Immune Changes in Patients with Locally Advanced Breast Cancer Receiving Neoadjuvant Chemotherapy

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ABSTRACT
Object: The effect of neoadjuvant chemotherapy on cellular immunity was investigated in patients with locally advanced breast cancer by evaluating the changes in peripheral blood lymphocyte (PBL) subpopulations and natural killer cell activity (NKA) after chemotherapy.

Materials and Methods: Blood samples were obtained from 13 advanced breast cancer patients before and after 3 or 4 cycles of the combination chemotherapy 5-fluorouracil, epirubicin, and cyclophosphamide. PBLs including CD3+, CD4+, CD8+, CD19+, CD25+, CD45RA+, CD45RO+, CD56+ and γδ-T cells were evaluated by flow-cytometric analyses by using specific monoclonal antibodies. NKA was assessed by anti-candidal indexes.

Results: After chemotherapy, the CD19+ B and CD45RA+ naive T-lymphocyte percentages and the CD4/CD8 ratio were decreased (p<0.001, p=0.06, p=0.04, respectively), while the CD8+ cytotoxic-supressor T cells were increased (p<0.001). In subgroup analysis, the anti-candidal indexes were found to be decreased (p=0.04), while the CD56+ (p=0.005), and CD45RO+ (p=0.05) memory T-cell percentages were increased in the responsive-group (n=7) after chemotherapy. However, the anti-candidal indexes (p=0.01) were observed to be increased, and the CD25+ activated T-cell percentages (p=0.004) were decreased in the chemotherapy non-responsive group (n=6) unlike the other groups. No statistically significant changes were observed in CD3+, CD4+, and γδ-T cell percentages after chemotherapy in the whole group and subgroup analyses.

Conclusion: Our results suggest that the anthracycline-based chemotherapy regimen induced increases in CD45RO+ memory T-cell percentages may enhance the efficacy of chemoimmunotherapy trials in chemotherapy-responsive patients. Detailed phenotypical and functional characterization of intratumoral lymphocytes warrants further investigation in terms of contrary findings on PBL percentages and NK cell function after chemotherapy.

Note: Part of this study was presented in ASCO 1998.

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Key Word: Cellular immunity, lymphocytes, peripheral blood, natural killer cells, lymphocyte subpopulations, natural killer cell activity, locally advanced breast cancer, neoadjuvant chemotherapy

INTRODUCTION

There are many host factors that influence the growth and the metastatic potential of tumors. Among these factors, the immune system may play an important role in the control of cancer spread. It has been suggested that natural killer (NK) cells may provide a first line defense against newly developed malignancies as immunological effector cells1-4. The activity of NK cells is mediated primarily by large granular lymphocytes, which are capable of lysing tumor cells without prior sensitization1.
The other pathway of the immune system to eradicate the cancer cells is by recognizing cancer cells by activating lymphocytes and driving the cancer cell to becoming susceptible to kill. Numerous studies have demonstrated that both CD8 and CD4 subsets of lymphocytes must be activated to elucidate a tumoricidal effect. Impaired cell-mediated immunity is a poor prognostic factor in addition to the presence of uncontrolled cancer cell growth and metastases. Furthermore, some studies showed elevated levels of tumor antigen-specific autoantibodies in cancer patients. Other reports demonstrated strong infiltration of primary tumors and draining lymph nodes by tumor-specific cytotoxic T lymphocytes. Mccoy et al. also demonstrated that cell-mediated immunity to tumor-associated antigens is a stronger prognostic factor than stage, grade, and lymph node status.

The most widely used anticancer agents for breast cancer are anthracyclines including doxorubicin and epirubicin that were shown to improve survival in patients in adjuvant setting. Both of these drugs exert antitumor effects through interference with DNA synthesis and function, especially during the S and G2 phases of the cell cycle. Furthermore, they also cause cytocidal activity by intercalation between nucleotide base pairs resulting in inhibition of nucleic acid (DNA and RNA) and in DNA cleavage by topoisomerase II. In general, administration of cytotoxic chemotherapy is characterized by bone marrow suppression that results in reduced numbers of peripheral blood immune effector cells including neutropenia. These effects are mostly reversible, and do not lead to permanent alterations in immune function.

