Featured Article

Down-Regulation of Intratumoral Aromatase Messenger RNA Levels by Docetaxel in Human Breast Cancers

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ABSTRACT

Purpose: The reason why chemotherapy induces resistance to subsequent hormonal therapy remains to be clarified in postmenopausal breast cancers. We hypothesized that chemotherapy might down-regulate the intratumoral biosynthesis of estrogens. Thus, we have studied the influence of chemotherapy (docetaxel) on intratumoral aromatase mRNA expression because aromatase is a key enzyme for intratumoral biosynthesis of estrogens.

Experimental Design: The mRNA levels of aromatase and its inducers [tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and cyclooxygenase 2 (COX-2)] were determined by a real-time polymerase chain reaction assay in breast cancer tissues obtained before and after neoadjuvant chemotherapy with docetaxel (four cycles of 60 mg/m² every 3 weeks) in 16 postmenopausal patients with estrogen receptor (ER)- and/or progesterone receptor (PR)-positive breast cancers. ER and PR levels in tumor tissues were also determined by enzyme immunoassay before and after chemotherapy.

Results: The intratumoral aromatase mRNA levels decreased significantly (P < 0.05) after chemotherapy from 0.84 ± 0.28 (mean ± SE) to 0.47 ± 0.28. The intratumoral TNF-α mRNA levels also decreased significantly (P < 0.05) after chemotherapy from 2.40 ± 0.52 to 0.95 ± 0.25. On the contrary, the intratumoral IL-6 and COX-2 mRNA levels showed a marginally significant increase (P = 0.07) and a significant increase (P < 0.05), respectively, after chemotheraoy. PR levels showed a marginally significant decrease (P = 0.08) after chemotherapy, whereas ER levels were almost constant before and after chemotherapy.

Conclusions: Antitumor activity of docetaxel is mediated, at least in part, through a down-regulation of aromatase expression in tumor tissues, resulting in the suppression of intratumoral estradiol synthesis. Aromatase expression seems to be regulated mostly by TNF-α, but not IL-6 and COX-2.

INTRODUCTION

In premenopausal breast cancer patients, chemotherapy is known to exert its antitumor effects not only directly through cytotoxic effects on tumor cells but also indirectly through suppression of ovarian function, as evidenced by the fact that 40% to 70% of breast cancer patients treated with chemotherapy develop amenorrhea (1) and that prognosis of estrogen receptor (ER)-positive breast cancer is better in patients with chemotherapy-induced amenorrhea than in those without it (1, 2). Thus, chemotherapy is considered to work as hormonal therapy in premenopausal women as well. On the other hand, in postmenopausal patients, it has been shown that serum estrogens levels are stable during and/or after chemotherapy (3), suggesting that estrogen production in the adipose tissue through aromatization of the adrenal gland-derived androgens to estrogens is not affected by chemotherapy. Thus, it has been thought that chemotherapy works as only chemotherapy in postmenopausal patients, unlike premenopausal patients, in whom chemotherapy works both as chemotherapy and hormonal therapy.

It is well known that the response rate to tamoxifen decreases when it is given after chemotherapy in metastatic postmenopausal patients (4, 5), and the recent results of the Arimidex and Tamoxifen: Alone or in Combination Trial (6) comparing tamoxifen with anastrozole have shown that anastrozole is superior to tamoxifen in postmenopausal patients untreated with adjuvant chemotherapy, but not in those treated with adjuvant chemotherapy. These results indicate a possibility that resistance to hormonal therapy can be induced by chemotherapy, although only limited clinical data are available thus far. Recent studies have disclosed the importance of intratumoral estradiol (E₂) synthesis in the growth of breast cancers in postmenopausal patients (7–9), and aromatase inhibitors are thought to exert their effects mainly by inhibiting the production of intratumoral E₂ in postmenopausal patients (10, 11). Thus, it is possible that chemotherapy also works as hormonal therapy even in postmenopausal patients by down-regulating intratumoral aromatase levels, resulting in low intratumoral E₂ levels.

