Increased Nuclear Localization of Transcription Factor Y-Box Binding Protein 1 Accompanied by Up-Regulation of P-glycoprotein in Breast Cancer Pretreated with Paclitaxel

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Abstract

Purpose: The Y-box binding protein 1 (YB-1) regulates expression of P-glycoprotein encoded by the MDR1 gene. There have been no previous studies regarding the involvement of YB-1 in the development of resistance to paclitaxel. The present study was done to examine how paclitaxel affects the localization and expression of YB-1 in breast cancer.

Experimental Design: We evaluated the expression and localization of YB-1 and P-glycoprotein in breast cancer tissues obtained from 27 patients before and after treatment with paclitaxel. The effect of paclitaxel on localization of cellular YB-1 was examined by using GFP-YB-1. Interaction of YB-1 with the Y-box motif of the MDR1 promoters was studied by electrophoretic mobility shift assay. The effects of paclitaxel on MDR1 promoter activity were examined by luciferase assay.

Results: Of 27 breast cancer tissues treated with paclitaxel, nine (33%) showed translocation of YB-1 from the cytoplasm to the nucleus together with increased expression of P-glycoprotein during the course of treatment. Twelve breast cancer tissues (44%) showed neither translocation of YB-1 nor increased expression of P-glycoprotein. Nuclear translocation of YB-1 was correlated significantly with increased expression of P-glycoprotein (P = 0.0037). Confocal analysis indicated that paclitaxel induced nuclear translocation of green fluorescent fused YB-1 in MCF7 cells. Furthermore, binding of YB-1 to the Y-box of MDR1 promoter was increased in response to treatment with paclitaxel. In addition, MDR1 promoter activity was significantly up-regulated by paclitaxel in MCF7 cells (P < 0.001).

Conclusions: The results of the present study suggested that YB-1 may be involved in the development of resistance to paclitaxel in breast cancer.

Paclitaxel is an anticancer agent that is effective against a variety of human tumors, including non-small cell lung cancer, ovarian cancer, breast cancer, head and neck cancer, and melanoma (1–5). Since the mid-1990s, paclitaxel has been used widely for locally advanced, metastatic, and recurrent breast cancers and has shown significant efficacy (3, 6). However, as is frequently observed with other chemotherapeutic agents, many of the patients who initially respond to paclitaxel later relapse. Furthermore, some tumors have been shown to be entirely resistant to paclitaxel even during initial treatment.

P-glycoprotein is a membrane glycoprotein of Mr 170,000 that functions as an ATP-dependent efflux pump. This molecule, encoded by the human MDR1 (ABCB1) gene, has been shown to reduce drug accumulation in cancer cells (7), and P-glycoprotein overexpression is closely associated with multidrug resistance in human cancers (8).

MDR1 is one of the target genes for Y-box binding protein 1 (YB-1). YB-1 is a member of a family of DNA-binding proteins that contain a highly conserved, cold shock domain and interact with inverted CCAAT boxes (Y-boxes) in the promoter regions of various eukaryotic genes, especially growth-related genes (9). YB-1 is localized mainly in the cytoplasm but is translocated into the nucleus when cells are exposed to either UV irradiation or to anticancer agents (10). Moreover, YB-1 levels directly alter the genotoxic stress-induced activation of the MDR1 promoter (11). Recent studies have indicated that the nuclear localization of YB-1 is closely associated with MDR1 gene expression and that P-glycoprotein levels were high in breast cancer, osteosarcoma, and ovarian serous adenocarcinoma cells in which YB-1...
was localized within the nucleus but low in those in which YB-1 was localized only within the cytoplasm (12–14).

To date, the mechanisms by which tumor cells develop resistance to paclitaxel are not fully understood. Early studies showed that the drug is a substrate for P-glycoprotein, and that cancer cell lines selected for paclitaxel have elevated levels of P-glycoprotein (15). However, little is known about the role of YB-1 in breast cancer treated with paclitaxel.

In the present study, to elucidate whether YB-1 is involved in the development of drug resistance to paclitaxel in breast cancer, we examined the correlation between the nuclear expression of YB-1 and the expression of P-glycoprotein in breast tumors from patients treated with paclitaxel. Furthermore, we investigated whether nuclear YB-1 expression was associated with objective response of breast cancer to paclitaxel.

