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PTEN/MMAC1 enhances the growth inhibition by anticancer drugs with downregulation of *IGF-II* expression in gastric cancer cells

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Abbreviation: IGF, insulin-like growth factor; IGF-IR, insulin-like growth factor-I receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphinyltetrazolium bromide; PI3-kinase, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-PCR

Abstract

PTEN/MMAC1 is a tumor suppressor gene that is mutated in a variety of advanced and metastatic cancers. Its major function is likely to be the phosphatase activity that regulates the phosphotidylinositol (PI)3-kinase/ Akt pathway. On the other hand, IGF system plays an important role in cell proliferation and cell survival *via* PI3-kinase/Akt and mitogen-activated protein kinase pathways in many cancer cells. To evaluate effect of *PTEN* on cell growth and IGF system in gastric cancer, human gastric adenocarcinoma cells (SNU-5 & -216) were transfected with human PTEN cDNA. Those PTEN-transfected gastric cancer cells had a lower proliferation rate than the pcDNA3-transfected cells. PTEN overexpression induced a profound decrease in the IGF-II and IGF-IR expression levels, and downregulation of *IGF-II* expression by PTEN was mediated through the regulation of the IGF-II promoter. In addition, a PI3-kinase inhibitor, LY294002, induced the downregulation of IGF-II expression. The PTEN-overexpressing SUN-5 and -216 cells were more sensitive to death induced by etoposide and adriamycin that induce DNA damage than the *pcDNA3*-transfected cells. These findings suggest that PTEN suppresses the cell growth through modulation of *IGF* system and sensitizing cancer cells to cell death by anticancer drugs.

Keywords: genes, tumor supprressor; insulin-like growth factor II; receptor, IGF type I; stomach neoplasms

Introduction

There is a high frequency of mutations in the tumor suppressor gene encoding *PTEN* in many primary human cancers and several familial cancer predisposition disorders (Liaw *et al.,* 1997; Teng *et al.,* 1997; Steck *et al.,* 1999). *PTEN* contains a sequence motif that is highly conserved in members of the protein tyrosine phosphatase family (Furnari *et al.,* 1997; Maehama *et al.,* 1998). Many cancer-related mutations have been mapped within the conserved catalytic domain of *PTEN*, suggesting that the phosphatase activity of *PTEN* is essential for the tumor suppressor function. In addition, wild-type *PTEN*, but not the mutant derivatives lacking phosphatase activity, suppresses the growth of glioblastoma cells and their tumorigenecity (Li *et al.,* 1988), which confirms the functional relevance of the *PTEN* phosphatase domain for tumor suppression.

Insulin-like growth factor *(IGF)-I* and *-II* are potent mitogens in many cell types in autocrine, paracrine and endocrine pathways (Daughaday *et al.,* 1989). The biological effects of *IGFs* are specifically mediated by binding to the cell-surface *IGF-I receptor (IGF-IR)* and activating the receptor tyrosine kinase (LeRoith *et al.,* 1995). Many tumors including gastric cancer overexpress *IGF-II*, *IGF-IR* and specific *IGFBPs* (Macualay, 1992; Yi *et al.,* 2001). *IGF-II* expression results in an autocrine feedback loop activating *IGF-IR* and stimulating cancer cell proliferation (Chen *et al.,* 1998; Sung *et al.,* 2003).

An Akt pathway, which is known to induce cellular transformation, is activated by the growth factors involved in angiogenesis and metastasis, such as platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor-I (Hemmings, 1997). Recent studies have shown that, *PTEN* inhibits the downstream function mediated by the phosphatidylinositol 3 (PI3)-kinase/Akt pathway, such as activation of protein kinase B (PKB, also known as Akt),

cell survival and cell proliferation, presumably through dephosphorylation of phosphatidylinositol 3,4, 5-triphosphate *in vitro* (Maehama *et al.*, 1998). There are some evidences suggesting a possible connection between *PTEN* and the *IGF* system (Davies *et al.*, 1998; Stambolic *et al.*, 1998). Since *IGF* promotes cell proliferation and cell survival through the PI3 kinase pathway, *PTEN* can directly modulate the *IGF*-induced activation of PI3 kinase, thereby inhibiting the *IGF*-Induced cell proliferation. Recently, Zhao *et al.*, (2004) have reported that *PTEN* inhibits cell proliferation and induces apoptosis by downregulating *IGF-IR* expression.

