Overexpression of PTEN Increases Sensitivity to SN-38, an Active Metabolite of the Topoisomerase I Inhibitor Irinotecan, in Ovarian Cancer Cells

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ABSTRACT

Purpose: PTEN is a tumor suppressor gene that was identified on chromosome 10q23. In addition to its original function as a tumor suppressor, this gene product was recently reported to enhance the sensitivity of cancer cells to anticancer agents. It is for the purpose of this study to investigate its function and the mechanisms by which PTEN enhances the sensitivity of ovarian cancer to antitumor agents.

Experimental Design: PTEN cDNA was introduced into the ovarian cancer cell line SHIN-3 and a high-expression cell line (SHIN-3/PTEN) was established. This cell line and a control were further analyzed.

Results: SHIN-3 cells did not carry any mutations in its genome after sequencing. In vitro examination of sensitivity to anticancer agents showed that the 50% growth-inhibitory concentration value for irinotecan metabolite (SN-38) in SHIN-3/PTEN was 800 nM, a 6.6-fold higher sensitivity compared with that of the control (5300 nM). There were no differences in sensitivity to cisplatin, paclitaxel, or gemcitabine between SHIN-3/PTEN and the controls. The percent differences in sensitivity to cisplatin, paclitaxel, or gemcitabine compared with that of the control (5300 nM) were observed in SHIN-3/PTEN, compared with controls.

Conclusions: These results indicate that high PTEN expression enhances the sensitivity of ovarian cancer cells to irinotecan and the induction of apoptosis and the suppression of topoisomerase I activity in cancer cells are suggested as possible mechanisms attributable to high PTEN expression.

INTRODUCTION

PTEN
d2 is a tumor suppressor gene identified from 10q23 (1, 2). In the gynecological field, a relatively high rate of PTEN abnormalities is observed in endometrial cancer; therefore, the involvement of PTEN in the development and progression of this disease has attracted attention (3, 4). PTEN has been reported recently to have the function of enhancing the sensitivity of cancer cells to certain anticancer agents (5, 6). We focused our attention on this function of PTEN. To investigate in vitro the function and mechanism of PTEN enhancing the sensitivity of ovarian cancer to antitumor agents, we introduced PTEN cDNA into an ovarian cancer cell line and established a cell line with high expression of PTEN.

MATERIALS AND METHODS

Cell Culture. The human ovarian serous cystadenocarcinoma cell line SHIN-3 (7) was cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 units penicillin, and 100 μg/ml of streptomycin at 37°C under 5% carbon dioxide.

PTEN Sequence Analysis in SHIN-3. Total DNA was extracted from SHIN-3 cells by the standard SDS-protease K procedure. The DNA extracted from SHIN-3 cells was subjected to PCR-single strand conformational polymorphism analysis to search for mutations in all nine exons of PTEN gene. PCR-single strand conformational polymorphism analysis was performed according to Orita et al. (8) with Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The PCR products were diluted 50-fold with 95% formamide and denatured at 80°C for 5 min, followed by rapid cooling on ice. Denatured products were separated on 5% polyacrylamide gels containing 5% glycerol with an automated laser fluorescence DNA sequencer ALF express (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and analyzed with software Allele link (Amersham Pharmacia Biotech).

Plasmid Construction and Transfection into SHIN-3. The cDNA of PTEN was cloned by PCR using human liver cDNA library (Stratagene, La Jolla, CA) as a template, and the primers were described previously (9). Cloned PTEN cDNA was inserted into the Smal site of pCMV-IRE-s-bsr vector (10). pCMV-PTEN-RES-bsr and the control vector pCMV-LUC-RES-bsr (10) encoding LUC were transfected into SHIN-3.
cells by the standard calcium phosphate precipitation method (11). In our previous experiments, we confirmed that gene introduction using pCMV-LUC-IRES-bsr does not affect cell growth, migration, invasion, anticancer drug sensitivity, or radiosensitivity (12, 13). The cells were selected with the concentration of 10 μg/ml blasticidin S hydrochloride (Funakoshi, Tokyo, Japan). Resistant clones were obtained after 4 weeks as SHIN-3/PTEN and SHIN-3/LUC. The cells were subsequently maintained in the presence of 10 μg/ml blasticidin S hydrochloride.

