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PTEN augments staurosporine-induced apoptosis in PTEN-null Ishikawa cells by downregulating PI3K/Akt signaling pathway

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Abstract

Staurosporine is a potent apoptosis inducer, but its mechanism remains to be clarified. We investigated the involvement of PTEN in staurosporine-induced apoptosis. Ishikawa cells, from an endometrial carcinoma cell line, expressed a high amount of PTEN mRNA but did not express the PTEN protein because of protein truncations. We isolated clones expressing the steady-state level of the PTEN protein from PTEN-null Ishikawa cells by transfection. The obtained clones showed reduced proliferative activity and reduced anchorage-independent cell growth with the augmented $p27^{Kip1}$. These cell lines were sensitized to apoptosis by staurosporine. A low concentration of UCN-01 did not affect apoptosis, but a high concentration augmented apoptosis in the PTEN-expressing clone. Alpha-sphingosine and H-7 did not affect apoptosis in these cell lines. PI3K inhibition augmented staurosporine-induced apoptosis in the parental cell line, but not in the PTEN-expressing clone. In the clone, phosho-Akt/PKB and phospho-Bad (Ser-136) were down-regulated. Staurosporine reduced the levels of phospho-Akt/PKB and phospho-Bad (Ser-136) in all the cell lines, but the reduction was most significant in the PTEN-expressing clone. These results suggest that inhibition of the PI3K/Akt/PKB signaling pathway might be associated with staurosporine-induced apoptosis in Ishikawa cells.
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Keywords: PTEN gene; endometrial carcinoma cell lines; transfection; growth suppression; staurosporine; apoptosis

Abbreviations: Akt/PKB, protein kinase B; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol-3,4,5-triphosphate

Introduction

PTEN (or MMAC1) is a tumor suppressor located at chromosome 10q23.3.[1,2] The gene is frequently mutated or heterozygously deleted in certain carcinomas. The PTEN protein can function as a protein phosphatase as well as a lipid phosphatase, but its major function as a tumor suppressor is considered to regulate the intracellular concentration of phosphatidylinositol-3,4,5-triphosphate (PIP3) by dephosphorylating its D3 position as a lipid phosphatase.[3] A deficiency of the PTEN protein increases the PIP3 level in cells and causes recruitment of cytosolic proteins such as Akt/PKB protein on the plasma membrane.[4] The translocated Akt/PKB protein undergoes a conformational change, resulting in the exposure of an activation loop that is then phosphorylated by PI3K-dependent serine/threonine kinase (PDK1).[5] Activated Akt/PKB phosphorylates proteins on serine/threonine residues to potentiate cell survival proteins such as Bad, caspase 9, GSK3, $p70^{S6K}$, 4E-BP1/PHAS-1, IKKα and members of the Forkhead transcription family.

PDK1 can mediate activation of atypical PKC (PKC zeta, PKC lambda).[6,7] Thus, deficiency of the PTEN protein can activate both Akt/PKB and some PKC signaling pathways. Furthermore, in mitochondria, a cooperative protection between Akt/PKB and PKC signaling pathways from apoptosis onset has been suggested.[8]

Staurosporine is a non-selective protein kinase inhibitor as well as a potent apoptosis inducer in a broad spectrum of cells. A variety of staurosporine analogs with less toxicity (e.g. UCN-01, 7-hydroxy-staurosporine) have been developed. The property of this kind of agent to induce apoptosis is thought to be deeply associated with its anticancer activity. It has been reported that the PKC signaling pathway is likely associated with staurosporine-induced apoptosis in HT-29 colon carcinoma cells[9] and in human embryonic lung fibroblast (HEL) cells,[10] whereas in rat thymocytes it is probably indirectly involved.[11] Thus, the involvement of PKC in staurosporine-induced apoptosis is still controversial.

Because the PTEN protein is a key enzyme in the Akt/PKB-associated signaling and in the PKC signaling pathways, we analyzed a possible involvement of the PTEN protein in staurosporine-induced apoptosis.