The influence of chemotherapy on intratumoral E₂ synthesis has not yet been studied, but it would be interesting to study this issue to clarify the possibility that chemotherapy could also work as hormonal therapy by down-regulating intratumoral aro-
matase in postmenopausal patients. Although its regulation is not fully understood, intratumoral aromatase expression is considered to be induced mainly by locally synthesized cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α and cyclooxygenase (COX)-2, which is involved in the production of prostaglandin E2 (12–16). It has been shown that the expression level of aromatase mRNA is enhanced in breast cancer tissue compared with normal breast tissue (17) and that IL-6, TNF-α, and prostaglandin E2 stimulate aromatase mRNA transcription using their specific promoters (18–20). Because switching from promoter I.4, which is predominantly used in normal breast tissue, to promoter I.3 and PII is frequently observed in breast cancer tissue (14), it is speculated that aromatase mRNA expression in breast cancer tissue is regulated differently than that in normal breast tissue, and an increase of these inducers has been thought to be responsible for up-regulation of aromatase mRNA expression in tumor tissue. In addition, the infiltrating lymphocytes and macrophages in tumors are thought to be responsible for the production of these inducers as well (21–23).

In the present study, the influence of chemotherapy on intratumoral aromatase mRNA expression levels has been studied by comparing expression levels before and after neoadjuvant chemotherapy with docetaxel in breast cancer patients, and in addition, the change in expression levels of IL-6, TNF-α, and COX-2 mRNA has also been studied to elucidate which factor is most responsible for the regulation of intratumoral aromatase mRNA levels.

**MATERIALS AND METHODS**

**Patient Characteristics and Tumor Specimens.** Sixteen postmenopausal patients with ER- and/or progesterone receptor (PR)-positive (hormone receptor-positive) locally advanced breast cancers who were treated with neoadjuvant chemotherapy were consecutively recruited in this study (Table 1). Tumor specimens were obtained before chemotherapy by vacuum-assisted core needle biopsy. Mastectomy or breast-conserving surgery was conducted 3 to 4 weeks after the last chemotherapy. Half of the tumor specimen collected intraoperatively was snap frozen in liquid nitrogen and kept at −80°C until use, and the other half was subjected to histologic examination to confirm the existence of viable cancer cells in the specimen. Patients who showed a pathological complete response (complete disappearance of tumor cells) were excluded from this study.

**Chemotherapy and Response Evaluation.** Four cycles of docetaxel (60 mg/m² intravenously every 3 weeks) were given to the patients as neoadjuvant chemotherapy. Response of breast tumor to chemotherapy was assessed clinically by measuring tumor size using ultrasonography and/or magnetic resonance imaging according to the World Health Organization criteria (24). Patients who showed complete response or partial response were classified as responders, and those who showed no change or progressive disease were classified as nonresponders.

**RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction.** Frozen tumor specimens were used for total RNA extraction, and cDNA synthesis was carried out using 3 μg each of total RNA with oligo(dT)₁₅ primer. Primers and probes for real-time polymerase chain reaction (PCR) amplification of aromatase and the conditions of PCR reactions using ABI Prism 7700 (Perkin-Elmer Applied Biosystems, Foster City, CA) have been described previously (25, 26). The primer and probe mixture for TNF-α, IL-6, and COX-2 was purchased from Perkin-Elmer Applied Biosystems, and PCR was performed following the manufacturer’s protocol. To quantify gene transcripts precisely, β-glucuronidase transcripts were monitored as a quantitative control, and each sample was normalized on the basis of its β-glucuronidase transcript content. We used the primer probe mixture for β-glucuronidase (Perkin-Elmer Applied Biosystems) and followed the method described in the manufacturer’s protocol. Finally, mRNA levels of aromatase, TNF-α, IL-6, and COX-2 were shown as ratios to β-glucuronidase mRNA levels when 10⁻¹⁰ μg of PCR product for aromatase, 10⁻¹² μg of PCR product for IL-6, 10⁻¹³ μg of PCR product for TNF-α and COX-2, and 10⁻⁸ μg of PCR product for β-glucuronidase were defined as 1. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for calculation of mRNA expression levels.