This study has found that *PTEN* inhibits the cell growth of gastric cancer cells with the downregulation of *IGF-II* and *IGF-IR* expression. In addition, *PTEN* overexpression results in the increased sensitivity of tumor cells to anticancer drugs.

Materials and Methods

Materials

The cell culture media (RPMI-1640) were purchased from Life Technologies Inc (Grand island, NY). The etoposide, adriamycin, and LY294002 were purchased from Sigma (St. Louis, MO). Polyclonal anti-*IGF-IR* and monoclonal anti-*PTEN* antibodies, and all secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell lines and cell culture

Two established human gastric adenocarcinoma cell lines from Korean (SNU-5 and-216) were purchased from the Korea Cell Line Bank (Seoul, Korea). The cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), 300 μg/ml L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Construction of *PTEN* plasmids, transfections

The *PTEN* plasmid was constructed as previously described (Hwang *et al.*, 2001). The *pcDNA3/PTEN* plasmid was transfected to SNU-5 and SNU–216 cells using a calcium phosphate method (Promega, Madison, WI) according to the manufacturer's protocol. To obtain *PTEN* expressing cells, cells were cultured in 400 μg/ml G418 (Life Technologies, Grand Island, NY) for 2 weeks and three single colonies were selected using glass-cloning rings directly on plate and used for further chracteristics. Cells from clone #1 of SNU-5 and clone #3 of SNU-216 were analyzed for their expression of *PTEN* by Western blot analysis.

MTT assay

Growth of SNU-5 (clone #1) and SNU-216 (clone #3) cells transfected with empty vector or wild-type *PTEN* and cell growth inhibition after the treatment of anticancer drugs was evaluated by MTT assay as previously described (Lee *et al.*, 2002). In brief, SNU-5, SNU-216 cells carrying either the empty vector *pcDNA3* or *pcDNA3/PTEN* were plated in 10% FBS-RPMI at a density of 1×10^5 cells/well in 12-well plates until 60% confluency and changed to media for 12 h, and then maintained in media in the presence of etoposide (10 μM) or adriamycin (10 μM). After the indicated periods of culture, 1 ml (2 mg) of MTT solution was added to each well and incubated for 4 h. Then, plates were centrifuged, and DMSO was added, and the plates were vigorously shaken to solubilize the MTT-formazan products. Absorbance at 540 nm was measured by spectrophotometer.

Protein preparation and Western blot analysis

Protein was extracted from cultured SNU-5 and SNU-216 cells transfected with empty vector *pcDNA3* or *pcDNA3/PTEN* using lysis buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tri-HCl (pH 8.0), 1%-NP 40, 1 mM aprotinin, 0.1 mM leupeptin, and 1 mM pepstatin and quantified based on the Bradford dye-binding procedure (Bio-Rad) as described previously (Hwang *et al.*, 2001). Total proteins (10 μg) were electrophoresed in 8% SDS-polyacrylamide gels under reducing conditions and electroblotted onto nitrocellulose membranes. The membranes were blocked using 5% skim milk in PBS, and then incubated with the primary antibodies at 1:1,000 dilution for 2 h. The proteins were visualized using the enhanced chemiluminescence (NEN, Boston, MA).

RNA extraction and RT-PCR

Total RNA was extracted from cultured SNU-5 and SNU-216 cells transfected with empty vector *pcDNA3* or *pcDNA3/PTEN* using Trizol (Life Technologies) according to the manufacturer's instructions. Reverse transcription (RT) and agarose gel electrophoresis were performed as previously described (Lee *et al.*, 2002). Primer sequences were: 1) *IGF-I* receptor sense, 5'-TTG CCC GAA GGT CTG TGA-3'; antisense, 5'-CCC GTT GTT CCT GGT GTT-3'; 2) *IGF-II* sense, 5'-CGA TGC TGG TGC TTC TCA-3'; antisense, 5'-GGG GTC TTG GGT GGG TAG-3'. In PCR amplification step, a standard cDNA for *IGF-II*, in which 113 base pairs had been removed from the *IGF-II* PCR product, was added and PCR amplification was carried out as described above.

