiltration and presence of tumor infiltrating lymphocytes (TILs). The cumulative apoptotic response in the tumor after the first dose of paclitaxel was also studied in 10 of 25 patients.

Results: Pretreatment lymphocytic infiltrate in the tumor was minimal in the majority of patients and showed no relationship with clinical response. In the patients without TILs before treatment, development of TILs after treatment was noted in 0/3 (0%) patients with stable disease, 3/12 (25%) patients with clinical partial response, and 4/6 (67%) patients with clinical complete response and pathological residual disease. These correlated with the tumor cell apoptotic response to the first dose of paclitaxel.

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Conclusions: These results suggest that development of TILs after treatment correlates with clinical response to neoadjuvant paclitaxel therapy. The possible mechanism(s) whereby neoadjuvant chemotherapy may lead to induction of antitumor T cells is discussed. Immunological processes may influence the response of breast cancer patients to neoadjuvant treatment.

INTRODUCTION

Many patients with breast cancer are currently suitable candidates for neoadjuvant (preoperative, induction) chemotherapy to facilitate the complete surgical excision of the tumor and to initiate early systemic treatment of distant micrometastases (reviewed in Ref. 1). This approach provides an opportunity to assess tumor sensitivity/resistance to the preoperative regimen by measuring the residual disease in the resection specimen obtained at surgery. Evidence is emerging that a good pathological response (*i.e.*, complete or nearly complete destruction of the tumor in the surgical specimen) after neoadjuvant chemotherapy is a strong predictor for survival (2–4). Indeed, breast cancer patients with a complete pathological response have an excellent DFS and overall survival, but they represent only a minority of the patients treated with chemotherapy alone (3–5).

Induction of tumor antigen-specific CTLs⁴ can be efficacious in the prevention and treatment of experimental tumors (reviewed in Ref. 6). However, poor immunogenicity of tumor cells often prevents development of an effective antitumor immune response. Recent data strongly suggest that presentation of tumor-derived antigens by DCs is a necessary step in the induction of an immune response to the tumor (reviewed in Ref. 7). DCs can acquire antigens from apoptotic cells and stimulate antigen-specific MHC class I-restricted CTLs (8). Apoptotic death is a critical requirement for antigen presentation on MHC class I molecules of DCs, because antigens from necrotic cells cannot enter this pathway (8). Importantly, a high ratio of apoptotic cells:DCs can also induce DC maturation, thus enhancing DC ability to efficiently present antigens derived from the apoptotic cells to T cells (9).

The chemotherapeutic agent paclitaxel induces apoptosis in susceptible cells and is effective against breast cancer. In experimental murine breast cancer models, the degree of paclitaxel-induced apoptosis correlates with its antitumor effect (10). Recently, we have shown that the apoptotic response to the first dose of paclitaxel administered as neoadjuvant chemotherapy to breast cancer patients can be measured in the tumor from serial

⁴ The abbreviations used are: CTL, cytolytic T cell; DC, dendritic cell; FNA, fine needle aspiration biopsy; TIL, tumor infiltrating lymphocyte; SD, stable disease; CPR, clinical partial response; DFS, disease-free survival; CCR, clinical complete response; PRD, pathological residual disease; PCR, pathological complete response.

FNAs (11). The apoptotic response appeared to predict the amount of cancer reduction from the treatment (11).

Several studies in breast and other cancers support the notion that tumor infiltration by lymphocytes indicates an anti-tumor cellular immune response (12–16). In addition, the degree of lymphocytic infiltration and, especially, the presence of lymphocytes within tumor cell nests has been shown to correlate with a better prognosis in many tumor types (17–22). Therefore, TILs indicate a cellular antitumor immune response that may contribute to control growth and spread of some tumors.