Due to the conflicting results in the literature, the present study investigated the effect of the neoadjuvant combination chemotherapy regimen including epirubicin on cellular immunity in patients with locally advanced breast cancer by evaluating the differential changes in peripheral blood lymphocyte (PBL) subpopulations and natural killer cell activity (NKA) after chemotherapy.

**MATERIALS AND METHODS**

**Patients and Study design**

Patients were evaluated by physical exam and radiologic imaging including mammography and breast ultrasound. A neoadjuvant (preoperative) chemotherapy was planned for those patients who were considered to have locally advanced breast cancer according to the TNM staging (T2N1-N3, or T3 or T4, and any N). Fine needle aspiration or trucut needle biopsy or incisional biopsy were performed for histopathological diagnosis. Further staging work-up included a chest X-ray, a bone scan and abdominal ultrasound or thorax & abdominal CT for detection any systemic metastases.

All patients received 3 or 4 cycles of the combination chemotherapy, 5-fluorouracil (500 mg/m²), epirubicin (80 mg/m²), and cyclophosphamide (500 mg/m²) (FEC) every 3 weeks as primary chemotherapy before surgery. The response to neoadjuvant chemotherapy was evaluated every 2 cycles by physical examination, and by ultrasonography or by mammography along with physical exam once the neoadjuvant chemotherapy was completed. Clinical responses were based on the primary tumor and axillary lymph node response and were categorized as follows: complete response (CR) = total resolution of the breast mass and axillary adenopathy on physical and radiographic examination; partial response (PR) = 50% or greater diminution of bidimensional tumor or axillary lymph nodes; stable disease (SD) = no more than 25% increase or decrease in tumor size or no change in lymph node; progressive disease (PD) = more than 25% increase in tumor. Blood samples in heparinized tubes (5-10 cc) were obtained from 13 consecutive locally advanced breast cancer patients before and after 3 or 4 cycles of FEC.

A modified radical mastectomy was performed after the neoadjuvant chemotherapy. The histopathologic type of tumor, surgical margin involvement and axillary involvement was detected in the specimen. Hormone receptors including estrogen and progesterone receptors were studied in the biopsy specimen which was performed before the neoadjuvant chemotherapy. The pathologic response was also evaluated in the mastectomy specimen and absence of invasive cancer was considered a pathologic complete response.

Patients completed their chemotherapy regimen following surgery by additional 3 or 4 cycles of FEC, or by Taxotere alone (75 mg/m²) if the chemotherapy response was considered as no response or progression. After completion of chemotherapy, patients had...
radiation treatment including 50 Gy to the thorax and to the lymphatic basins (axilla, supraclavicular nodes and internal mammarial chain). Radiotherapy was administered as 20 fractions over a 4 week periods. Tamoxifen (20 mg/day) was given to estrogen receptor positive patients for five years. Patients were followed up by the surgeon and oncologists periodically after completion of their radiotherapy.

**Analyses of Peripheral Blood Lymphocyte Subsets**

Blood samples for analysis of the PBL subsets and NKA were drawn before and 2 weeks after the completion 3 cycles neoadjuvant chemotherapy. Samples from each patient were processed immediately for the phenotypic analysis and NKA. Phenotypic analyses were performed using a FACScan flow cytometer (Becton Dickinson, USA) equipped with a 15-mW air-cooled argon-ion laser. The percentages of peripheral blood lymphocyte subsets including CD3+, CD4+, CD8+, CD19+, CD25+, CD45RA+, CD45RO+, CD56+, γδ-T cells were measured by using specific monoclonal antibodies. FACScan analyses were performed by 3 investigators (S.A., N.G. and B.K.) who were blinded to clinical data and NKA results.