Results

Status of PTEN in the cells

We analyzed the status of PTEN in Ishikawa cells (from an endometrial carcinoma cell line). In Western blot analysis, we could not detect the PTEN protein, but PTEN mRNA was abundantly expressed in Northern blot analysis (Figure 1). To

search for the reason, we studied the copy number of the PTEN transcript and possible mutations of each copy. To screen for possible mutations, PCR–SSCP analysis from genomic DNA was performed. In the SSCP analysis, shifted bands from the control were found only in exon 8. Next, the region spanning exon 8 in the PTEN transcript was amplified by RT–PCR, and the amplified DNA fragment was subcloned into pCR2.1, a TA cloning vector, to analyze copy number and mutations of the transcript. We found that there were two copies of PTEN transcript. One copy lacked 4-nucleotides (TTAC) from codon 318 to codon 319, and the other lacked A in codon 289 (Figure 2). These sequence alterations were compatible with the results of the sequence analyses on the genomic DNA. Either mutation would produce a stop codon adjacent to the mutation site.

Stable transfection of PTEN and characterization

After plasmid transfection and G418 selection, a number of clones resistant to G418 had appeared. We screened 27 clones and obtained four clones expressing the steady-state level of the PTEN protein (PTEN-IK5, PTEN-IK14, PTEN-IK18, and PTEN-IK23). In the clones, the expression level of $p27^{Kip1}$ was increased as compared to that of the parental Ishikawa cells (Figure 3).

Among the clones expressing the PTEN protein, the malignant phenotype including proliferation activity and anchorage-independent cell growth was evaluated. All the clones showed significantly reduced cell growth potential and significantly reduced anchorage-independent cell growth activity as shown in Figure 4.

Apoptosis induction by staurosporine

The involvement of PTEN in staurosporine-induced apoptosis was analyzed. After 48 h exposure to 1 μM of staurosporine, the percentage of apoptotic cells was calculated. It was 23.4% (SD, 4.9) in parental Ishikawa cells, while it was 56.7% (SD, 8.7) in PTEN-IK5, 54.9%

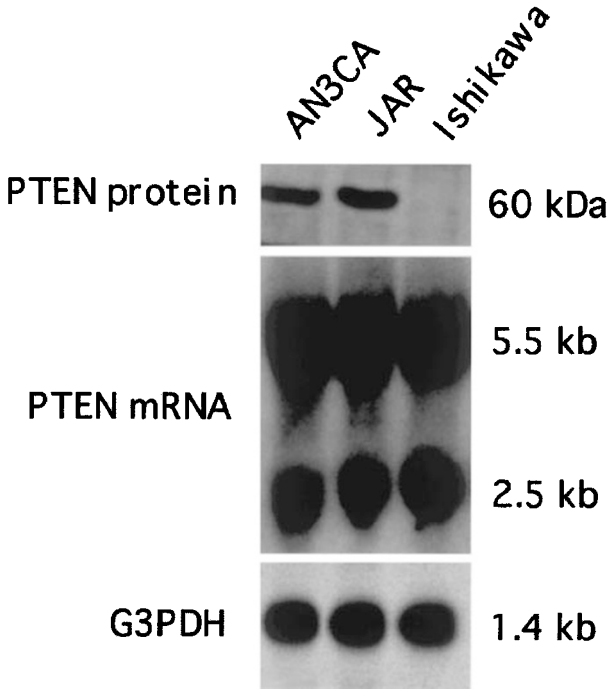

Figure 1 Expressions of PTEN protein and PTEN mRNA in AN3CA, JAR and Ishikawa cells. AN3CA cells and JAR cells express PTEN protein and mRNA, whereas Ishikawa cells do not express PTEN protein in spite of abundant expression of PTEN mRNA