The study of tumor molecular characteristics that play a role in response to neoadjuvant therapy is an area of active investigation (reviewed in Ref. 1). Histological changes after neoadjuvant chemotherapy of breast cancer have been reported previously (23–26). However, the effects of treatment on tumor/host interactions have not been assessed. To test whether increased apoptosis in the tumor promotes an antitumor immune response, we evaluated the extent of lymphocytic infiltration and presence of TILs in histological sections from pre- and post-treatment surgical specimens from breast cancer patients who received neoadjuvant paclitaxel chemotherapy. Our data suggest that antitumor T-cell responses are triggered in the host when chemotherapy-mediated killing of the cancer cells in the primary tumor is efficient. Elicited tumor-specific CTLs may then contribute to eliminate cancer cells that have survived neoadjuvant treatment.

PATIENTS AND METHODS

A neoadjuvant paclitaxel protocol was offered to women with primary breast cancer if the tumor was T2 or greater and there was no evidence of systemic metastatic disease. Four pretreatment core biopsies (14-gauge) were obtained from different parts of the tumor mass for tissue diagnosis and immunohistochemical assays of selected biomarkers. After obtaining informed consent for treatment from the patients, paclitaxel (200 mg/m²) was administered for 3 h as an i.v. infusion and was given every 2 weeks for a total of four cycles. Surgery was usually performed 2–3 weeks after the last dose of paclitaxel. The post-treatment surgical specimen was thoroughly examined and the tumor bed extensively sampled. Postoperative chemotherapy (doxorubicin and cyclophosphamide) and radiation therapy were administered as adjuvant treatment. Tamoxifen was subsequently added for patients who had tumors positive for estrogen and/or progesterone receptors.

Clinical response to neoadjuvant paclitaxel chemotherapy was determined as reported previously (11, 27). Women who opted for neoadjuvant paclitaxel chemotherapy were also offered participation in a clinical trial using serial FNAs to assess apoptotic response to the first dose of paclitaxel as reported previously (11). Ten of the 25 patients consented to participate. Briefly, the primary tumor was sampled by FNA before the core biopsy (before chemotherapy) to obtain a baseline value and at 24, 48, 72, and 96 h after the first paclitaxel infusion. H&E-stained cytology smears prepared from this material were analyzed microscopically, and the number of apoptotic cells in 1000 cancer cells was recorded as percentage (index). The apoptotic index at each time point was divided by the pretreatment index and expressed as a ratio to correct for variability in baseline

indices from different tumors. The cumulative apoptotic response for the first 96 h of treatment was then calculated as sum of ratios. For more details see Symmans *et al.* (11).

To evaluate the extent of lymphocytic infiltration in the primary tumor and the presence of TILs, H&E-stained histological sections from the pretreatment biopsy and post-treatment surgical specimens from 25 patient breast cancers were analyzed. A grading system for semiquantitative scoring of lymphocytic infiltration in breast cancer has been established by Black *et al.* (17). In this system, grade 0 corresponds to absence of lymphocytes and grades 1–4 to increasing degree of lymphocytic infiltration from a few scattered cells (grade 1) to marked infiltrate that mimics a lymphoid organ (grade 4). Two components of lymphocytic infiltration have been recognized: lymphocytes scattered in the stroma surrounding the tumor are defined as tumor-associated lymphocytes, whereas lymphocytes present within tumor cell nests are defined as TILs (reviewed in Ref. 12). Recent studies suggest that only TILs may correlate with an efficacious antitumor response (21, 22). In Black's scoring system tumor-associated lymphocytes and TILs are not clearly defined. Therefore, a modification of Black's scoring system was used in the present study. First, to improve interobserver reproducibility, intermediate degrees of lymphocytic infiltrate were grouped into a single category: the lymphocytic infiltrate was scored as absent (grade 0), minimal (grade 1), moderate (grades 2 and 3), and brisk (grade 4; Fig. 1). Second, presence of TILs (lymphocytes infiltrating within carcinoma cell nests) was assessed in at least 10 fields at high power magnification ($\times 400$). TILs were always absent when the infiltrate was minimal and present in association with brisk infiltrate. However, in some cases with a moderate infiltrate, lymphocytes were confined to the stroma with no or only focal presence within tumor cell nests. These cases were considered to be negative for TILs. Grading of TILs (*e.g.*, number of lymphocytes relative to tumor cells and/or area) was not possible given the limited number of cases available for evaluation and the low frequency of this finding.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections with antibodies against the pan T-cell marker CD3 (Ventana), the cytolytic T-cell marker CD8 (Dako), and the cytotoxic granule marker TIA-1 (Beckman Coulter) using an avidin-biotin-peroxidase complex method with diaminobenzidine as chromogen according to the manufacturer's instructions. Sections from both the pre- and post-treatment surgical specimens of 10 patients, including 7 of the 8 cases showing TILs after treatment, were stained with anti-CD3 and anti-CD8. In one case the focus of residual tumor was small and disappeared from deeper cuts of the block. Only sections from the post-treatment specimens were stained with anti-TIA-1.

