Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells

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Abstract. The aim of this study was to assess paclitaxel resistant-epithelial ovarian carcinoma (EOC) cells for cellular morphology, motility, and molecular changes consistent with epithelial-mesenchymal transition (EMT). The human EOC cell lines NOS-2, TAOV and SKOV-3 were continuously exposed to increasing doses of paclitaxel to establish three stable cell lines resistant to paclitaxel (NOS-PR, TAOV-PR, and SKOV-PR cells, respectively). Using these cell lines, cellular functions such as motility, invasive ability, and proliferative potential were assessed. Several molecules involved in EMT or cell invasiveness were assessed using Western blot analysis. In a peritoneal metastasis model using mice inoculated with NOS-2 or NOS-PR cells, we investigated the differences of peritoneal dissemination and survival time. NOS2-PR cells showed phenotypic changes consistent with EMT; with spindle-shaped morphology and enhanced pseudopodia formation. Western blot analysis revealed decreased expression of the epithelial adhesion molecule, E-cadherin and an increase in mesenchymal markers such as vimentin, fibronectin and smooth-muscle actin in NOS-PR cells compared to NOS-2 cells. The NOS-2-PR cells displayed increased expression of Snail and Twist, EMT-regulatory transcription factors. Migratory potential in a wound assay and metastatic potential for epithelial ovarian carcinoma cells were assessed using several cell lines. Using these cell lines, epithelial ovarian carcinoma (EOC) is a major cause of death among the gynecological malignancies (1). EOC has a poor prognosis, primarily due to its late symptomatology, and is often associated with multiple intraperitoneal disseminations, and frequently with distant metastases. Treatment for advanced EOC is difficult because of both the inability to completely resect diffuse tumor involvement on the peritoneal surface and the eventual resistance of the tumor cells to chemotherapy. Paclitaxel is a first-line chemotherapeutic agent that is effective for the treatment of EOC. Paclitaxel exerts its effect through stabilization of microtubules, induction of cell cycle arrest in G2-M, and activation of proapoptotic signaling (2,3). However, in spite of the comparatively high sensitivity of EOC to paclitaxel, the prognosis of advanced or recurrent cases remains poor since most deaths are the result of metastasis that is refractory to conventional chemotherapy. To overcome the paclitaxel-resistance, a variety of additional molecular-targeting therapies combined with paclitaxel have been investigated (4,5).

Tumor cells progress from a non-invasive to an invasive and malignant phenotype through a series of metastatic steps (6,7). These steps involve a change in morphology of the epithelioid or cobblestone appearance to a fibroblastic form through a process referred to as the epithelial-mesenchymal transition (EMT) (8-10). EMT is a process initially observed during embryonic development in which cells lose epithelial characteristics such as downregulation of E-cadherin and gain mesenchymal properties that increase motility and invasion (11). According to several reports, the EMT is induced by several growth factors involved in tumor-progression processes such as VEGF, TGF-ß, or EGF (12,13). Furthermore, with respect to chemoresistance, recent studies demonstrated that there is a close link between EMT and insensitivity to several growth factors or chemotherapeutic agents (12,14,15). Hiscox et al demonstrated that Tamoxifen-resistant MCF7 cells showed loss of association between β-catenin and E-cadherin, and additionally showed increased cytoplasmic and nuclear β-catenin and elevated transcription of β-catenin target genes known to be involved in tumor progression and EMT (15).

Clinically, EOC frequently shows sudden and multiple metastasis following continuous treatment with chemotherapeutic agents, including paclitaxel. Based on this
observation, in the current study, we examined the EMT-like change in paclitaxel-resistant EOC cells, which display enhanced motile and invasive behavior in vitro and increased formation of peritoneal metastasis in a mouse model compared to their paclitaxel-sensitive counterparts. Our data demonstrate that the development of paclitaxel-resistance in EOC cells is accompanied by a transition from an epithelial to a mesenchymal phenotype.