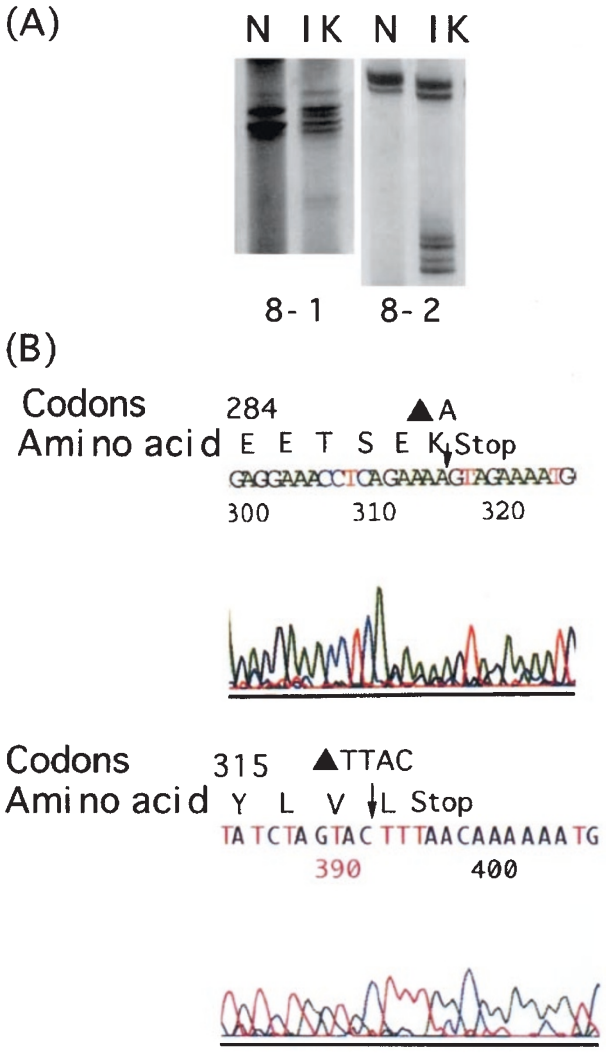

Figure 2 (**A**) SSCP analysis of PTEN exon 8 of Ishikawa cells. As a control, normal endometrium was used. Exon 8 of the PTEN gene (225 nucleotides long) was divided into 2 parts (8-1, 8-2) for the analyses. N, normal endometrium; IK, Ishikawa cells. (**B**) Sequence analysis of the PTEN transcript. Five hundred and sixty nucleotides spanning exon 8 were amplified by RT–PCR. PCR products were subcloned into pCR2.1, a TA cloning vector. The sequence of the insert was analyzed in 10 clones. One copy deletes A in codon 289; subsequently the expected amino acid sequence from the mutation site is changed into 'K and a stop signal'. The other copy deletes TTAC from codon 318 to codon 319; therefore the expected amino acid sequence is changed into 'L and a stop signal'

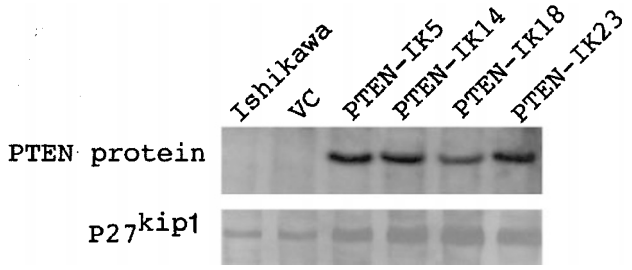

Figure 3 Western blot analyses of PTEN protein and $p27^{kip1}$ protein expression. VC, vector control