RESULTS

H&E-stained histological sections from the pretreatment biopsy and post-treatment surgical specimens of 25 patients (Table 1) treated between May 1997 and October 1999 were evaluated for the morphological equivalent of a cellular antitumor immune response. At surgery, 5 patients had SD (<50% diameter reduction), 13 had CPR, 6 had CCR/PRD, and 1 had

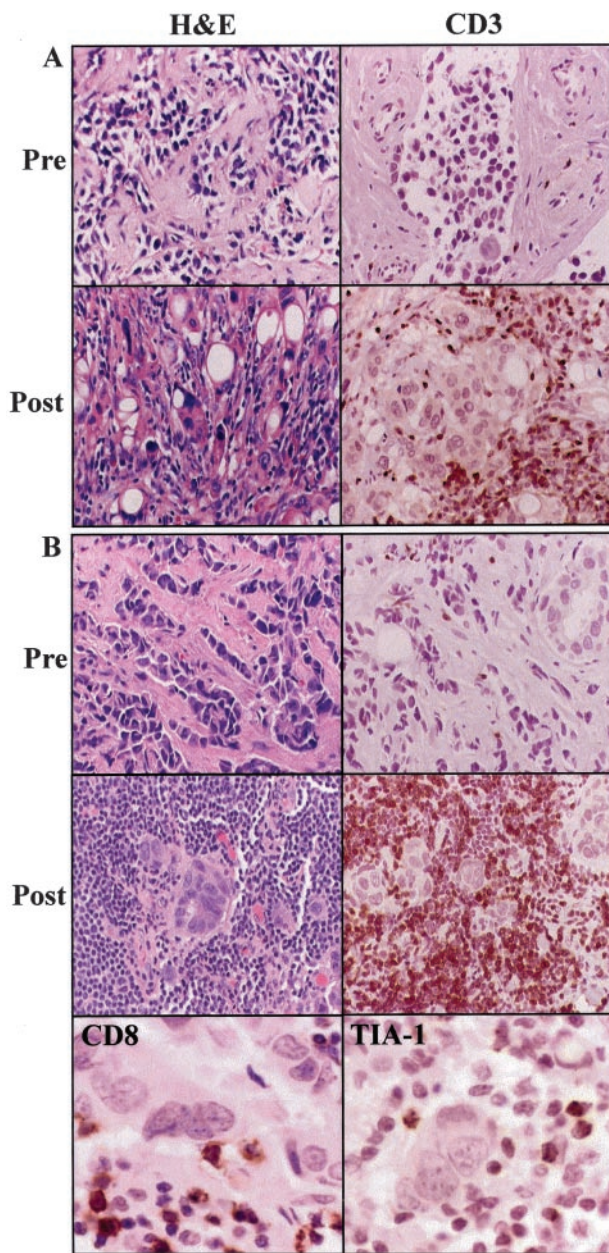


Fig. 1 TILs within the residual breast cancer after neoadjuvant chemotherapy. Histological sections from the pretreatment biopsy (*Pre*) and post-treatment surgical specimens (*Post*) were stained with H&E or immunohistochemically stained for CD3, CD8, or TIA-1. **A**, a patient with CPR showing minimal lymphocytic infiltrate without TILs pretreatment and development of TILs after treatment accompanied by an increase of lymphocytic infiltration to moderate. Rare T cells (CD3+) are scattered in the stroma in the pretreatment specimen, whereas after treatment they are infiltrating tumor cell nests ($\times 400$). **B**, a patient with CCR/PRD showing minimal pretreatment and brisk post-treatment lymphocytic infiltrate with development of TILs. T cells (CD3+) are seen infiltrating and disrupting residual tumor cell nests ($\times 400$). CD8+ and TIA-1+ TILs interacting with the tumor cells are seen after treatment ($\times 1000$).