**Immunohistochemical Staining of Aromatase, Tumor Necrosis Factor α, Interleukin-6, and Cyclooxygenase-2.** Immunohistochemical staining was performed using formalin-fixed, paraffin-embedded tumor tissues. After deparaffinization, antigen was retrieved by heating the 4-μm-thick paraffin sections at 120°C for 15 minutes (aromatase) or at 95°C for 20 minutes (TNF-α and IL-6) in 10 mmol/L citrate acid buffer (pH 6.0). Endogenous peroxidase activity was blocked by treatment with 3% H₂O₂ for 30 minutes. The primary antibodies used in this study were a rabbit polyclonal anti-aromatase antibody [working dilution, 1:400; established by Harada et al. (27)], a rabbit polyclonal anti–TNF-α antibody (P-300A; working dilution, 1:3;200; Endogen, Woburn, MA), a goat polyclonal anti–IL-6 antibody (RC206; working dilution, 1:250; DakoCytomation, Kyoto, Japan), and a rabbit polyclonal anti–COX-2 antibody (18516; working dilution, 1:25; Immuno-Biological Laboratories, Gunma, Japan). Primary antibodies were incubated with the sections overnight at 4°C (aromatase and COX-2) or for 15 minutes at room temperature (TNF-α and IL-6). An avidin-biotin-peroxidase complex technique was used for visualization of aromatase activity.
and COX-2, and additional treatment with catalyzed signal amplification (DAKO, Kyoto, Japan) was used for visualization of TNF-α and IL-6.

**Estrogen Receptor and Progesterone Receptor Assays.** ER and PR levels in tumor tissues were measured by enzyme immunoassay using the kit provided by Abbott Research Laboratories (Chicago, IL). The cutoff value for ER and PR was 5 fmol/mg protein.

**Statistical Methods.** Aromatase, TNF-α, IL-6, and COX-2 mRNA expression levels before and after neoadjuvant chemotherapy were compared by paired Student’s t test. Statistical significance was assumed for \( P < 0.05 \).

**RESULTS**

**Intratumoral Messenger RNA Levels of Aromatase, TNF-α, IL-6, and COX-2 before and after Chemotherapy.** Intratumoral aromatase mRNA levels after chemotherapy (0.47 ± 0.28, mean ± SE) were significantly lower \( (P < 0.05) \) than those before chemotherapy (0.84 ± 0.28), as shown in Fig. 1A. Intratumoral TNF-α mRNA levels also showed a significant decrease \( (P < 0.05) \) after chemotherapy \( (2.40 \pm 0.52 \) versus \( 0.95 \pm 0.25 \); Fig. 1B). On the other hand, intratumoral IL-6 and COX-2 mRNA levels showed a nonsignificant increase \( (P = 0.07 \) for IL-6) and a significant increase \( (P < 0.05 \) for COX-2 mRNA) after chemotherapy, respectively (Fig. 1C and D).

Results of the subset analysis of the change in aromatase, TNF-α, IL-6, and COX-2 mRNA levels after chemotherapy according to the response to chemotherapy are shown in Table 2. The intratumoral aromatase mRNA and TNF-α mRNA levels decreased significantly after chemotherapy in the responders [0.09 and 0.38 (median values) of the levels before chemotherapy, respectively], but not in the nonresponders (Table 2). The intratumoral IL-6 and COX-2 mRNA levels did not change significantly in either the responders or nonresponders, but a nonsignificant trend toward an increase in IL-6 mRNA level and a significant increase in COX-2 mRNA level after chemotherapy was seen in both the responders and nonresponders.

**Table 2** Change in intratumoral mRNA levels of aromatase, TNF-α, IL-6, and COX-2 after chemotherapy according to response to chemotherapy

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Responders</th>
<th>Nonresponders</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aromatase</strong></td>
<td>0.28 (0–7.95) *‡</td>
<td>0.09 (0–0.77) *‡</td>
<td>0.66 (0.02–7.95) *</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>0.31 (0.08–8.25) †</td>
<td>0.38 (0.22–3.79) †</td>
<td>0.24 (0.08–8.25)</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>5.79 (0.26–614) ‡</td>
<td>4.27 (0.86–614)</td>
<td>8.47 (0.26–116)</td>
</tr>
<tr>
<td><strong>COX-2</strong></td>
<td>4.20 (0.48–47.1) †</td>
<td>7.63 (0.48–47.1)</td>
<td>3.09 (0.50–28.0)</td>
</tr>
</tbody>
</table>

* Median (range) of ratios of mRNA levels of aromatase, TNF-α, IL-6, and COX-2 after chemotherapy to mRNA levels before chemotherapy.
‡ \( P < 0.05 \).
‡ \( P = 0.07 \).
Fig. 2 Intratumoral ER levels (A) and PR levels (B) before and after docetaxel therapy. Bars, mean ± SE.