Luciferase assay

The −574 to +136 region of the *IGF-II* promoter, which was a generous gift from Dr. Charles Robert, Jr. (Dept. of Pediatrics, OHSU, Portland, Oregon) was recombined into the pGL2-basic vector (Promega). The *pcDNA3/PTEN* and *IGF-II* promoter-pGL2-basic vectors were co-transfected into the SNU-5 cells using a calcium phosphate precipitation method (Promega). After 72 h transfection, the cells were washed with PBS and lysed using a reporter lysis buffer (Promega) according to the manufacturer's protocol. The luciferase activity was measured with a Lumat LB 96P luminometer (Berthold Systems, Aliquippa, PA).

DNA fragmentation and nuclear condensation assay

Cells transfected with the *pcDNA3* or *PTEN* (1×10^6) were cultured in 150 mm culture dish until they reached 90% confluence and then treated with etoposide (10 μM) for 24 h. After the etoposide treatment, the cells were washed with cold PBS, and DNA fragmentation assay was performed as described previously (Yi *et al.*, 2002). For nuclear condensation assay, the *pcDNA3* or *PTEN*-transfected cells were incubated in a 6 well plate with or without 10 μM etoposide for 24 h. After washing with PBS, cells were fixed with methyl alcohol/acetic acid, and stained with Hoechst 2495 solution (Sigma) as previously described (Yi *et al.*, 2002).

Statistical analysis

The results are expressed as the mean ± S.E. Statistical analysis of the data was performed using a Student's *t* test, and *P* values of < 0.05 were considered significant.

Results

Stable transfection of *PTEN* and growth of *PTEN*-transfected gastric cancer cells

To characterize role of *PTEN* in gastric cancer, we stably overexpressed *PTEN* in SNU-5 and -216 gastric cancer cells. As shown in Figure 1, *PTEN*-transfected SNU-5 and -216 cells produced high levels of 54 kDa of *PTEN* protein, which showed only a weak signal in *pcDNA3*-transfected cells at 72