**Western Blotting.** SHIN-3, SHIN-3/LUC, and SHIN-3/PTEN, 5 × 10⁵ cells each, were plated in 3.5-cm plastic dishes and cultured in 10% serum-supplemented DMEM. After 24 h, the cells were washed with PBS and were dissolved in 100 ml of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 100 IU/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride]. Protein concentrations were measured using the Bio-Rad (Veemendaal, the Netherlands) protein assay kit. Western blotting was performed under standard procedures (14) using anti-PTEN polyclonal antibody (N-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the ECL Detection System (Amersham Pharmacia Biotech).

**Cell Growth Kinetics.** SHIN-3, SHIN-3/LUC, and SHIN-3/PTEN, 1 × 10⁵ cells each, were plated in 3.5-cm plastic dishes and cultured in 10% serum-supplemented DMEM. Every 24 h, the cells were dislodged using 0.05% trypsin-EDTA and counted by a hemocytometer.

**Colorimetric Assay.** The sensitivity of SHIN-3/LUC and SHIN-3/PTEN to four chemotherapeutic agents: cisplatin, paclitaxel (the above two drugs: Bristol-Myers Squibb Co., Ltd., Tokyo, Japan), SN-38 (active metabolite of irinotecan; Daiichi Pharm., Tokyo, Japan), and gemcitabine (Eli Lilly, Kobe, Japan) was investigated by colorimetric assay using Cell Proliferation Kit II (XTT; Boehringer Mannheim Biochemica, Mannheim, Germany). SHIN-3/LUC and SHIN-3/PTEN were exposed to each drug at concentrations of 1–10⁵ nM for 72 h. The viable cell count measured by colorimetric assay was presented as a percentage ratio to the count of the control untreated with anticancer drugs. A dose-response curve was prepared, and the 50% growth-inhibitory concentration (IC₅₀) was obtained for each anticancer drug.

**Analysis of SN-38-induced Apoptosis.** SHIN-3/LUC and SHIN-3/PTEN were treated with SN-38 at a dose of IC₅₀ (800 nM). To assess apoptosis morphologically, Hoechst 33258 (Molecular Probes, Inc., Eugene, OR) staining was performed as described by Oberhammer et al. (15). Over 2000 cells were counted in each experiment. The AI was defined as: AI (%) = 100 × apoptotic cells/total cells.

**Assessment of AKT Phosphorylation.** AKT phosphorylation was assayed by Western blotting. Cell lysates were immunoprecipitated with an anti-AKT antibody (New England Biolabs, Inc., Beverly, MA) for probing with the same antibody. Membrane was then stripped, reprobed for phospho-AKT (Ser473; New England Biolabs), and visualized again by ECL.

**Assessment of Topo-I Activity.** Topo-I activity was assayed by relaxation of superhelical plasmid DNA described by Liu and Miller (16). DNA relaxation activity was evaluated by minimum amount of nuclear extract that showed the relaxed form completely.

**Statistical Analysis.** Each experiment was performed in triplicate. All of the significances of difference were analyzed by unpaired Student’s t test and ANOVA. P < 0.05 was defined as statistically significant.

**RESULTS**

**PTEN Sequence Analysis in SHIN-3 Cells.** Mutations in the PTEN gene were not found in SHIN-3 in any of the nine coding exons.

**Expression of PTEN in SHIN-3 Cells.** The PTEN expression vector pCMV-PTEN-IRES-bsr and the control vector pCMV-LUC-IRES-bsr were transfected into SHIN-3 cells. As shown in Fig. 1, PTEN expression was detected by Western blotting at the position corresponding to a molecular weight of Mₚ 55,000.

**Cell Growth Kinetics.** The growth curves of SHIN-3, SHIN-3/LUC, and SHIN-3/PTEN are shown in Fig. 2, showing no significant difference among the three groups. Therefore, enhanced expression of the PTEN did not affect the cell growth in vitro.
Chemosensitivity. The IC\textsubscript{50} (nM) of each anticancer drug on SHIN-3/LUC and SHIN-3/PTEN obtained from colorimetric assay are shown in Table 1. The IC\textsubscript{50} for SN-38 in SHIN-3/PTEN was 800 nM, a 6.6-fold higher sensitivity compared with that of the control (5300 nM). Therefore, enhanced expression of the PTEN increased sensitivity to SN-38 in SHIN-3.