Materials and Methods

Patient and tumor samples. Between February 2001 and February 2003, 27 patients with primary breast cancer were treated with preoperative chemotherapy using paclitaxel followed by resection at four institutions belonging to the Kitakanto Koshinetsu Breast Clinical Oncology Group. The clinical features of the patients are summarized in Table 1. Clinical stages were determined according to the Union Internationale Contra Cancrum—tumor-node-metastasis classification and stage grouping system. The cancer specimens from the patients were obtained by core needle biopsy before initiation of chemotherapy. One course of treatment consisted of administration of paclitaxel (80 mg/m²) on days 1, 8, and 15 followed by a 2-week interval without drug administration. Three courses of treatment were done before surgical resection. The specimens were immediately cut into small pieces after resection, snap-frozen in liquid nitrogen, and stored at −80°C in a freezer. Informed consent was obtained from all patients, and the study protocol was approved by the Ethics Committee of Shinshu University. The characteristics and response to preoperative chemotherapy of the 27 patients are listed in Table 1. All patients underwent two or three courses of paclitaxel chemotherapy without severe adverse events. The overall clinical response (partial response and complete response) rate was 59.3%.

Clinical criteria for assessment of chemotherapy response. We evaluated clinical response to paclitaxel according to the following WHO clinical criteria: complete response, disappearance of all known disease; partial response, ≥50% decrease in tumor size; no change, <50% decrease, or <25% increase in tumor size; and progressive disease.
≥25% increase in tumor size or appearance of new lesions. Chemotherapeutic histologic response was evaluated according to the following Japanese Breast Cancer Society criteria: no response (grade 0), almost no change in cancer cells after treatment; mild response (grade 1a), mild change in cancer cells regardless of the area, or marked changes seen in less than one third of cancer cells; moderate response (grade 1b), marked changes in one third or more but less than two thirds of tumor cells; marked changes in two thirds or more of tumor cells; complete response (grade 3), necrosis or disappearance of all tumor cells. Replacement of all cancer cells by granuloma-like and/or fibrous tissue (16).

**Antibodies and immunohistochemical analysis.** Immunohistochemical analysis of YB-1 and P-glycoprotein expression was carried out using anti-human YB-1 antibody (17) and anti-human P-glycoprotein antibody (JSB-1, Sanbio, Uden, the Netherlands). Anti-YB-1 was diluted 1:12,000 in PBS plus 0.1% bovine serum albumin, JSB-1 was diluted 1:20 in PBS. For staining with anti-YB-1 anti-P-glycoprotein, sections were pretreated with 0.01 mol/L citrate buffer (pH 6.0) twice for 6 minutes each time at 100°C in a microwave oven. The sections were treated at 4°C overnight with primary antibodies followed by staining with a DAKO Envision System (Carpinteria, CA), then stained with freshly prepared diaminobenzidine solution and counterstained with hematoxylin. On each section, the five microscopic fields that had the greatest accumulation of positive signals (hotspots) were selected under a microscope. The ratios of positive cells in these areas were calculated, and the mean values of five fields were used as the values of YB-1 and P-glycoprotein expression. For further analysis, the value of each sample was assigned to one of two groups (0-10%, negative and >10%, positive for YB-1 and P-glycoprotein). The hormone receptors were detected by immunohistochemistry; in cases in which >10% of cancer cells in cancer tissues showed positive staining for the receptors, they were regarded as positive. HER2 expression was determined using Herceptest according to the manufacturer’s protocol (DAKO). In addition, immunostaining was semiquantitatively scored for another extent, intensity (absent, score = −; weak, score = 1+; moderate, score = 2+; strong, score = 3+).

**Drugs and cell culture.** Paclitaxel and cisplatin were obtained from Sigma (St. Louis, MO) and dissolved in DMSO and 0.9% physiologic saline (pH 3.0), respectively. The MCF7 human breast cancer cell line was maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO₂ atmosphere.

**Cytotoxic assays.** MCF7 cells were plated at a density of 2 × 10⁴ per well in 96-well plates, and indicated concentration of drugs was added the following day. After 72 hours, surviving cells were assayed with TetraColar ONE (Seikagaku Corp., Tokyo, Japan) for 2 hours at 37°C according to the protocol provided, and absorbance was measured at 450 nm. IC₅₀ of paclitaxel and cisplatin were 0.002 and 2 μmol/L, respectively (data not shown).

**Confocal analysis.** Green fluorescent protein (GFP) fused YB-1 expression plasmid was obtained previously (10). MCF7 cells were plated on glass coverslips in six-well plates at a density of 1 × 10⁵/cm². The following day, cells were transfected with GFP-YB-1 or GFP expression plasmid using SuperFect according to the manufacturer’s protocol (Qiagen, Tokyo, Japan). After 3 hours, culture medium was changed to a fresh one. Twenty-four hours after transfection, cells were treated with 0.002 μmol/L paclitaxel or 2 μmol/L cisplatin and cultured for 6 hours. For confocal analysis, cells were then washed twice with PBS and fixed in PBS containing 5% freshly prepared paraformaldehyde. Alternatively, coverslips were mounted directly on slides for observation. The samples were analyzed using a Leica TCS SP5 system.
were examined under a Leica TCS SP laser scanning confocal imaging system (Tokyo, Japan).