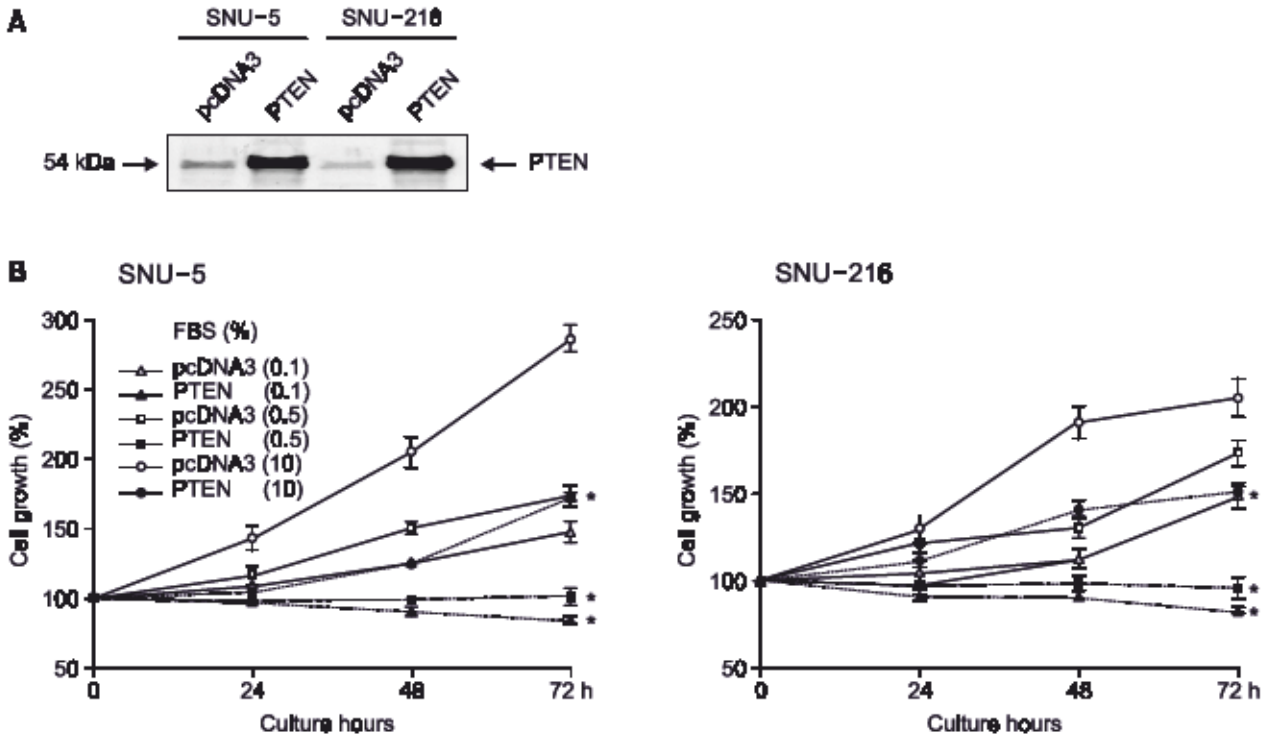

Figure 1. Expression of *PTEN* protein and effect of *PTEN* overexpression on cell growth. (A) Total proteins from the cell lysates of the stable SNU-5 and SNU-216 cells transfected with empty vector *pcDNA3* or *PTEN* were extracted and subjected to immunoblotting as described in the Materials and Methods. (B) Stable cells transfected with the *pcDNA3* or *PTEN* were seeded into 24 well plates at 1×10^5 cells per plate and were grown under various FBS concentration, over a 72 h period. At the indicated times, the number of viable cells was determined by a MTT assay and is presented as the percentage of cell growth. The results are a mean ± S.E. of three separate experiments performed in duplicate wells. *$P < 0.05$ compared with the *pcDNA3*-transfected cells.

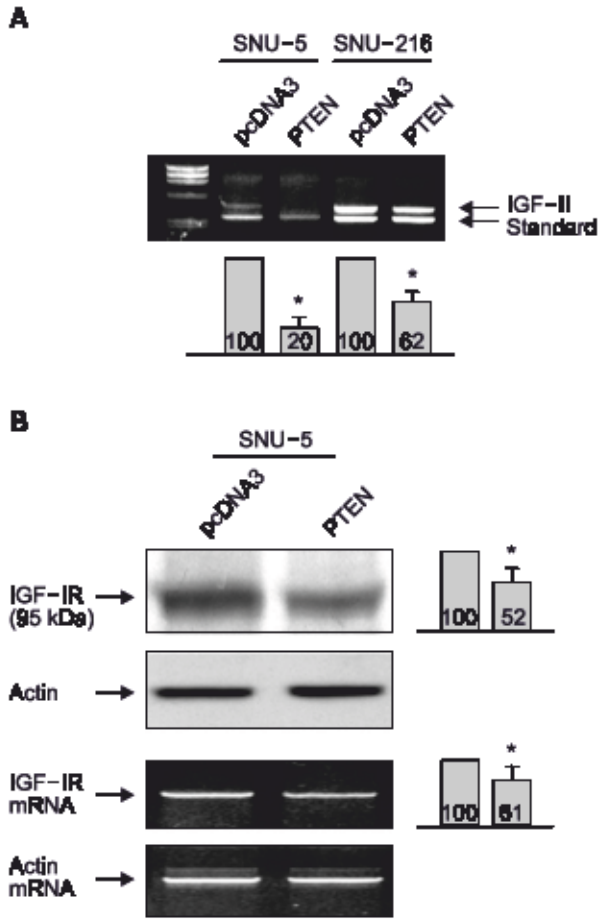

Figure 2. Effect of *PTEN* overexpression on *IGF-II* and *IGF-I* receptor expressions. Stable cells transfected with the *pcDNA3* or *PTEN* were grown for 3 days. (A) Total RNA of each clone was extracted and analyzed by competitive-PCR, as described in the Materials and Methods. The bar graphs were generated from the densitometric analysis of the *IGF-II* mRNA level from three separate experiments, and the results represent a mean ± S.E. **P* < 0.05 compared with the *pcDNA3*-transfected cells. (B) Total RNA and protein of each clone were extracted and analyzed by RT-PCR and immunoblotting with anti-*IGF-IR* antibody. *IGF-IR* mRNA levels were normalized to the corresponding *actin* mRNA levels. **P* < 0.05 compared with the *pcDNA3*-transfected cells.