**NK Cytotoxic Cell Activity**

NK cytotoxicity was assessed as the anticandidal colorimetric index, which was found to be a simple method to assess NKA. Ten-milliliter blood samples were taken in standard tubes with heparin and then centrifuged by Ficoll Isopaque. Lymphocyte layers were diluted with RPMI-1640, and living cells stained with trypan blue were counted.

By using lymphocytes as effector cells and Candida (Candida stellatoidea) as target cells, cells were mixed in the ratios of 1/5 or 1/25 target:effector cells. As the control, 0.025 ml Candida solution in phosphate-buffered saline, neutral pH, was inoculated into blood agar. After 48 h, colonies were counted in triplicate and the anti-candidal index was calculated: anti-candidal index (%) = 1 - experimental colony unit/control colony unit x 100.

**Statistical Analyses**

Statistical tests were performed by using SPSS 11.00 for Windows statistical software (SPSS Inc, Chicago, IL). Comparison between the means of the two groups were made by using the paired Student’s t test. All p values were two-sided in tests and p values <0.05 were considered significant.

**RESULTS**

The median age was 43 (range, 24–75). According to the TNM staging classification, 12 patients were clinically T4 N0-N2 as stage IIIB and one patient was T2N2 as stage IIIA without any systemic metastases before neoadjuvant chemotherapy. Partial response was obtained in 7 patients whereas no significant response to chemotherapy was observed in 6 patients who therefore were considered as non-responsive patients. When all the patients’ immunological results before and after chemotherapy were analyzed (Table 1), the CD8+ cytotoxic-suppressor T cell percentages were found to be increased following chemotherapy (p<0.001). Furthermore, the CD4/CD8 ratio (p=0.04), and the CD19+B lymphocyte (p<0.001), and CD45RA+ non-activated T cell percentages were decreased after chemotherapy even though the decrease in CD45RA+ population did not reach the statistical significance (p= 0.06).

In subgroup analyses (Table 1), in the chemotherapy-responsive group, the CD8+ cytotoxic-suppressor T cell percentages were increased (p<0.001), whereas the CD4/CD8 ratio (p<0.04), and the CD19+B lymphocyte percentages (p<0.001) were decreased similar to the whole group analyses. Interestingly, however, CD56+ NK cells (p=0.005), and CD45RO+ memory T-cells (p=0.05) were found to be increased, whereas the NKA (p=0.04) were decreased in this group after chemotherapy.

In the chemotherapy nonresponsive group (Table 1), the CD8+ cytotoxic-suppressor T cell per-

<table>
<thead>
<tr>
<th>Monoclonal Antibodies</th>
<th>Peripheral Blood Lymphocyte Subpopulations</th>
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<tr>
<td>CD3</td>
<td>Total mature T cells</td>
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<tr>
<td>CD4</td>
<td>T-helper cells</td>
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<tr>
<td>CD8</td>
<td>Suppressor-cytotoxic T-cells</td>
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<tr>
<td>CD25</td>
<td>Activated T-cells (IL-2 receptor)</td>
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<tr>
<td>CD19</td>
<td>Total B-lymphocytes</td>
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<tr>
<td>CD56</td>
<td>Natural Killer Cells</td>
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<td>CD45RA</td>
<td>Non-activated T cells</td>
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<tr>
<td>CD45RO</td>
<td>Memory T-cells</td>
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<td>T-cell γδ</td>
<td>γδ T lymphocytes</td>
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percentages were increased (p=0.03), whereas the CD19⁺ B lymphocyte percentages (p=0.04) were decreased similar to the whole group and to the chemotherapy-responsive group analyses. Nevertheless, unlike the other groups, the CD25⁺ activated T-cell percentages (p=0.004) were decreased, and the NKA (p=0.01) was found to be increased in this group analyses after chemotherapy.

However, no statistically significant changes were observed in other PBL subpopulation percentages including CD3⁺, CD4⁺, and γδ-T cell populations in the whole group and subgroup analyses after chemotherapy.