**Nuclear extracts and Western blot analysis.** Preparation of nuclear extracts of MCF7 cells and Western blotting were described previously (18). Briefly, 10 μg of nuclear extracts were subjected by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membrane was incubated with antibody against YB-1 (1:5,000) for 1 hour at 25°C and visualized by the enhanced chemiluminescence protocol (Amersham Biosciences, Piscataway, NJ).

**Electrophoretic mobility shift assay.** Sequences of the oligonucleotides used as probes were as follows: MDR1 Y-box, 5'-GGTTAGGCTGATTGGCTGGGCAGGA-3'; Nuclear factor-κB consensus, 5'-TCAAGGGAGAGAAGGGACTTTCCCAAC-3'; and GC consensus, 5'-GGGCATCGGCATCGCCAGGGGGGGG-3'. Double-stranded oligonucleotides were labeled with [γ-32P]ATP using T4 polynucleotide kinase and purified on PAGE gels. Electrophoretic mobility shift assay was done as described previously (19). Briefly, 10 μg of nuclear extract were incubated for 30 minutes at 4°C in a final volume of 20 μl containing 25 mmol/L HEPES (pH 7.9), 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mg/ml bovine serum albumin, 5% glycerol, 0.05% NP40, 0.1 μg of poly(deoxyinosinic-deoxycytidylic acid), and 4 ng of 32P-labeled Y-box oligonucleotide as a probe in the presence or absence of competitors. For supershift assays, preincubation was done in the presence of 2 μL of anti-YB-1 antibody or rabbit IgG for 30 minutes at 4°C before the addition of radiolabeled probes. Products were analyzed on nondenaturing 4% polyacrylamide gels using a bioimaging analyzer (BAS 2000, Fuji Photo Film, Tokyo, Japan).

**Reporter assays.** Construction of MDR-Luc and MDR-m-Luc reporter plasmids and luciferase assay were described previously (18). Briefly, 10 μg of reporter plasmid was cotransfected with 2 μg of poly(deoxyinosinic-deoxycytidylic acid) and 4 ng of 32P-labeled Y-box oligonucleotide as a probe in the presence or absence of competitors. For supershift assays, preincubation was done in the presence of 2 μL of anti-YB-1 antibody or rabbit IgG for 30 minutes at 4°C before the addition of radiolabeled probes. Products were analyzed on nondenaturing 4% polyacrylamide gels using a bioimaging analyzer (BAS 2000, Fuji Photo Film, Tokyo, Japan).

**Preparation of nuclear extracts and Western blot analysis.** Nuclear extracts of MCF7 cells and Western blotting were described previously (18). Briefly, 10 μg of nuclear extracts were subjected by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membrane was incubated with antibody against YB-1 (1:5,000) for 1 hour at 25°C and visualized by the enhanced chemiluminescence protocol (Amersham Biosciences, Piscataway, NJ).

**Table 2. Expression of P-glycoprotein and YB-1 in breast cancers before and after treatment with paclitaxel**

<table>
<thead>
<tr>
<th>P-glycoprotein expression after treatment with paclitaxel</th>
<th>Negative or unchanged (%)</th>
<th>Increased (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YB-1 nuclear translocation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (n = 14)</td>
<td>12 (85)</td>
<td>2 (15)</td>
</tr>
<tr>
<td>Positive (n = 13)</td>
<td>4 (31)</td>
<td>9 (69)</td>
</tr>
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**Correlation between YB-1 nuclear expression and P-glycoprotein expression in breast cancer patients.** Nine (69%) of the 13 tumors positive for nuclear YB-1 translocation during treatment with paclitaxel showed increased expression of P-glycoprotein during the course of treatment. In addition, nuclear translocation of YB-1 was observed in 9 of 11 (82%) tumors that showed increase of P-glycoprotein expression during treatment with paclitaxel. On the other hand, the levels of P-glycoprotein expression maintained negative or unchanged during the course of treatment in 12 of the 14 (85%) tumors in which YB-1 translocation from cytoplasm to nucleus was not detected. Thus, nuclear translocation of YB-1 was correlated significantly with increased expression of P-glycoprotein (P = 0.0037; Table 2).