PCR. Clinical results and correlates with apoptotic response have been reported previously (11, 27). No significant change in morphological appearance of the tumors was noted after treat-

Table 1 Patient characteristics before treatment

No. of patients	25
Age (yr)	
Mean	50.6
Range	30–73
Tumor diameter (cm)	
Mean	7.3
Range	2.5–20
Patients w/axillary lymph node involvement (clin.)	10
Tumor histology	
Ductal, well differentiated	1
Ductal, moderately differentiated	5
Ductal, poorly differentiated	15
Ductal, medullary features (atypical medullary)	3
Lobular	2 ^a

^a One patient had two separate infiltrating carcinomas, one lobular and one ductal type.

Table 2 The degree of lymphocytic infiltrate pretreatment does not correlate with response to treatment

Lymphocytic infiltrate	Response to neoadjuvant paclitaxel chemotherapy			
	SD	CPR	CCR/PRD	PCR
Absent (5)	1	3	1	0
Minimal (15)	3	8	4	0
Moderate (5)	1	2	1	1
Brisk (1)	1	0	0	0

ment. Necrosis was seen pre- and post-treatment in a few very large tumors (7–11 cm) suggesting that its presence was related to tumor size more than treatment.

The pretreatment lymphocytic infiltrate in the tumor was absent in 4 (16%), minimal in 15 (60%), moderate in 5 (20%), and brisk in 1 patient (4%). The extent of lymphocytic infiltration before treatment did not appear to correlate with response (Table 2).

Pretreatment, TILs were present in 4 patients, of which 3 had a subtype of infiltrating ductal carcinoma characterized histologically by a predominant component of anaplastic cells growing in syncytial sheets with a prominent lymphoplasmacellular infiltrate but with infiltrative borders and/or dense fibrous stromal bands (ductal carcinoma with medullary features or atypical medullary carcinoma; Ref. 28). Of these patients, 1 had CPR and 2 had SD. No significant change was noted after treatment in the extent of infiltration or presence of TILs. As reported previously (29), TILs in these atypical medullary carcinomas included cells positive for CD3, CD8, and TIA-1 by immunohistochemistry (not shown). The fourth patient with TILs pretreatment had a poorly differentiated ductal carcinoma and CPR to treatment. The degree of lymphocytic infiltration was increased after treatment from moderate to brisk, and TILs were accordingly more prominent (not shown).

After treatment, TILs were identified in 7 of the 21 patients with residual tumor that had no TILs before treatment (Fig. 1). An increase in the degree of lymphocytic infiltration to moderate or brisk always accompanied the development of TILs, whereas there was no increase in the degree of lymphocytic

Table 3 Development of TILs following neoadjuvant chemotherapy correlates with response to treatment^a

Response	Presence of TILs after treatment (no. of patients)
SD	0/3
CPR	3/12
CCR/PRD ^b	4/6
PCR	NA ^c (1)

^a The four patients with TILs before treatment are not included.

^b The residual tumor in the patients who developed TILs consisted of a single (3 mm) (1 case) or multiple microscopic foci (2 cases). The fourth patient had a 1.2-cm residual tumor. The two patients who did not develop TILs both had a 2-cm residual tumor, and one had metastases in the axillary nodes.

^c NA, not assessed. There was only scar tissue at the site of the tumor.

infiltration without TILs. Development of TILs was not seen in patients with SD but was noted in 25% of patients with CPR and 67% of patients with CCR/PRD (Table 3). Interestingly, when the patients with CCR/PRD were additionally stratified based on the amount of residual cancer determined pathologically, the 2 patients that did not develop TILs had the largest residual tumor and 1 had lymph node metastases. Therefore, there is an apparent relationship between response to neoadjuvant treatment and development of an antitumor cellular response that can be appreciated by histopathology. Immunohistochemical staining showed that the majority of TILs were T cells and that CD8+ CTL were present among cancer cells (Fig. 1B). Moreover, many TILs containing TIA-1-positive cytotoxic granules were seen interacting with residual cancer cells (Fig. 1B). This suggests the possibility that at least some of the cancer cells surviving chemotherapy may be killed by antitumor CTLs.