**DISCUSSION**

It is well established that intratumoral E2 synthesis by aromatase plays a very important role in the growth of breast cancers in postmenopausal patients (7–9). Treatment with an aromatase inhibitor decreases E2 synthesis in tumor tissues, resulting in growth inhibition (10, 11). Our present observation that docetaxel significantly down-regulates intratumoral aromatase mRNA levels suggests that docetaxel exerts its antitumor effects, at least in part, by decreasing the intratumoral E2 levels. Because chemotherapy does not affect serum estrogens levels (3) in postmenopausal patients (unlike premenopausal patients, who often develop ovarian dysfunction due to chemotherapy), our present observation that PR (E2-inducible protein) levels decreased after docetaxel therapy strongly indicates that such a decrease in PR levels is induced by a decrease in the intratumoral E2 synthesis. These results indicate that docetaxel can exert its antitumor effects not only directly through cytotoxic effects on tumor cells (chemotherapy) but also indirectly through down-regulation of aromatase mRNA (hormonal therapy).

The importance of TNF-α, IL-6, and COX-2 as an inducer of aromatase has been well documented in several in vitro and in vivo experiments (13, 22, 28, 29) but has yet to be elucidated in human breast cancers in vivo. In the present study, we demonstrated that the intratumoral mRNA levels of TNF-α significantly decreased in parallel with those of aromatase after docetaxel therapy, but the IL-6 and COX-2 mRNA levels, on the contrary, showed a nonsignificant tendency toward an increase. Our results on COX-2 mRNA levels are consistent with those reported by Subbaramaiah *et al.* (30), who showed that COX-2 mRNA levels increased in a mammary epithelial cell line treated with docetaxel. Therefore, these results, taken together, suggest that TNF-α plays the most important role in induction of aromatase in breast cancer tissues among these three inducers and that the roles of IL-6 and COX-2 are not as important as expected from the previous experimental studies (13). Furthermore, the facts that COX-2 expression is lower in breast cancer tissues than in the adjacent normal tissues (31) and, inversely, that aromatase expression is higher in breast cancer tissues than in adjacent normal tissues (32) seem to suggest that COX-2 is unlikely to play a pivotal role in the induction of aromatase in breast cancer.

Tsavaris *et al.* (33) have reported that serum TNF-α levels decrease significantly in breast cancer patients after chemotherapy with taxanes. Although the mechanism of reduction of serum TNF-α levels after chemotherapy is currently unknown, it is unlikely to be explained by reduction of macrophage-derived TNF-α because TNF-α mRNA levels in macrophages have been reported to be unaffected by chemotherapy with paclitaxel (34). Rather, it seems to be more plausible to speculate that the reduction of tumor cell-derived TNF-α results in a decrease in the serum TNF-α levels, based on our observation that immunohistochemical examination has localized TNF-α predominantly in tumor cells.