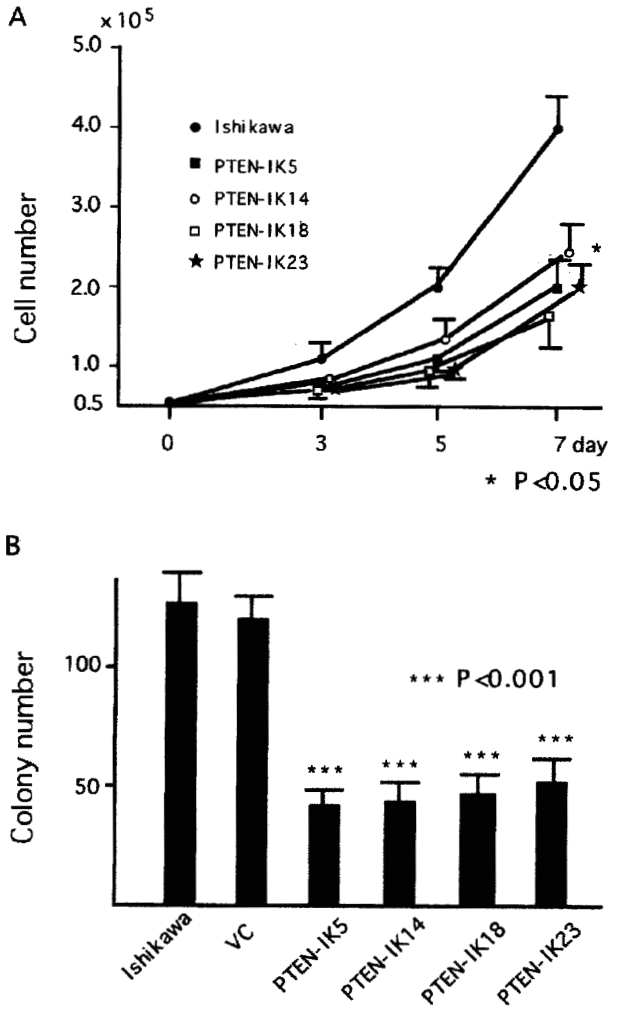

Figure 4 (**A**) Cell growth of the PTEN-expressing clones. 5×10^4 cells were seeded in culture dishes and cell number was monitored for 7 days. The cell growth of the clones is significantly reduced on the 7th day. (**B**) Colony formation of the PTEN-expressing clones in soft agar. One thousand cells were seeded in soft agar and the formed colonies were counted on the 20th day. Experiments were performed in triplicate. *VC*, vector control

(SD, 7.4) in PTEN-IK14, 52.7% (SD, 5.1) in PTEN-IK18, and 47.8% (SD, 5.9) in PTEN-IK23. Apoptotic cells were scarcely observed in all the respective untreated cell lines (Figure 5).

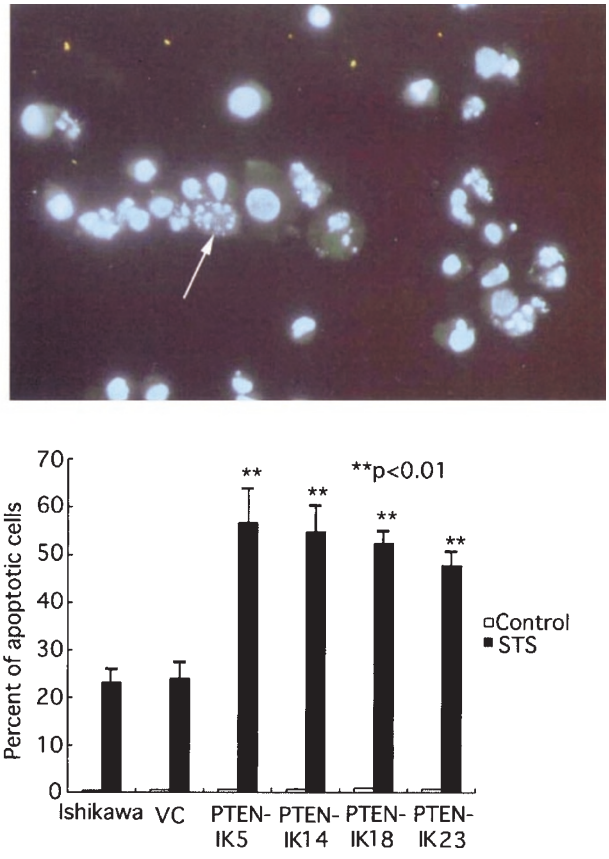

Figure 5 Staurosporine-induced apoptosis in the clones. The cells were exposed to 1 μM of staurosporine for 48 h and then stained with Hoechst 33258. Upper: this multinucleated cell is considered to be an apoptotic cell (arrow). Lower: the percentage of apoptotic cells is shown. Experiments were performed in triplicate. STS, staurosporine; Control, without exposure to staurosporine; VC, vector control