Materials and methods

Cell culture. We used three human EOC cell lines (SKOV-3, NOS-2, and TAOV) in this study. SKOV-3, NOS-2, and TAOV cells were cultured and maintained as described previously (6,16). The cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO2.

Establishment of paclitaxel-resistant EOC cells. The above cells were washed thoroughly with PBS, and transferred to RPMI-1640 medium containing 10% FCS and penicillin-streptomycin. The cells were continuously exposed to paclitaxel for more than 6 months, during which time the medium was replaced every 3 days and the cell cultures were passaged by trypsinization after 70% confluency was reached. Gradually, these cells displayed resistance to the growth-inhibitory properties of paclitaxel. These cell lines, designated paclitaxel-resistant, were cultured for a further 3 months in medium containing paclitaxel before characterization studies. Through this process, we generated three paclitaxel-resistant EOC cell lines (NOS-PR, TAOV-PR, and SKOV-PR cells).

Wound assay. Cells were grown in 10-cm culture dishes. When the cells became confluent, the medium was replaced with fresh RPMI-1640 containing 1% FCS. Confluent cells were wounded with a uniform scratch using sterile pipette tips, rinsed to remove debris, then incubated in culture medium containing 1% FCS for 18 h. Quantitative analysis of the wound healing index expressed as percentage was calculated using 20 randomly chosen distances across the wound at 0 h and 18 h, divided by the distance measured at 0 h.

In vitro invasion assay. Cell invasiveness was evaluated using 24-well Matrigel invasion chambers (Becton Dickinson Labware). Cells were suspended in the upper chamber at a final concentration of 10x10⁴/ml in 200 μl of RPMI-1640 supplemented with 1% FCS. The lower chamber contained 700 μl of RPMI-1640 supplemented with 1% FCS. After 18 h of incubation, the tumor cells remaining on the upper surface of the filters were removed by wiping with cotton swabs, and the invading cells on the lower surface were stained with May-Grünwald Giemsa staining. The number of cells on the lower surface of the filters was counted under a microscope at a magnification of x200. The invasion assay was performed in triplicate in four individual experiments.

In vitro cell proliferation assay. Cells were plated in triplicate at a density of 1500 cells in a 200-μl volume in 96-well plates, and cultured for 1 to 4 days. Cell viability was assayed using a modified tetrazolium salt MTT assay performed using a CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega) according to the manufacturer’s instructions. Absorbance was measured at 492 nm using a microplate reader (Labsystems, Multiskan Bichromatic).

Paclitaxel chemosensitivity assay. Cells were plated in triplicate in 96-well plates at a density of 5000 cells in a volume of 200 μl of culture medium containing 10% FCS. After incubation for 24 h at 37°C, the medium was replaced by fresh medium with or without various concentrations of paclitaxel (Bristol Myers Squib, Tokyo, Japan). After an additional 72 h, cell viability was assayed using a modified MTT assay as described above. IC50 values were defined as the concentrations resulting in a 50% reduction in growth as compared with control cell growth. Experiments were performed in triplicate.

Western blot analysis. Western blot analysis was performed as described previously (16). Briefly, 30 μg of cell lysates were electrophoresed in a sodium dodecyl sulfate polyacrylamide gel under reducing conditions. After electrophoresis, the proteins were transferred electrophoretically to an Immobilon membrane (Millipore, Bedford, MA). After blocking, the membrane was incubated for 1 h with the respective anti-human primary antibody at the recommended dilution [anti-E-cadherin, fibronectin, vimentin, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-II (TIMP-II), Snail, Twist, β-actin (Santa Cruz), smooth muscle actin (SMA) (Progen), membrane-type 1-MMP (MT-MMP) (Chemicon), TIMP-I (Oncogene)]. The membrane was washed 3 times with TWEEN/PBS for 15 min each time, and then incubated with the appropriate secondary antibody for 1 h. After washing with TWEEN/PBS, the membrane was treated with ECL-Western blotting detecting reagent (Amersham Biosciences K.K., Tokyo, Japan).