The assessment of lymphocytic infiltrate and TILs pretreatment was done on needle core biopsies, whereas the whole residual tumor was available for assessment after treatment. Therefore, the possibility that the increase in TILs after treatment could be an artifact of sampling was considered. To estimate the likelihood that sampling would lead to an apparent increase in the degree of infiltration and/or presence of TILs, a group of control tumors was analyzed. Selection criteria were a tumor size of ≥ 2 cm and adequate biopsy material available for evaluation (*e.g.*, cases with only or predominantly *in situ* tumor in the core biopsy or less than two cores containing invasive carcinoma were excluded). Among patients who underwent needle core biopsy of a breast tumor followed by surgery (without preoperative treatment), 11 cases at our institution during the last 2 years met the selection criteria. The average number of cores examined per patient was 4.7 and the average interval between biopsy and surgical resection was 3 weeks (range, 2–5). Patients mean age was 52.5 (range, 37–73), and mean tumor diameter was 4.1 cm (range, 2–5.5 cm). All of the tumors were ductal carcinomas, 6 poorly and 5 moderately differentiated. The frequency of lymphocytic infiltrate as estimated from analysis of the biopsy specimens in this control group of untreated tumors was similar to that observed in the study group before treatment: the lymphocytic infiltrate was absent in 2 (18%), minimal in 5 (45%), moderate in 3 (27%), and brisk in 1 patient (9%). TILs were present only in the 1 case

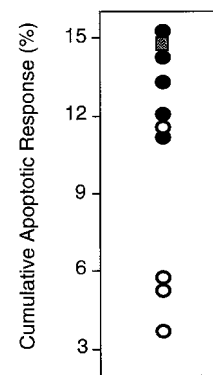


Fig. 2 Relationship between apoptotic response of tumor cells to neoadjuvant chemotherapy and development of TILs. The patients studied generally showed an apoptotic response over the first 4 days after the first dose of paclitaxel, which was usually complete by day 4. The apoptotic index at each time point was divided by the pretreatment baseline index (0 h) and expressed as a ratio. The cumulative apoptotic response was calculated as sum of ratios (for more details see Ref. 11). ○, cases showing no change in the lymphocytic infiltrate after treatment; ●, cases showing development or increase in the extent of TILs after treatment; and ◻, 1 case with no residual tumor (only scar tissue) after treatment. The cumulative apoptotic response after the first dose of paclitaxel shows a correlation with development of TILs.

with brisk infiltrate. No significant change was noted between the biopsy and resection specimens. These results suggest that, when sampling is adequate, the biopsy reflects accurately the composition of a relatively large tumor and support the conclusion that changes observed in patients after neoadjuvant paclitaxel chemotherapy truly reflect a change in tumor-host interactions.

Data from 10 patients were available on the apoptotic response in the tumor 96 h after the first dose of paclitaxel. As reported previously by Symmans *et al.* (11) the apoptotic response to paclitaxel strongly correlated with tumor reduction. There was a tendency for those patients who had a significantly strong apoptotic response after chemotherapy to develop TILs in the residual tumor (Fig. 2). In contrast, no increase in TILs was seen in any of the patients with poor or no response to chemotherapy. Because the apoptotic response was measured shortly after administration of the first dose of paclitaxel, it is unlikely that killing of tumor cells by TILs could have influenced the amount of observed apoptosis. Therefore, these data suggest that the extent of cell death induced in a tumor by paclitaxel chemotherapy may contribute to the development of an antitumor immune response.

DISCUSSION

In this study we analyzed pre- and post-treatment surgical specimens of 25 breast cancer patients treated with neoadjuvant paclitaxel chemotherapy for evidence of a local cellular antitumor immune response. Although the small number of patients does not permit statistical analysis or definitive conclusions, our findings suggest that the development of TILs after treatment may be related to the clinical response and to the apoptotic response to neoadjuvant chemotherapy.