Next, we analyzed a possible mechanism by which staurosporine augmented apoptosis in the PTEN-expressing clones. In preliminary experiments, we determined the IC50 of UCN-01 against PKC activity. It was 47 nM in Ishikawa cells and 45 nM in PTEN-IK5. A concentration of 50 nM of UCN-01, at which PKC was preferentially inhibited, caused apoptosis in the parental cells and in PTEN-IK5, and the percentage of apoptotic cells was equivalent between them. A 20-fold higher concentration of UCN-01 (1 μM), at which a number of protein kinases would be inhibited, augmented apoptosis in the PTEN-expressing clone. At this concentration, the percentage of apoptotic cells in PTEN-IK5 increased significantly compared with the percentage in the parental Ishikawa cells and in the vector control cells ($P<0.01$) (Figure 6). Alpha-sphingosine (1–50 μM) and H-7 (1–50 μM), PKC inhibitors, scarcely caused apoptosis in either cells (data not shown). Next we analyzed the effects of wortmannin, a PI3K inhibitor, on staurosporine-induced apoptosis. Pre-treatment with 1 μM of wortmannin significantly augmented staurosporine-induced apoptosis in the parental cells (from 23.4% to 53.7%), but not in PTEN-IK5 (Figure 7).

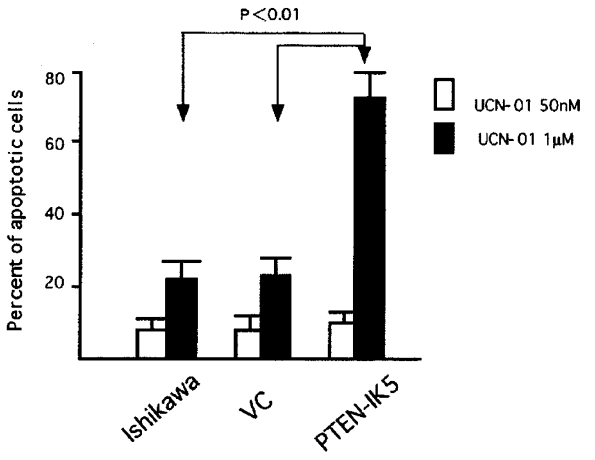

Figure 6 UCN-01-induced apoptosis in the cells. The cells were exposed to UCN-01 (50 nM or 1 μM) for 48 h. The percentage of apoptotic cells is shown. Experiments were performed in triplicate. A higher concentration of UCN-01 augmented apoptosis in PTEN-IK5, but not in the parental Ishikawa cells or vector control. VC, vector control

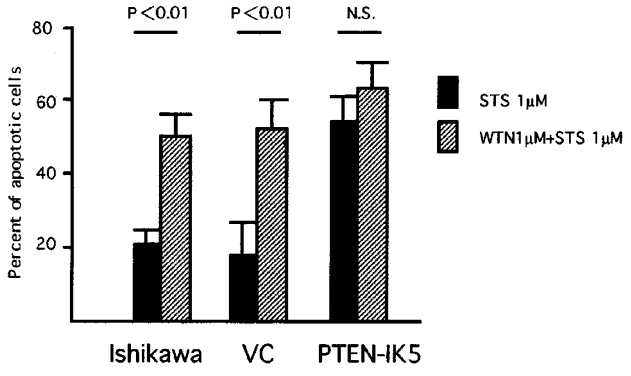

Figure 7 Effects of wortmannin on staurosporine-induced apoptosis. Note that pre-treatment with wortmannin augments apoptosis in parental Ishikawa cells and the vector control, but not in PTEN-IK5. The percentage of apoptotic cells is shown. Experiments were performed in triplicate. STS, staurosporine; WTN, wortmannin; VC, vector control; N.S., not significant

To study whether the PI3K/Akt/PKB signaling pathway is associated with staurosporine-induced apoptosis, we analyzed the phosphorylation status of the Bad protein and Akt/PKB. The levels of Bad and Akt/PKB were constant in all the cell lines, but phospho-Bad (Ser-136) and phospho-Akt/PKB in the PTEN-expressing clone were downregulated (Figure 8).

Staurosporine reduced the expression levels of phospho-Bad (Ser-136) and phospho-Akt/PKB in all the cell lines, regardless of constant levels of Bad and Akt/PKB proteins. The reduction of the phosphoproteins was most significant in PTEN-IK5 (Figure 8).

The other clones showed similar results.