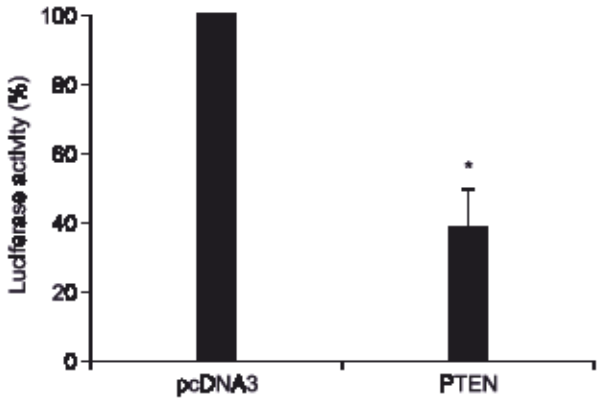

Figure 3. Effect of *PTEN* overexpression on *IGF-II* promoter activity. *pcDNA3/PTEN* and *IGF-II* promoter-pGL2-basic vectors were transiently cotransfected into the SNU-5 cells. Cell extracts were prepared after 72 h transfection and assayed for their luciferase activity as described in the Materials and Methods, and the results are presented as the percentage luciferase activity. The results are presented as a mean ± S.E. of three separate experiments performed in duplicate wells. **P* < 0.05 compared with the *pcDNA3*-transfected cells.

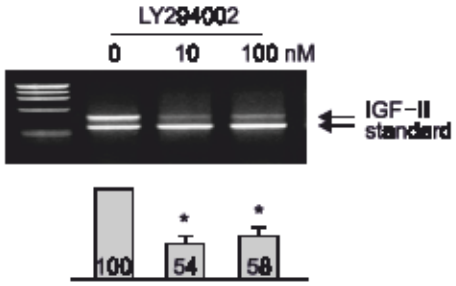

Figure 4. Effect of LY294002 on *IGF-II* expression. Stable SNU-5 Cells were grown until they were 90% confluent and incubated in the absence or presence of 10 or 100 nM LY294002 for 24 h. Total RNA was extracted and analyzed by competitive-PCR. The Bar graphs are generated from densitometric analysis of the *IGF-II* mRNA levels from three separate experiments, and results represent the mean ± S.E. **P* < 0.05 as compared with the cells without LY294002 treatment.

h after transfection.

Because *PTEN* has been characterized as a tumor suppressor, this study examined the effect of *PTEN* overexpression on growth of the gastric cancer cells. Growth rate of the *PTEN*-overexpressing SNU-5 cells was considerably lower than that of the *pcDNA3*-transfected cells with a maximum 2-fold reduction at 72 h after transfection regardless of FBS concentrations ranging from 0.1 to 10% (Figure 1B, $P < 0.05$). The growth pattern of the *PTEN*-overexpressing SNU-216 cells was similar to that of the *PTEN*-overexpressing SNU-5 cells. However, the growth inhibition of SNU-216 cells was more prominent in the low FBS concentration (0.1 and 0.5%), compared with the SNU-5 cells (Figure 2, $P < 0.05$).

Downregulation of *IGF-II* and *IGF-IR* by *PTEN*

Because *IGFs* play an important role in tumorigenesis and tumor progression in a variety of tumors and are potent mitogens in cancer cells, effects of *PTEN* on the expression of *IGF-II* and *IGF-IR* in gastric cancer cells were examined. As shown in Figure 2A and B, *IGF-II* and *IGF-IR* were expressed in the *pcDNA3*-transfected SNU-5 and −216 cells. However, in the *PTEN*-overexpressing SNU-5 cells, *IGF-II* expression was significantly reduced by up to 5-fold ($P < 0.05$). In addition, *IGF-IR* mRNA and

protein expressions were significantly reduced by *PTEN* overexpression in the SNU-5 cells (Figure 3B, $P < 0.05$).