Purohit *et al.* (35) have reported that paclitaxel and 2-methoxyestradiol, both microtubule-stabilizing agents, inhibit basal- and TNF-α-stimulated aromatase activity in cultured fibroblasts. Furthermore, it has also been shown that...
2-methoxyestradiol inhibits the aromatase activity in the aromatase gene-transfected MCF-7 cells (36). Thus, the ability of docetaxel, which is also a microtubule-stabilizing agent, to reduce aromatase activity may result not only from a decrease of TNF-α expression but also from direct inhibition of aromatase activity. It is unlikely, however, that the down-regulating effect on intratumoral aromatase mRNA expression is specific to microtubule stabilizers because resistance to hormonal therapy can be induced after chemotherapy with doxorubicin + cyclophosphamide (4) and with cyclophosphamide + methotrexate + 5-fluorouracil (5) in postmenopausal patients; in addition, we have obtained a preliminary result that epirubicin + cyclophosphamide therapy is able to down-regulate the intratumoral aromatase and TNF-α mRNA levels comparably with docetaxel therapy (data not shown). Taken together with our observation that a significant decrease in intratumoral aromatase and TNF-α mRNA levels was seen in responders but not in nonresponders, it is speculated that tumor cells suffering from cytotoxic damage from any type of chemotherapy fail to maintain expression of aromatase mRNA due to the decrease of TNF-α or degradation process of tumor cells, resulting in the low intratumoral E₂ levels. Thus, any type of chemotherapy is considered to have the potential to work as hormonal therapy as well. The reason why chemotherapy induces resistance to subsequent hormonal therapy in postmenopausal patients seems to be explained by this dual effect of chemotherapy, i.e., tumors that become resistant to chemotherapy are speculated to have also acquired resistance to hormonal therapy, to some extent. We think this dual effect is also operative in premenopausal patients. Thus, it is speculated that in premenopausal patients, chemotherapy can work as hormonal therapy in two ways, i.e., suppression of ovarian function and intratumoral E₂ synthesis.

We have found that aromatase and TNF-α mRNA levels showed a nonsignificant trend toward a decrease in the nonresponders as well. It is speculated that, even in nonresponders, tumor cells might suffer from slight cytotoxic damage, so that they show some reduction in aromatase and TNF-α mRNA levels. It is also possible that chemotherapy might affect the tumor-infiltrating lymphocytes and macrophages so as to reduce the TNF-α mRNA levels, leading to the reduced aromatase mRNA expression in tumor cells because immunohistochemical examination has shown that, although TNF-α is localized predominantly in tumor cells, TNF-α is also observed in lymphocytes and macrophages in tumor tissues. The influence of chemotherapy on tumor-infiltrating lymphocytes and macrophages needs to be studied in more detail in future studies. Although it remains to be determined whether aromatase is localized predominantly in tumor cells or stromal cells, the immunohistochemical study in the present study has shown that aromatase is localized predominantly in tumor cells, and thus the decrease in aromatase expression after chemotherapy in tumor tissue is considered to be mainly attributable to the decrease of aromatase expression in tumor cells. A discrepancy in the results on immunohistochemical localization of aromatase could be explained by the difference in the antibodies used in the studies (37).

![Fig. 3 Immunohistochemical staining of aromatase, TNF-α, IL-6, and COX-2 in tumor tissues before and after docetaxel therapy.](image-url)
A limitation of the present study is a lack of data on E₂ concentrations in breast tumor tissues before and after chemotherapy. Because it is very difficult to measure such a low level of E₂ in tumor tissues with high accuracy and because a relatively large volume of tumor tissues is required for the assay, we did not investigate the change of E₂ concentrations in tumor tissues in the present study. Ideally, a decrease of E₂ concentrations in tumor tissues after chemotherapy needs to be proven, but we feel that a decrease of intratumoral aromatase mRNA levels as well as PR levels after chemotherapy can be strongly supporting evidence that the intratumoral E₂ concentrations actually decrease after chemotherapy.

In conclusion, we have shown that intratumoral aromatase mRNA levels significantly decrease after chemotherapy, suggesting that antitumor activity of chemotherapy is mediated, at least in part, through suppression of intratumoral E₂ synthesis. Because TNF-α mRNA levels, but not IL-6 and COX-2 mRNA levels, significantly decreased in parallel with the aromatase mRNA levels after chemotherapy, a decrease in TNF-α, but not in IL-6 and COX-2, is considered to lead to down-regulation of aromatase mRNA levels by chemotherapy. Taken together with the fact that both aromatase and TNF-α were localized predominantly in tumor cells by immunohistochemical analysis, it is speculated that aromatase expression is regulated mostly by TNF-α in an autocrine manner and that chemotherapy down-regulates aromatase mRNA expression through down-regulation of TNF-α. Our present results seem to provide a fresh insight for better understanding of the mechanism of docetaxel action as well as the mechanism of acquisition of resistance to hormonal therapy after chemotherapy. Our preliminary results need to be confirmed by a future study including a larger number of patients treated with various chemotherapeutic regimens.

ACKNOWLEDGMENTS

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