Discussion

We demonstrated that Ishikawa cells have two copies of the PTEN transcript and that the expression level of PTEN mRNA

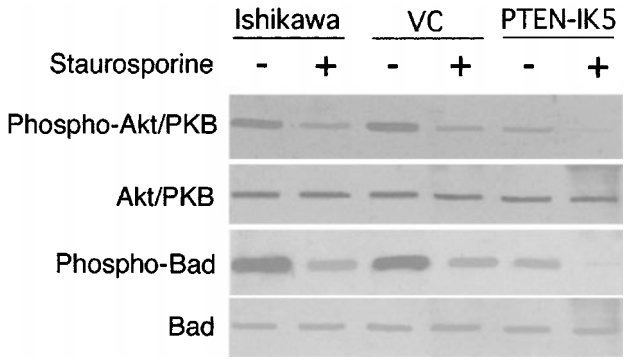

Figure 8 Effects of staurosporine on the phosphorylation of Akt/PKB and Bad (Ser-136). The cells were exposed to 1 μM of staurosporine for 24 h and then Akt/PKB, Bad or the respective phospho-proteins were analyzed by immunoprecipitation and the subsequent Western blot analyses. The levels of phospho-Akt/PKB and phospho-Bad (Ser-136) of PTEN-IK5 were downregulated, irrespective of constant levels of Bad and Akt/PKB proteins. Staurosporine reduced the level of the phospho-proteins in all the cell lines. Note that the level of phospho-Bad (Ser-136) in PTEN-IK5 was markedly lower than in the parental Ishikawa cells after staurosporine exposure. VC, vector control

is very high. However, one copy lacked A in codon 289, resulting in the formation of a stop codon one codon downstream, and the other copy lacked 4-nucleotides from codon 318 to 319, resulting in the formation of a stop codon two codons downstream from the mutation site. The PTEN protein consists of 403 amino acids, and the core phosphatase motif of this protein exists in the middle portion of the peptide. The expected PTEN proteins of Ishikawa cells would possess the core phosphatase motif, but would lack the C-terminal. The antibody we used for the PTEN protein detection recognizes an epitope located at the C-terminal of this protein (388–400 amino acid residues). This is why we could not detect the PTEN protein by Western blot analysis. It has been shown that C-terminal truncation of the PTEN protein deprives it of its stability and phosphatase activity.[12] Therefore, we may conclude that Ishikawa cells do not possess functional PTEN protein.

The successful transduction of wild-type PTEN gene decreased cell growth potential and colony formation in soft agar in Ishikawa cells, and induced upregulation of $p27^{Kip1}$. These data agree with reports by others.[13–16] We found that the level of phospho-Akt/PKB protein was clearly reduced in the PTEN-expressing clone. This implies that exogenous PTEN would function well in the cells.

We found that PTEN could sensitize the Ishikawa cells to staurosporine-induced apoptosis. Staurosporine was originally isolated from Streptomyces as a potent antifungal agent.[17] Thereafter, its broad spectrum of inhibitory activity against protein kinases has been found. Currently staurosporine analogs such as UCN-01 and CGP 41251 are being evaluated as anticancer drugs in clinical phase I trials.[18] UCN-01 was originally identified as a selective inhibitor of PKC.[19] Recent studies have demonstrated that it interacts with cyclin-dependent kinase 2[20,21] and Chk1, a DNA damage checkpoint kinase.[22,23] At a higher concentration of UCN-01, however, a number of protein kinases are inhibited.[24] In the present study, at a concentration of

50 nM of UCN-01, at which the kinases including PKC would be preferentially inhibited, the percentage of apoptotic cells was roughly equal between PTEN-null Ishikawa cells and the PTEN-expressing clone from Figure 6. At the higher concentration (1 μM), at which a number of protein kinases would be inhibited, the percentage of apoptotic cells was significantly higher in the PTEN-expressing clone than in PTEN-null Ishikawa cells. In addition, PKC inhibitors such as alpha-sphingosine and H-7 scarcely caused apoptosis in any of the cell lines regardless of the status of PTEN, suggesting that the PKC signaling pathway via PTEN is not involved in apoptosis of Ishikawa cells.