SN-38-induced Apoptosis. The Hoechst 33258 staining showed that AI 24 h after SN-38 exposure was significantly greater in SHIN-3/PTEN (16.6\%\,H_1\,100\,0.7\%) than in SHIN-3/LUC (8.6\%\,H_1\,100\,0.9\%; \textit{P} < 0.01; Fig. 3). Therefore, enhanced expression of PTEN increased apoptosis induced by SN-38.

AKT Phosphorylation. AKT phosphorylation in SHIN-3/LUC and SHIN-3/PTEN was assessed by immunoblotting with anti-phospho-AKT. As shown in Fig. 4, the level of AKT phosphorylation was not significantly different between SHIN-3/LUC and SHIN-3/PTEN. Therefore, the enhanced expression of PTEN did not affect the level of AKT phosphorylation.

Topo-I Activity. The minimum amount of extracted nuclear protein showing complete DNA relaxation was greater in SHIN-3/PTEN (200 ng) than in SHIN-3/LUC (50 ng; Fig. 5). Therefore, enhanced expression of PTEN suppressed Topo-I activity.

DISCUSSION

In this study, we transfected \textit{PTEN} cDNA into an ovarian cancer cell line SHIN-3 and established a cell line (SHIN-3/PTEN) with high expression of PTEN to investigate its function. We confirmed that compared with control cells, SHIN-3/PTEN showed no difference in \textit{in vitro} cell growth, \textit{i.e.}, the high expression of PTEN did not influence the growth of ovarian cancer cells. However, SHIN-3/PTEN exhibited high sensitivity to the irinotecan metabolite (SN-38), indicating that the high expression of PTEN enhances the sensitivity of ovarian cancer cells to irinotecan. Several studies that involved transfecting \textit{PTEN} into cell lines with \textit{PTEN} abnormalities have reported that PTEN enhances the sensitivity of cancer cells to anticancer agents such as doxorubicin and paclitaxel (5, 6). Because it has been reported that mutations in the \textit{PTEN} gene were not found in any of the 50 primary ovarian cancers or 11 immortalized ovarian cancer cell lines (17), in this study, we used the ovarian cancer cell line SHIN-3 having no \textit{PTEN} mutations as a clinical ovarian cancer model. However, to make the experimental results more convincing, it would be useful to introduce \textit{PTEN} into cell lines with \textit{PTEN} abnormalities and, conversely, to observation of change of sensitivity of ovarian cancer cells that did knock out \textit{PTEN}.

Although the mechanisms of the irinotecan sensitivity-enhancing function of PTEN remains to be elucidated, we suggest the following two possibilities. The present finding that SHIN-3/PTEN showed a higher apoptotic index after irinotecan administration compared with that in controls suggests the in-

<table>
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<th>Table 1</th>
<th>The 50% growth-inhibitory concentration (IC\textsubscript{50}) value (nM) of each anticancer drug on SHIN-3/LUC and SHIN-3/PTEN obtained from colorimetric assay</th>
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<td></td>
<td>SN-38</td>
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<td>SHIN-3/LUC</td>
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<td>SHIN-3/PTEN</td>
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Fig. 3 Photomicrographs of SHIN-3/LUC and SHIN-3/PTEN stained with Hoechst 33258 to evaluate apoptosis after treatment for 24 h with SN-38. The number of apoptotic cells was greater in SHIN-3/PTEN than in SHIN-3/LUC. Arrows, apoptotic cells. ×100.

Fig. 4 AKT phosphorylation in SHIN-3/LUC and SHIN-3/PTEN. Lysates were immunoprecipitated with AKT and immunoblotted with anti-AKT (upper panel) or anti-phospho-AKT (lower panel). There was no difference in AKT phosphorylation between the two groups.