**Association of clinical response and Y-box binding protein-1 expression.** Partial response and complete response to treatment with paclitaxel were obtained in 10 of 14 patients (71%) negative for YB-1 nuclear translocation and in 6 of 13 (46%) positive for YB-1 nuclear translocation, respectively. Thus, significantly more patients negative for nuclear YB-1 translocation responded to paclitaxel than those positive for nuclear YB-1 translocation (P = 0.048). As for P-glycoprotein expression after treatment with paclitaxel.

**Table 3. Clinical response to paclitaxel and expression of YB-1 and P-glycoprotein**

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>PR + CR</th>
<th>P</th>
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<tbody>
<tr>
<td>YB-1 nuclear translocation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (n = 14)</td>
<td>4</td>
<td>10</td>
<td>0.048</td>
</tr>
<tr>
<td>Positive (n = 13)</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>P-glycoprotein expression after treatment with paclitaxel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative or unchanged (n = 17)</td>
<td>7</td>
<td>10</td>
<td>0.347</td>
</tr>
<tr>
<td>Increased (n = 10)</td>
<td>4</td>
<td>6</td>
<td></td>
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</table>
expression, more patients who did not show increased expression of P-glycoprotein tended to show better clinical response than those with increased P-glycoprotein expression (Table 3).

Nuclear translocation of Y-box binding protein-1 induced by paclitaxel. We examined the effects of paclitaxel on cellular YB-1 using MCF7 cells. GFP-YB-1 transfected in MCF7 cells was localized in cytoplasm before any treatment (Fig. 2B). However, nuclear translocation of GFP-YB-1 was clearly observed following treatment of 2 μmol/L (IC50) cisplatin (Fig. 2F) known to induce nuclear translocation of YB-1 (17). Next, MCF7 cells transfected with GFP-YB-1 were treated with 0.002 μmol/L (IC50) paclitaxel. Translocation of GFP-YB-1 from the cytoplasm to the nucleus was observed as well as cisplatin (Fig. 2D). When MCF7 cells were transfected with GFP expression plasmid, green fluorescence tended to accumulate in nucleus compared with cytoplasm. The ratio of nucleus/cytoplasm was not affected by treatment of neither cisplatin nor paclitaxel (Fig. 2A, C, and E). These results indicate that paclitaxel induces nuclear translocation of YB-1 in breast cancer cells.

In parallel, we analyzed whether paclitaxel increased the nuclear expression of endogenous YB-1 by Western blot analysis. MCF7 cells were treated with 0.002 μmol/L (IC50) paclitaxel, and nuclear extracts were prepared. Figure 3 showed that an increase of about 1.5-fold in nuclear YB-1 expression in MCF7 cells was observed after 6 hours treatment with paclitaxel. An almost identical result was obtained by treatment with 2 μmol/L (IC50) cisplatin.

Interaction of the transcription factor Y-box binding protein-1 with Y-box in the MDR1 promoter. To determine whether paclitaxel induces the binding of YB-1 to the Y-box motif of the MDR1 promoter, nuclear extracts of human breast carcinoma MCF7 cells were analyzed by electrophoretic mobility shift assay using a double-stranded Y-box oligonucleotide in sequence to the MDR1 promoter (Fig. 4). First, we investigated binding of nuclear extract of MCF7 cells treated with paclitaxel to the Y-box oligonucleotide. A few DNA-protein complexes were observed under mock treatment, but the major DNA-protein complex was increased around 3-fold after 6 and 12 hours of treatment with 0.002 μmol/L (IC50) paclitaxel as well as 2 μmol/L (IC50)
cisplatin (Fig. 4A). Major DNA-protein complex was markedly reduced by addition of unlabeled double-stranded Y-box oligonucleotide in a dose-dependent manner, whereas either nuclear factor-κB or GC box oligonucleotides showed no competition (Fig. 4B). Furthermore, incubation with anti-YB-1 antibodies reduced partially the major DNA-protein complex, whereas incubation with a corresponding IgG had no effect (Fig. 4C). These data show that major DNA-protein complex contained YB-1, DNA-YB-1 complex bound to the MDR1 promoter via Y-box motif, and DNA-YB-1 complex was increased by treatment of paclitaxel in MCF7 cells.

Up-regulation of MDR1 promoter activity induced by paclitaxel. We did MDR1 promoter reporter assay to evaluate whether paclitaxel is capable of modifying MDR1 promoter activity (Fig. 5). An increase in relative luciferase activity of 1.4-fold was observed after exposure to 0.002 μmol/L paclitaxel for 24 hours in the presence of Y-box-positive MDR1 promoter (P < 0.001). On the other hand, paclitaxel did not affect the MDR1 promoter activity in cells transfected with Y-box-negative MDR1 promoter. These results were corresponding to those of cisplatin and suggest that paclitaxel could induce MDR1 promoter activity via the Y-box motif.