Bad belongs to a proapoptotic member of the bcl-2 family. Dephosphorylated Bad binds to and inactivates Bcl-XL and to a lesser extent Bcl-2, resulting in a proapoptotic state. The phosphorylation of Bad at either of two sites, serine residues 112 and 136, dissociates Bad from Bcl-2 and Bcl-XL, and renders Bad to interact with 14-3-3, resulting in the liberation of antiapoptotic proteins such as Bcl-2 and Bcl-XL and the promotion of cell survival. Serine 136 of the Bad protein can be phosphorylated by Akt/PKB.[25,26] In the present study, we observed that the expression levels of phospho-Akt/PKB and phospho-Bad (Ser-136) were reduced in the PTEN-expressing clone. Staurosporine reduced the expressions of phospho-Bad (Ser-136) and phospho-Akt/PKB in all the cell lines, but the reduction was most significant in the PTEN-expressing clone. This suggests that staurosporine may exert its apoptosis-induced effect on Ishikawa cells by downregulation of the PI3K/Akt/PKB signaling pathway.

Wortmannin, a PI3K inhibitor, significantly augmented staurosporine-induced apoptosis in parental Ishikawa cells. Wortmannin reduces the PIP3 level by inhibiting PI3K and subsequently reducing the level of phospho-Akt/PKB protein, resulting in the inactivation of the downstream signaling pathway. This physiological situation is quite the same as that of the PTEN-expressing clone. In fact, we observed a diminished level of the phospho-Akt/PKB protein in the parental Ishikawa cells treated with wortmannin (data not shown). With such a status, staurosporine would significantly inhibit the Akt/PKB signaling pathway to augment apoptosis.

We hereby demonstrated that PTEN was deeply involved in staurosporine-induced apoptosis. PKC modulation by PKC inhibitors scarcely affected apoptosis either in PTEN-null Ishikawa cells or in the PTEN-expressing clone. Taken together, we conclude that the augmentation of staurosporine-induced apoptosis in the PTEN-expressing clone is independent of the PKC signaling pathway, but is associated with the PI3K/Akt/PKB signaling pathway.

Materials and Methods

Cell lines and chemicals

Ishikawa and AN3CA cells, from endometrial carcinoma cell lines, and JAR, from a choriocarcinoma cell line, were used in this study. The cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. UCN-01 was gifted from Kyowa-Hakko Kogyo, Inc. (Tokyo, Japan). Staurosporine, H-7, alpha-sphingosine and wortmannin were purchased from Sigma Chemical Co. (St Louis, MO, USA).

PTEN status of the cells

Genomic DNA was extracted from the cells, and PTEN exons 1–9 were amplified by PCR as previously described.[27] The PCR products were further analyzed by the technique of single-strand conformational polymorphism (SSCP) using a GeneGel Excel (Pharmacia Biotech, Inc., Uppsala, Sweden). PCR products were loaded onto gel and electrophoresed at 30 W for 90 min at 15°C. After silver staining by a Hoefer Automated Gelstainer (Pharmacia Biotech, Inc.), the bands showing aberrant mobility shift were excised from the gel, and re-amplified by PCR under the same conditions. Sequencing analysis was performed on the PCR products with an ALFexpress™ DNA Sequencer (Pharmacia Biotech, Inc.).

Mutational analysis of PTEN transcript of Ishikawa cells

Messenger RNA was isolated from Ishikawa cells using a Quickprep Micro mRNA Purification Kit (Pharmacia Biotech, Inc.) following the manufacturer's instructions, and then reverse-transcribed using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Inc, Piscataway, NJ, USA). The region spanning exon 8 was amplified with the following set of primers: 5′-ATCCTCAGTTTGTGGTCTGC-3′ and 5′-GTGTATGCTGATCTT-CATCAAA-3′. Amplified DNA was subcloned into pCR2.1 plasmid (Invitrogen Corp., San Diego, CA, USA), a TA cloning vector. In 10 arbitrarily chosen clones, the sequence of the insert was determined by the sequence analyses described earlier.

Production of wild-type PTEN cDNA

Messenger RNA extraction and cDNA synthesis were performed on the normal endometrium as described earlier. PCR amplification of the PTEN full-length coding region was performed with a set of PCR primers: 5′-ATGACAGCCATCATCAAAGAG-3′ and 5′-TATTTT-CATGGTGTTTTATCCCTC-3′ under the conditions of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 25 cycles. PCR products (1252 base pairs) were subcloned into the pCR2.1 cloning vector. The insert was analyzed by the sequence reaction to obtain wPTEN/pCR2.1. Wild-type PTEN cDNA was excised with *Hind*III/*Xho*I restriction enzymes from the wPTEN/pCR2.1. The extracted fragment was subcloned into the *Hind*III/*Xho*I sites of pcDNA3 vector (Invitrogen Corp.). The resulting vector was designated 'PTEN/pcDNA3'.