In order to address a molecular mechanism of *PTEN* activation in *IGF-II* expression, this study investigated whether *PTEN* activates the promoter of the *IGF-II* gene. The *IGF-II* promoter activity was decreased up to half after being transfected with *PTEN*, compared with that of the *pcDNA3*-transfected cells (Figure 3, $P < 0.05$). This result suggests that *PTEN* overexpression induces a reduction in *IGF-II* expression at transcriptional level. Furthermore, there was a significant decrease in the *IGF-II* expression level as a result of a treatment with LY29002, PI3-kinase inhibitor (Figure 4, $P < 0.05$). This result suggests that *PTEN* downregulates *IGF-II* expression *via* Akt-mediated pathway in gastric cancer cells.

Sensitization of cancer cells to anticancer drug by *PTEN*

In order to examine whether growth inhibitory effect of anticancer drugs is enhanced by *PTEN*, this study investigated the antiproliferative effects of etoposide and adriamycin in *PTEN*-overexpressing SNU cells. Following a 10 μM etoposide treatment for 72 h, the growth of *PTEN*-overexpressing the SNU-5 cells was inhibited more than approximately 3-fold compared with that of the *pcDNA3*-transfected cells (Figure 5, $P < 0.05$). A similar growth inhibitory effect was observed with the 10 μM adriamycin treatment ($P < 0.05$).

DNA fragmentation and nuclear condensation assays were performed to determine whether the enhancement of cellular death in the *PTEN*-overexpressing gastric cancer cells was due to apoptosis. As shown in Figure 6A, treatment with 10 μM etoposide for 24 h induced nucleosomal DNA fragmentation in the *PTEN*-overexpressing SNU cells but not in the *pcDNA3*-transfected cells. In addition, when the SNU-5 cells were treated with 10 μM etoposide for 24 h, condensed and fragmented nuclei were observed in the *PTEN*-overexpressing SNU-5 cells only (Figure 6B, lower right panel). Overall, these results suggest that *PTEN* overexpression results in an increased sensitivity of SNU cells to anticancer drugs due to the enhancement of the apoptotic pathway.

Discussion

Gastric cancer is one of the leading causes of cancer death throughout the world, although its incidence has declined in many countries. The etiology of gastric cancer is still unclear. However, the remarkable technical advances in molecular biology seen in recent years have enhanced the understanding of carcinogenesis and the progression of cancer. Normal growth and differentiation of cells in the gastrointestinal tract are regulated by autocrine and paracrine secretion of peptide growth factors which are responsible for controlling maturation, differentiation and apoptosis (Moss, 1998; Park *et al.*, 2005). In gastric cancer, where there is unrestricted growth, it is likely that there are abnormalities of secretion or response to those peptides. IGFs and their receptors may be important in the regulation of epithelial cell growth, but few data are available on