Immunofluorescence staining. NOS-2 and NOS-PR cells were grown in chamber slides (Nalge Nunc International). They were fixed for 15 min with 4% paraformaldehyde and washed several times with PBS. Coverslips were incubated in blocking solution containing 2% BSA in PBS for 1 h, and incubated with the appropriate primary antibodies [anti-mouse E-cadherin and anti-rabbit β-catenin antibodies (Santa Cruz Biotech), anti-human E-cadherin, fibronectin, vimentin, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-II (TIMP-II), Snail, Twist, β-actin (Santa Cruz), smooth muscle actin (SMA) (Progen), membrane-type 1-MMP (MT-MMP) (Chemicon), TIMP-I (Oncogene)]. The membrane was washed with Tween/PBS, then incubated with the appropriate secondary antibody for 1 h. After washing with Tween/PBS, the membrane was treated with ECL-Western blotting detecting reagent (Amersham Biosciences K.K., Tokyo, Japan).

In vivo studies. Female nude mice (BALB/c) at 5 weeks of age were obtained from Japan SLC (Nagoya, Japan). The treatment protocol followed the guidelines for animal experimentation adopted by Nagoya University. NOS2 and NOS2-PR cells (1x10⁶ cells/0.5 ml of medium/mouse) were injected into mice intraperitoneally to generate peritoneal metastasis of EOC in the mouse model. To examine the metastatic potential of the cells, mice were sacrificed 25 days after the injection of carcinoma cells, and the production of ascites and formation...
of intraperitoneal dissemination were evaluated (n=4). In addition, survival time was compared between the two groups (n=7).

Statistical analysis. For data of in vitro experiments, statistical comparisons among groups were performed using the non-paired Student's t-test. Differences between groups were considered statistically significant at P<0.05. Data are expressed as mean ± SD. Moreover, the Kaplan-Meier method was used to generate survival curves, and comparisons were performed using log-rank tests. P<0.05 was considered significant.

Results

Acquisition of paclitaxel resistance induces morphologic changes consistent with EMT in EOC cells. We first confirmed the resistance to paclitaxel of NOS-PR cells. Fig. 1A shows the paclitaxel-sensitivity assay of parental NOS-2 and resistant NOS-PR cells. Chemoresistance to paclitaxel was found in NOS-PR cells compared to NOS-2 cells. The IC50 values of NOS-2 and NOS-PR cells were 9.3 ng/ml and 367.3 ng/ml, respectively. In parental NOS-2 cells, the shape of marginal cells was rounded, showing little formation of pseudopodia (panel a and c). In contrast, the phenotypic changes observed in NOS-PR cells included loss of cell polarity causing a spindle-shaped morphology, and increased formation of pseudopodia (a and c). Bars, 30 μm (a and b) and 60 μm (c and d).

Enhanced migratory and invasive potential of NOS-PR cells. We next compared various cellular functions between the NOS-PR and parental NOS-2 lines, such as migratory ability, invasiveness, and proliferative potential. Wound assays were performed to compare the migratory potential of NOS-PR and parental NOS-2 cells. At 18 h, the NOS-PR cells showed a 2.7-fold increase in the number of cells migrating across the wound (P<0.001; Fig. 2A and B). Increased formation of pseudopodia was observed in the edge of the wounds of NOS-PR cells compared to those of NOS-2 cells (data not shown). In addition, we compared the invasive potential between NOS-PR cells and NOS-2 cells using a Matrigel Boyden chamber. The capacity of NOS-PR cells to invade through a Matrigel-coated membrane was 2.4-fold greater compared with that of the parental NOS-2 cells (Fig. 2C, P<0.005).