Discussion

Paclitaxel has been widely used for treatment of a variety of cancers, including locally advanced, metastatic, and recurrent breast cancers. However, the mechanisms by which tumor cells show resistance to paclitaxel have not been determined in detail. Recent studies have shown that YB-1 plays an important role in modification of drug sensitivity of cancer cells by increasing the expression of P-glycoprotein, p21, and other proteins (20). However, there have been no previous reports regarding whether taxanes affect the localization of YB-1 in cancer cells. To our knowledge, this is the first report indicating that paclitaxel can affect localization of YB-1 both in vitro and in vivo.

Previous studies have shown a plausible association between YB-1 and drug resistance both in cultured cancer cells and in numerous clinical human tumor samples (12–14, 17). With regard to breast cancer, Bargou et al. (12) reported that YB-1 expression in the nuclei of untreated primary breast cancers showed an almost perfect correlation between YB-1 and P-glycoprotein expression. Saji et al. (21) also reported that nuclear YB-1 expression showed a significant correlation with P-glycoprotein expression in breast cancer tissues. In the present study, we observed a positive correlation between YB-1 nuclear...
YB-1 and P-glycoprotein in Breast Cancer

localization and positive P-glycoprotein expression, although the ratio of positive nuclear YB-1 expression in the pretreated breast cancer was lower than that reported in other studies. However, translocation of YB-1 from the cytoplasm to the nucleus in cancer cells during the course of treatment with paclitaxel in the same patients was observed in the present study. Furthermore, we also showed a positive correlation between YB-1 nuclear translocation and up-regulation of P-glycoprotein expression in breast cancer tissues treated with paclitaxel. Our observations in clinical samples support the hypothesis that nuclear translocation of YB-1 is involved in the up-regulation of P-glycoprotein in breast cancers, both in those untreated and treated with anticancer drugs. In ovarian cancer, alteration of negative nuclear YB-1 expression in primary lesions to positive nuclear YB-1 expression in recurrent lesions was detected in ~30% of tumors treated with regimens containing cisplatin, suggesting that nuclear YB-1 plays an important role in acquired cisplatin resistance in ovarian cancer (22). In the present study, the patients with breast cancer that showed translocation of YB-1 from the cytoplasm to the nucleus showed a significantly reduced response to paclitaxel. Our results suggest that nuclear YB-1 may be involved in modification of the sensitivity of breast cancer to paclitaxel. Janz et al. (23) reported that high levels of nuclear YB-1 expression in breast cancer tissues were associated with an unfavorable clinical course and showed that YB-1 expression is associated with clinical drug resistance as well as tumor aggressiveness. Our results are in agreement with this previous study in terms of drug-resistant phenotype of YB-1-positive tumors.

The involvement of YB-1 in mediating the effects of different external stimuli has been reported for a variety of chemicals and drugs, and also for UV light and hyperthermia (10, 11, 17, 24). However, as for anticancer agents, direct induction of translocation of cellular YB-1 from the cytoplasm into the nucleus has been shown only by treatment with cisplatin. In the present study, translocation of YB-1 was shown to be induced by treatment with paclitaxel in breast cancer cells transfected with YB-1, demonstrating that paclitaxel may function as an external stress factor that causes activation of YB-1.

Previous studies showed that YB-1 is involved in the regulation of P-glycoprotein expression in breast cancers and osteosarcomas (13). The promoter of the MDR1 gene contains a Y-box, which is responsible for basal MDR1 expression. In the present study, we showed that treatment with paclitaxel amplified the binding activity and interaction of YB-1 with the Y-box motif of the MDR1 gene, moreover, we showed that treatment with paclitaxel resulted in enhanced MDR1 promoter activity. Our results thus directly show that paclitaxel can up-regulate MDR1 expression in a Y-box-dependent manner.

In conclusion, our present data provide new information regarding the involvement of YB-1 in the development of resistance to paclitaxel in breast cancer both in vitro and in vivo. However, further experiments are needed to fully elucidate the role of YB-1 in the development of drug resistance because many factors are thought to be involved in the development of the drug-resistant phenotype in vivo. Nevertheless, the results presented here add to a growing body of evidence that YB-1 represents a promising target molecule for novel therapeutic strategies to overcome multidrug resistance in cancer.

Acknowledgments

We thank Kamijo and Sakamoto for technical assistance and Dr. Susan P.C. Cole for helpful suggestions.

References

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