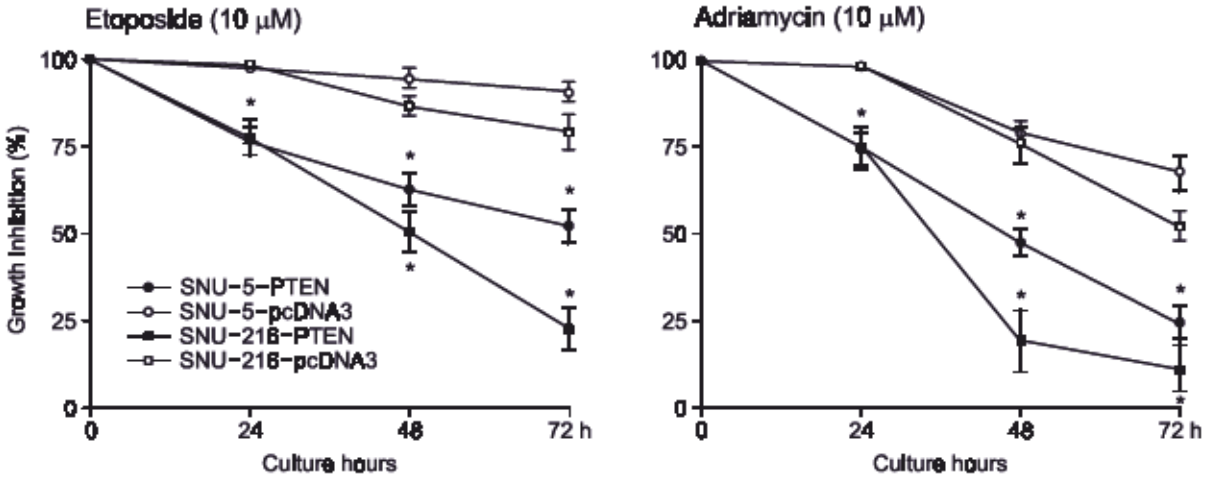

Figure 5. Effect of *PTEN* overexpression on cellular sensitivity to anticancer drug. Stable cells transfected with the *pcDNA3* or *PTEN* were grown until they reached 60% confluence, and were incubated in the presence of 10 μM etoposide or adriamycin over a 72 h period. After the indicated period of time, the level of growth inhibition was measured by a MTT assay, and is presented as the percentage of growth inhibition. The results are the mean ± S.E. of three separate experiments performed in triplicate wells. *$P < 0.05$ as compared with the *pcDNA3*-transfected cells.

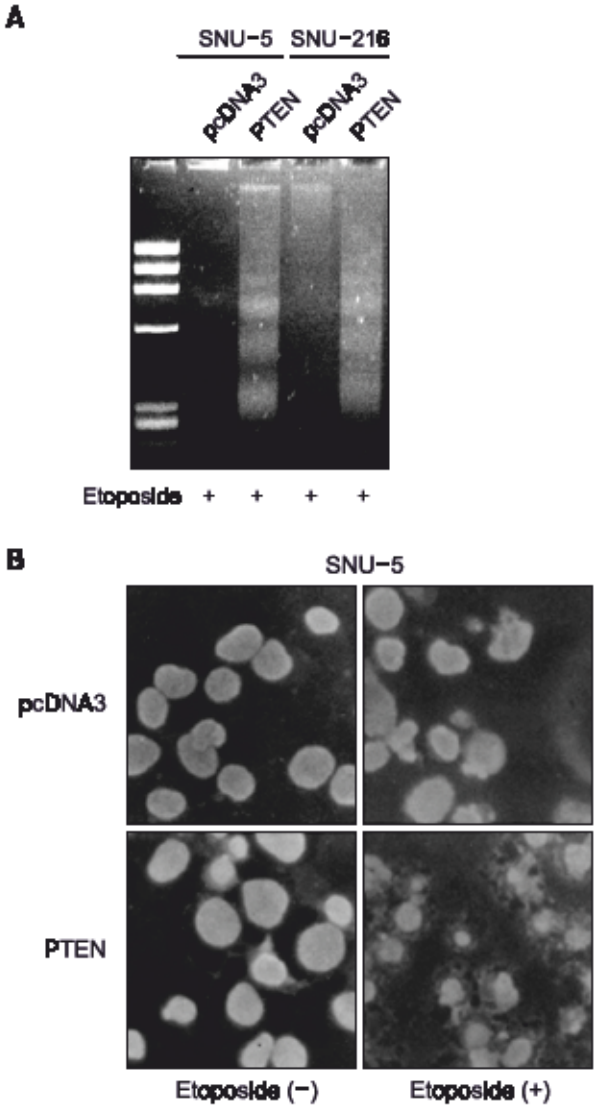

Figure 6. Effect of *PTEN* overxpression on apoptosis. The stable cells transfected with the *pcDNA3* or *PTEN* were grown until they were 90% confluent and incubated in the presence or absence of 10 μM etoposide for 24 h. (A) DNA fragmentation assay: genomic DNA was extracted, separated on a 1.5% agarose gel, and stained with ethidium bromide, as described in Materials and Methods. A representative data of three separate experiments is shown. (B) Nuclear condensation assay: fixed cells on the slide were observed under a fluorescence microscope, as described in Materials and Methods.

the expression and biological function of the IGF system in gastric cancer. The *IGF-II* gene plays an important role in lymph vessel permeation especially in expanding-type gastric cancers (Shiraishi *et al.,* 1998). Pavelic and colleagues (Pavdic *et al.,* 2003) have reported that increased expression of *IGF-II* and *IGF-IR* genes is found in gastric cancer as compared with non-tumor tissue. Furthermore, there is a significant difference between *IGF-II* expression in the more aggressive diffuse type of gastric cancer than in the intestinal type.