The expression of EMT- and cell invasiveness-related molecules in NOS-2 and NOS-PR cells. To determine whether the acquisition of paclitaxel resistance induced specific molecular changes consistent with EMT, Western blotting was performed on cell lysates from the NOS-PR and parental NOS-2 cells (Fig. 3A). Expression of the epithelial adhesion molecule, E-cadherin was decreased in NOS-PR cells compared with NOS-2 cells. The expression of mesenchymal markers such as vimentin, SMA, and fibronectin was increased in NOS-PR cells. In contrast, the expression of TIMP-1 and TIMP-2 was downregulated in NOS-PR cells (Fig. 3A). Moreover, the expression of several proteolytic proteins involved in cellular invasiveness, including MMP-2 and MT1-MMP was concurrently increased in NOS-PR cells. In contrast, the expression of TIMP-1 and TIMP-2 was downregulated in NOS-PR cells (Fig. 3B). Furthermore, the expression of EMT-related transcription factors Snail and Twist was increased in NOS-PR cells compared with NOS-2 cells (Fig. 3C). To further investigate the possibility that
paclitaxel-resistance leads to changes in the cellular localization of E-cadherin and β-catenin, immunofluorescence staining was performed on NOS-PR and NOS-2 cells. As shown in Fig. 4, E-cadherin and β-catenin were localized and organized in the membrane in NOS-2 cells. In contrast, in NOS-PR cells, the cell-shape was disorganized and E-cadherin and β-catenin seemed to be dispersed throughout the cytoplasm.

The occurrence of EMT and increased expression of EMT-related molecules in PAC-resistant TAOV and SKOV-3 cells. We further examined whether the occurrence of EMT and enhanced expression of EMT-related proteins were observed in other EOC cell lines resistant to paclitaxel (TAOV-PR, SKOV-PR cells). The IC50 values of TAOV, TAOV-PR, SKOV-3, and SKOV-PR cells were 6.7, 452.2, 50.7, and 532.1, respectively. As shown in Fig. 5A, the parental TAOV cells showed an epithelioid, cobblestone appearance, like NOS-2 cells. On the other hand, the morphology of TAOV-PR cells was mixed type, showing an epithelioid and a short-bipolar/fibroblastic pattern with formation of pseudopodia. In addition, a remarkable morphological change was observed in SKOV-PR cells. While the parental SKOV-3 cells showed a short spindle-shaped morphology, SKOV-PR cells had a long spindle/fibroblastic morphology with a scattered and
unorganized growth pattern (Fig. 5B). Furthermore, increased levels of mesenchymal markers such as vimentin, SMA, and Twist were observed in both TAOV-PR and SKOV-PR cells compared to the respective parental cells (Fig. 5C and D).

Enhanced metastatic potential of NOS-PR cells to the murine peritoneum. Finally we investigated the ability to metastasize to the peritoneal cavity using a mouse intraperitoneal model. Carcinomatous peritonitis with a large amount of bloody ascites was observed approximately 25 days after the inoculation of NOS-PR cells into mice (Fig. 6A). Fig. 6B shows the intra-abdominal appearance of the mice 25 days after the inoculation of NOS-PR and NOS-2 cells. In the former, a number of disseminated tumors were observed throughout the whole
peritoneal cavity. In contrast, a mouse injected with NOS-2 cells had no macroscopically visible disseminated tumor, and had a significantly smaller amount of ascites at the same time of autopsy (Fig. 6C). Fig. 6D shows the survival curves of these two groups. All mice finally died of carcinomatous peritonitis. However, the mice injected with NOS-PR cells had significantly shorter survival than those injected with NOS-2 cells [mean survival, 46.7±2.43 days (NOS-2) and 31.4±3.61 days (NOS-PR), respectively, P=0.0004].