**DISCUSSION**

It is well known that chemotherapy induces bone marrow suppression that may cause myelosuppression and leucopenia. We therefore investigated in this study whether the FEC regimen which is the most common chemotherapy regimen used in breast cancer could affect the natural killer cell activity and peripheral blood lymphocyte subsets in patients. We briefly found that even though there were decreases in the CD19⁺ B-lymphocyte and CD45RA⁺ naive T-lymphocyte percentages and the CD4/CD8 ratio and increases in the CD8⁺ cytotoxic-suppressor T cells, the NKA did not show any change after chemotherapy. In the subgroup analyses, the CD45RO⁺ memory T-cell percentages were interestingly found to be increased in the chemotherapy responsive-group whereas the NKA was observed to be increased in the chemotherapy non-responsive group following chemotherapy.

In concordance with previous studies, decreased CD19⁺ B-lymphocyte percentages were obtained following chemotherapy in patients with breast cancer²⁶,²⁷. Furthermore, similar to our results, Murta et al.¹ observed increased levels of CD8⁺ cytotoxic-suppressor T cells in patients after FEC regimen as neoadjuvant chemotherapy. In subgroup analyses according to the response to chemotherapy, however, they also observed an increased ratio CD4/CD8 ratio in chemotherapy-responsive patients compared to nonresponders unlike our findings.

It has been recently suggested that chemotherapy can impair cell function through the destruction of bone marrow environment. Solomayer et al.²⁰ reported reduced numbers of activated NK and NK-T cells 24 months after surgery and adjuvant chemotherapy in breast cancer patients indicating a long-lasting negative effect of chemotherapy on the bone marrow immune system. However, the mechanisms are not well understood. Another study demonstrated that the number of NK cells (CD16⁺ and CD56⁺) in cancer patients decreased after chemotherapy regimen including bleomycin, etoposide and cisplatin²¹. On the contrary, FEC regimen caused an increase in the percentages of NK cells and T-cytotoxic cells suggesting that different chemotherapy regimens may have different effects on immune cells.

| Table 2. Changes in Peripheral Blood Lymphocyte Subpopulation Ratios and Natural Killer Cell Activity in Patients with Locally Advanced Breast Cancer after Chemotherapy (meansSD) |
|------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| All patients (n=13)          | CD3    | CD4    | CD8    | CD4/CD8| CD19   | CD25   | CD56   | CD45RA | CD45RO | γδ-T cell| NKA    |
| Before CT                    | 69.4±9.0| 5.4±10 | 27.9±8.7| 1.3±0.4 | 9.3±3.7| 6.1±3.1| 20.4±13.6| 46.6±7.8| 45.6±6.1| 5.8±4.7 | 34.8±13.3|
| After CT                     | 73.4±7.0| 32.3±12.5| 37.7±7.6| 0.9±0.5 | 2.4±1.5| 4.1±3.0| 25.2±9.5 | 36±6.7 | 48.4±21.1| 12.5±15.8| 32.5±10.3|
| P-value                      | 0.2     | 0.6     | <0.001 | 0.04   | <0.001 | 0.3    | 0.1    | 0.06   | 0.7    | 0.3     | 0.7    |
| CT-responsive (n=7)          |        |        |        |        |        |        |        |        |        |        |
| Before CT                    | 69.8±8.6| 37.8±9.7| 27.6±9.6| 1.5±0.4 | 9.9±3.1| 6.75±4.5| 17.7±12.1| 45±8.7 | 48.7±7.6| 4±1.0   | 40.8±12.4|
| After CT                     | 73.6±6.6| 29.9±15.8| 37.4±9.1| 0.9±0.6 | 1.8±1.3| 6.75±1.7| 24.7±9.5 | 32.3±3.8| 63.7±10.3| 5.7±2.1 | 27.5±9.5 |
| P-value                      | 0.5     | 0.4     | 0.01   | 0.05   | 0.001  | 0.999  | 0.005  | 0.2    | 0.06   | 0.4     | 0.04   |
| CT-nonresponsive (n=6)       |        |        |        |        |        |        |        |        |        |        |
| Before CT                    | 69±10.5| 32.5±10.2| 28.4±8.3| 1.04±0.2| 8.6±4.6| 5.3±1.0| 24.3±16 | 47.8±7.9| 43.2±4.5| 8.5±7.8 | 25.5±5.1 |
| After CT                     | 74±8.2 | 35.8±5.3| 38±5.8 | 0.97±0.2| 3.2±1.5| 0.51±0.6| 25.8±10.7| 38±7.5 | 37±20.3 | 21.5±26.2 | 46.9±10.7|
| P-value                      | 0.2     | 0.3     | 0.03   | 0.6    | 0.04   | 0.004  | 0.8    | 0.3    | 0.6    | 0.5     | 0.01   |
Furthermore, previous studies showed a decreased activity of NK cells during chemotherapy including regimens with antracyclines, fluorouracil or cyclophosphamide or melphalan with methotrexate in patients with breast cancer even though they did not observe any change in NK cell percentages \(^{22,27}\). In our study, no change was seen in NK cell percentages or NKA after FEC chemotherapy. Interestingly however, in the subgroup analyses, there was an increase of NK cell percentages and a decrease in NKA in chemotherapy-responsive patients whereas an increased NKA was observed in chemotherapy-nonresponsive patients. The discrepancies in the findings of our study and other reports might be due to the different techniques used to assess NK cell activity since we used Candida cells as target cells for NK cells as opposed to K562 human erythroleukemia cell line used in the majority of other studies\(^{22,24,26}\).