In this study, we have found that overexpression of *PTEN* in a well-documented gastric cancer cell line inhibits cell growth and causes downregulation of *IGF-II* and *IGF-IR* expression. Furthermore, *PTEN* overexpression sensitizes these cells to the cytotoxic effect of anticancer drugs. *PTEN* is frequently deleted or mutated in a wide range of human tumors and tumor cell lines such as a glioblastoma, melanoma and gastric cancers, suggesting that *PTEN* plays important roles in tumorigenicity (Teng *et al.,* 1997; Chang *et al.,* 1999; Yi *et al.,* 2001). It has previously been reported that *PTEN* inhibits cell growth in glioma and melanoma cells, and its major function is likely to be the phosphatase activity of *PTEN* that regulates the PI3 kinase/Akt pathway, which is a survival pathway (Li *et al.,* 1988; Furnari *et al.,* 1997). In this study, the results demonstrate that *PTEN*-overexpressing gastric cancer cells exhibits a lower proliferation rate than the *pcDNA3*-transfected cells. This suggests that *PTEN* plays an important role in growth of gastric cancer cells.

Insulin-like growth factors, *IGF-I* and *-II*, are important mitogens in a number of different cell types (Daughaday *et al.,* 1989). *IGF-II*, a mitogenic peptide necessary for normal fetal growth, is overexpressed in a number of malignant human tumors including gastric cancer (Wang *et al.,* 1998; Yi *et al.,* 2001). Consistent with the role of *PTEN* in the PI3/Akt signaling pathway, several lines of evidence suggest that *PTEN* regulates *IGF-IGF-IR*-induced Akt activity, thereby modulating *IGF*-mediated cell proliferative and antiapoptotic effects in a variety of cells (Davies *et al.,* 1998; Stambolic *et al.,* 1998). Recent studies have demonstrated that *PTEN* downregulates *IGF-II* and *IGF-IR* expression in hepatoma and prostate cancer cells, respectively, suggesting that antiproliferative effects of *PTEN* is, at least in part, mediated through the regulation of expression of components of the *IGF* system (Kang-Park *et al.,* 2003; Zhao *et al.,* 2004). Our reslts have shown that *PTEN* overexpression results in a marked reduction in *IGF-II* and *IGF-IR* expression in gastric cancer cells, and the downregulation of *IGF-II* is due to the transcriptional activation of its promoter. Furthermore, a PI3-kinase inhibitor also induces a marked decrease in *IGF-II* expression. These findings suggest that *PTEN* may regulate *IGF-II* expression via PKB/Akt-dependent pathway in gastric cancer cells. By downregulating *IGF-II* and *IGF-IR* expression, *PTEN* reduces the *IGF/IGF-IR*-induced antiapoptotic signaling.

Variations in chemosensitivity of cells to different anticancer drugs is an important aspect of cancer research, and p53 and p21 have been shown to be

the major determinants of cellular chemoresistance (Wahl *et al.,* 1996; Waldman *et al.,* 1996). Wu *et al.,* (2000) have reported that increased expression of *PTEN* renders the cells more sensitive to apoptotic cell death. In this study, we have observed that cell death caused by etoposide or adriamycin is significantly enhanced in the *PTEN*-overexpressing cells compared with the *pcDNA3*-transfected cells. In addition, it has also been found that the enhancement of cell death by *PTEN* expression is due to apoptosis. This finding suggests that tumor suppressive effect of *PTEN* is mediated, in part, by the enhanced sensitivity towards apoptotic cell death.

In summary, *PTEN* overexpression downregulates *IGF-II* and *IGF-IR* expression levels and sensitizes cancer cells to chemotherapy. This study suggests that *PTEN* suppresses cell growth by 1) inducing apoptosis mediated through the modulation of IGF system, and 2) sensitizing cancer cells to cell death by anticancer drugs toward apoptotic cell death.

Acknowledgement

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