In the four cases with presence of TILs before treatment,

the response to neoadjuvant chemotherapy was poor: two patients had SD and two CPR. This could suggest that patients with pretreatment TILs may be less likely to respond to paclitaxel. However, three of the four cases were ductal carcinomas with medullary features, a subtype always associated with the presence of TILs but with other distinct cytological and histological characteristics. Therefore, it is possible that this tumor type has an “intrinsic” resistance to the action of paclitaxel, which is unrelated to the presence of TILs. Indeed, the observed correlation between tumor reduction and apoptotic response of tumor cells to the first dose of paclitaxel (11) suggests that the intrinsic sensitivity of cancer cells to paclitaxel is a primary determinant of response. If the tumor is sensitive to the chemotherapy, the accumulation of apoptotic cancer cells may trigger immune mechanisms that contribute to the elimination of surviving cancer cells. Unfortunately, chemotherapy alone leads to accumulation of apoptotic cancer cells in only a fraction of treated patients (11). Treatments capable of inducing sufficient accumulation of apoptotic tumor cells in a larger proportion of patients may be also more efficient at triggering antitumor immune responses. One promising approach is the use of concomitant local radiation and chemotherapy as neoadjuvant treatment for locally advanced breast cancer (30–32).

Recent reports suggest that the accumulation of apoptotic tumor cells above a certain threshold can trigger DC uptake and presentation of tumor antigens and ultimately result in the induction of antitumor T lymphocytes (9, 33). Thus, induction of apoptosis of tumor cells after paclitaxel treatment may also enhance tumor immunogenicity by providing tumor antigens for presentation by DC to T lymphocytes. In addition, paclitaxel may be distinctive among chemotherapeutic agents in its ability to stimulate production by monocytes and cancer cells of cytokines such as interleukin 1 β , a potent inducer of DC maturation (34, 35). The presence of cytolytic effector cells among residual tumor cells after treatment (Fig. 1B) does support a hypothesis that activation of a cytolytic T-cell response contributes to additional elimination of cancer cells. It is intriguing to consider whether a longer interval between completion of neoadjuvant treatment and subsequent surgery would convert some patients with pathological minimal residual disease and TILs into a PCR.

A limitation of the present study is that specificity and functional capacity of TILs could not be tested with the available material. Development of preclinical models is required to unequivocally test the hypothesis that induction of apoptosis of cancer cells by neoadjuvant therapy can enhance tumor immunogenicity and induce effective antitumor immune responses. We are currently working to develop such models. In addition, long-term follow-up of the patients should provide information about whether the development of TILs after chemotherapy is associated with an improved DFS. Such correlation would add support to the hypothesis that the lymphocytic infiltrate represents a correlate of a protective antitumor immune response (21, 22).

Recent data in an animal model strongly suggest that efficient killing of cancer cells by local radiation therapy of the primary tumor can provide tumor antigens for presentation by DC and trigger an immune response capable of controlling distant metastases when combined with immunomodulators to enhance DC function (36). The data presented here suggest that

chemotherapy-mediated killing of breast cancer cells, when efficient, may also activate antitumor T cells in some patients. If proven in larger studies, that would support the selection of neoadjuvant protocols with strong local antitumor effect (*e.g.*, chemotherapy combined with local radiation; Refs. 30–32) as initial treatment of breast cancer. In addition, because DC function is depressed in many breast cancer patients (37), concomitant use of cytokines enhancing DC function may be required to induce an effective and long-lasting antitumor immunity that would improve the survival of these patients. Interestingly, a recently reported clinical study indicates an encouraging improvement in DFS and overall survival in patients with locally advanced breast cancer treated with neoadjuvant chemotherapy combined with granulocyte-macrophage colony stimulating factor, which can enhance DC function, (38, 39). We are beginning new clinical protocols designed to augment the local pathological response to chemotherapy by including radiation therapy in the neoadjuvant treatment. An important part of this work will be studying the molecular and immunological events that accompany the response to treatment.

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