Fig. 5 Topo-I catalytic activity of SHIN-3/LUC and SHIN-3/PTEN. The minimum amount of extracted nuclear protein showing complete DNA relaxation was greater in SHIN-3/PTEN (200 ng) than in SHIN-3/LUC (50 ng).
duction of apoptosis by PTEN as a mechanism of sensitivity enhancement. For the pathway of apoptosis induction associated with PTEN, a pathway via AKT may be a candidate. It has been reported that activated AKT has the function of suppressing apoptosis, and that PTEN suppresses the activation of AKT. Thus, the high expression of PTEN induces the cell to undergo apoptosis by suppressing AKT activity (18). However, because the high expression of PTEN did not suppress the phosphorylation of AKT in this study, we postulate a pathway that does not involve AKT. The following apoptotic pathways other than that of AKT are possible. Matsushima-Nishiu et al. (19) introduced PTEN into endometrial carcinoma cells, analyzed them using cDNA microarrays, and found abnormal expression of genes related to the apoptosis induction-related tumor necrosis factor signaling pathway (20) and the Notch signaling pathway (21). Thus, these pathways may be related to apoptosis accelerated by PTEN. The second possibility is a signaling pathway (21). Thus, these pathways may be related to tumor necrosis factor signaling pathway (20) and the Notch signaling pathway (21).

Irinotecan suppresses Topo-I activity and exerts a cell-killing effect by forming stable Topo-I-DNA-cleavable complexes (22, 23). Because high PTEN expression and irinotecan presumably act through the suppression of Topo-I, the two may have functioned synergistically.

Of the above two possible mechanisms for the enhancement of SN-38 sensitivity by PTEN, the latter Topo-I inhibition is considered to predominate for the following reasons: (a) PTEN overexpression had little effect on sensitivity to anticancer drugs other than SN-38; (b) the observed difference in apoptotic index may simply reflect a difference in SN-38 sensitivity, because the survival rate of SHIN-3/PTEN after contact with the apoptosis-inducing 800 nm SN-38 was 50%, whereas that of SHIN-3/LUC with low SN-38 sensitivity was calculated at 84%.

Irinotecan is reportedly effective for patients with refractory or recurrent ovarian cancer who have previously undergone platinum-based combination therapy and has attracted attention as an anticancer agent for second-line chemotherapy (24). The present study showed that the high expression of PTEN had the function of enhancing the sensitivity of ovarian cancer cells to irinotecan.

In this study, the overexpression of PTEN did not affect cancer cell growth. However, Minaguchi et al. (9) reported that overexpression of PTEN inhibited ovarian cancer cell growth. An increased copy number of the PIK3 gene has been observed in some ovarian cancer cases (25). PIK3 phosphorylates PIP3 and promotes cell growth (26, 27). PIP3 is also one of the specific substrates of PTEN (27), and overexpression of PTEN may inhibit cell growth by dephosphorylation of PIP3. In the SHIN-3 cell line used in this study, PIK3 may not be amplified, which may be the reason why the cell growth was not inhibited. The inhibitory effect of PTEN on cell growth may be observed only in ovarian cancers carrying amplified PIK3.

Many fundamental studies (28) and several clinical trials (29, 30) of therapy with the tumor suppressor gene p53 have been conducted. Clinical trials have been performed in patients with tumors carrying p53 mutations. However, mutant p53 has been reported to inhibit the function of wild-type p53 (31); therefore, the therapeutic effect is limited in patients with these tumors. In contrast, gene therapy with PTEN for ovarian cancer may be promising, because mutant PTEN is rare in clinical tumors. Thus, our attempt to create a tumor cell line overexpressing a mutation-free tumor suppressor gene with resultant higher antitumor drug sensitivity has the potential for a strategy of gene therapy with a tumor suppressor gene.

This study showed that PTEN overexpression enhances the sensitivity of ovarian cancer cells to SN-38 and suggest the inhibition of Topo-I activity in the cancer cells by PTEN overexpression as a mechanism for this enhancement. The interactions between PTEN and Topo-I are still not clear, and there are no reports concerning the relationship. Although its mechanism is unknown, the use of the PTEN transfectants established by us may facilitate its elucidation and subsequent discovery of a new function of PTEN.

REFERENCES


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