Brittenden et al\(^{28}\) similarly reported that NK or lymphokine-activated killer cell activity was significantly suppressed in locally advanced breast cancer patients receiving CHOP chemotherapy that could be enhanced by L-arginine supplementation. However, optimal levels of antibody dependent cellular cytotoxicity of CD16\(^+\) NK cells could be obtained in patients with breast cancer against MCFHER2/neu in the presence of Herceptin, a humanized recombinant monoclonal antibody recognizing HER2/neu antigen in breast cancer cells, after chemotherapy completion\(^{29}\). All these results suggest that chemotherapy induced changes in natural immunity could be modulated by immunotherapy strategies to enhance the antitumoral response in patients.

Furthermore, we demonstrated increased percentages of CD45\(^+\) memory T-cells and CD8\(^+\) T lymphocytes in chemotherapy-responsive patients after chemotherapy. Eralp et al\(^{30}\) similarly found antigen-specific CD8\(^+\) T lymphocytes were induced by doxorubicin or paclitaxel chemotherapy and vaccination with gene vaccine to HER2/neu in mice that were injected with an aggressive breast tumor cell line that expresses HER2/neu. In this study, neither vaccination nor chemotherapy alone significantly reduced the tumor growth in mice, whereas chemotherapy followed by vaccination interestingly significantly inhibited the tumor growth. Similarly, effective antitumoral response could be obtained in mice inoculated with E0771 breast medullar adenocarcinoma cells by a combination chemoimmunotherapy consisting of a single dose of doxorubicin injection followed by daily injections of IL-2\(^{31}\). This combination therapy, not chemotherapy or immunotherapy alone caused a tumor-free long-term survival of 40% of mice and the majority of the surviving mice had specific memory cells for E0771 cells. The increased CD45RO\(^+\) memory T-cell percentages might contribute to the chemoimmunotherapy trials in breast cancer patients concomitant with chemotherapy or following chemotherapy.

**CONCLUSION**

Our results suggest that the anthracycline-based chemotherapy regimen-induced increases in memory T-cells in chemotherapy-responsive patients may further enhance the efficacy of chemoimmunotherapy and/or vaccination trials in patients\(^{32}\). Detailed phenotypical and functional characterization of intratumoral lymphocytes along with peripheral blood lymphocytes warrants further investigation to design a realistic treatment option regarding chemoimmunotherapy trials in terms of contrary findings on PBL percentages and NK cell function\(^{33}\).

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