Discussion

Metastatic processes require reduction of E-cadherin expression to disrupt the adherence junction complex and the upregulation of proteolytic enzymes degrading extracellular matrix complexes, resulting in the facilitation of invasion into the surrounding tissues. Thus, many studies have focused on the relationship between loss of E-cadherin expression and the invasive and metastatic process. The prognosis of patients with EOC is most likely related to the degree of peritoneal dissemination (8,10,11). Numerous immunohistochemical studies have demonstrated that loss of E-cadherin expression is frequently associated with a poorer survival rate in patients with EOC (17-19). Besides the enhanced metastatic ability induced by loss of E-cadherin, chemoresistance is the major cause of the poor prognosis of EOC patients. Cisplatin and paclitaxel are major chemotherapeutic agents whose use results in improved outcomes; however, five-year survival is only 20% in advanced EOC patients in spite of the fact that most tumors are chemosensitive during the initial treatment (20). Clinically, enhanced metastatic ability and chemoresistance are frequently concurrent during the therapeutic course of EOC and seem to be linked to each other in light of the evolution of tumors toward increasingly malignant characteristics. However, the molecular mechanisms associated with these changes have not been elucidated.

In our current analysis, we demonstrated that EOC cells acquired an increased capacity for in vitro migration and invasion during the development of resistance to paclitaxel. In addition, we demonstrated that paclitaxel-resistant EOC...
cells lost their epithelial features and acquired mesenchymal characteristics, which are events characteristic of the EMT. Furthermore, in our animal model, the mice injected with NOS-PR cells had significantly shorter survival and enhanced peritoneal dissemination compared to those injected with NOS-2 cells. According to this result, tumors that have acquired paclitaxel-resistance may have characteristics that favor easily spreading into the peritoneal cavity, resulting in an increased chance of adhering to the mesothelium and enhanced formation of microscopic or macroscopic peritoneal metastasis. Our current evidence seems to be associated with the clinical observation that sudden peritoneal recurrence or another type of lymphovascular metastasis occurs during continuous treatment with chemotherapeutic agents, including paclitaxel. Our data indicate a possible link between paclitaxel-resistance and facilitated metastatic potential of EOC.

We obtained molecular evidence that the EMT-like changes in the NOS-PR cell line were associated with an increase in the transcription factors Snail and Twist. Snail and Twist are major transcription factors responsible for the development of the EMT. They can bind directly to the E-boxes on the E-cadherin promoter to repress its transcription (21). Clinically, in our preliminary study in 82 EOC patients, Twist was not associated with any of the clinicopathological parameters examined. However, positive Twist expression significantly predicted poorer overall survival and progression-free survival when compared with negative expression. Wang et al demonstrated that increased Twist was responsible for the development of acquired paclitaxel-resistance in nasopharyngeal carcinoma cells and ectopic expression of Twist conferred resistance to microtubule-disrupting agents, including paclitaxel (22). Conversely, our present study revealed that paclitaxel-resistance led to upregulation of Twist expression and subsequent occurrence of EMT. Although the detailed mechanism is still under investigation, it is possible that the increased expression of Twist or Snail is involved in the mechanism of the occurrence of EMT in paclitaxel-resistant cells. Chemotherapeutic agents, including paclitaxel, generally induce tumor regression through apoptosis, which is modulated through a series of proto-oncogenes and tumor suppressor genes. However, alterations in the regulation of such apoptotic processes potentially may lead to increased expression of such EMT-inducible factors and result in the failure of therapy. Although the detailed mechanisms underlying paclitaxel-resistance and EMT are still under investigation, therapeutic strategies targeting these EMT-promoting molecules may restore the chemosensitivity of EOC cells to paclitaxel.

As far as we know, this is the first report showing that chronic paclitaxel-resistance may lead to the EMT and a subsequent increase of the potential to metastasize to the peritoneal cavity. An earlier study by Yang et al showed that oxalaplatin-resistant colorectal cells displayed EMT-like changes with decreased expression of E-cadherin and an increase in the mesenchymal markers vimentin and Snail (14). This evidence is consistent with our current results.

In conclusion, we have shown here that the development of paclitaxel-resistance in EOC cells is accompanied by inducible EMT-like changes with increased potential to metastasize to the peritoneal cavity of nude mice. Further elucidation of the relationship between paclitaxel-resistance and EMT should make it possible to develop new therapeutic approaches for the treatment of chemotherapeutic tumors in the future.

References

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