Transfection and isolation of clones

Ishikawa cells were transfected with PTEN/pcDNA3. As a vector control, pcDNA3 plasmid was used. Transfection was performed using 20 μl of Lipofectin reagent (GIBCOBRL Life Technologies) conjugated with 1 μg of plasmid DNA against approximately 5×10^4 cells. The transfected cells were grown in medium containing 1 mg/ml of G418 for 4 weeks.

Northern blot analysis

Total RNA was isolated using Isogen reagent (Nippongene Corp., Tokyo, Japan). Twenty μg of total RNA was electrophoresed in 0.66 M

formaldehyde-1.2% agarose gel and then transferred to a nitrocellulose membrane. Subsequent hybridization was performed with ^{32}P-labeled PTEN cDNA corresponding to the full-length coding region as described above. Blots were exposed to X-ray film at -80°C.

Western blot analysis

Cells were lysed with RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) containing aprotinin (30 μl/ml, Sigma Chemical Co.). The protein concentration was determined with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Protein (20 μg) was loaded on 8% SDS–PAGE gel for electrophoresis and transferred to a nitrocellulose membrane by electroblotting. The membrane was incubated with a primary antibody. The primary antibodies used were anti-PTEN (PTEN A2B1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-p27^{kip1} (Santa Cruz Biotechnology, Inc.). After extensive washes, the membrane was incubated with horseradish peroxidase-linked sheep antimouse-IgG (Amersham Pharmacia Biotoch Ltd., Tokyo, Japan) for both the PTEN and p27^{kip1} detections, and then rinsed with PBS. The membrane was stained using the Enhanced Chemiluminescence Western Blot Detection Kit (Amersham Pharmacia Biotoch Ltd.).

Cell growth

Approximately 5×10^4 cells were seeded onto a culture plate. The total cell number was monitored at various incubation times (3–7 days), and the cells were harvested by trypsinization. The viability of cells was evaluated by the trypan-blue exclusion test.

One thousand cells were suspended in tissue culture medium containing 0.7% agar and layered onto 1% agar in 90-mm tissue culture dishes. Cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Colony growth was assessed at 20 days.

Apoptosis assay

Approximately 1×10^4 cells were seeded onto a culture plate. Two days later, they were exposed to various agents for a further 48 h. For the study on the effects of wortmannin on staurosporine-induced apoptosis, 1 μM of wortmannin was added to the medium 3 h prior to the drug exposure. All cells were collected and centrifuged at $400 \times g$ for 5 min. One percent of glutaraldehyde solution was added to the cell pellet. Cells were rinsed with PBS and finally stained with Hoechst 33258 and observed under a fluorescence microscope.

Multi-nucleated cells were considered to be apoptotic cells. Two hundred arbitrarily chosen cells were evaluated. The apoptosis induction activity of the agent was expressed as the percent of apoptotic cells. Experiments were performed in triplicate.

Phosphorylation of Bad and Akt/PKB

Approximately 5×10^6 cells were treated with 1 μM of staurosporine for 24 h, washed once in Tris-buffered saline (TBS: 20 mM Tris, 140 mM NaCl, pH 7.4) and lysed with RIPA buffer. The cell lysates were immunoprecipitated with Bad antibody (New England Biolabs, Beverly, MA, USA) or Akt/PKB antibody (New England Biolabs) for 2 h before addition of 30 μL of protein A-sepharose beads (Sigma Chemical Co.). The mixture was then agitated for 1 h. Immunoprecipitates were washed, separated by SDS–PAGE and then Western blotted with anti-phospho-Bad (Ser-136) (New England Biolabs), anti-Bad, anti-phospho Akt/PKB (New England Biolabs) and anti-Akt/PKB antibodies, as described above.

Statistics

The Student's *t*-test was used for statistical analyses.

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