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The PTEN tumor suppressor inhibits telomerase activity in endometrial cancer cells by decreasing hTERT mRNA levels

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Abstract

Objectives. Loss of PTEN expression is one of the most prevalent and earliest molecular abnormalities associated with endometrial carcinogenesis. Given that PTEN is often absent and telomerase is overexpressed by endometrial cancers, we hypothesize that PTEN signaling is important in telomerase regulation.

Methods. PTEN expression was reconstituted in the PTEN-null Ishikawa endometrial cancer cells by adenovirus-mediated gene transduction. Cell proliferation was evaluated 12–96 h after infection. Western blot analysis was performed to assess PTEN status and phosphorylated Akt expression. Telomerase activity was determined by the telomeric repeat amplification protocol (TRAP) assay. hTERT mRNA levels were assessed by real-time RT-PCR. Ishikawa cells were also treated with LY294002, a PI3-kinase inhibitor.

Results. Infection of Ishikawa cells by replication-defective recombinant adenovirus expressing wild-type PTEN, but not control adenovirus or adenovirus expressing lipid phosphatase defective PTEN GE mutant, inhibited constitutive Akt activation and suppressed proliferation of Ishikawa cells. Infection by wild-type PTEN adenovirus, but not control adenovirus, inhibited telomerase activity 24 h after infection. This inhibition of telomerase activity was parallel to decreased hTERT mRNA levels. LY294002 treatment resulted in dose-dependent inhibition of Akt activation and cellular proliferation. LY294002 suppressed telomerase activity and decreased hTERT transcript levels in a dose-dependent manner.

Conclusions. Our data suggest that PTEN may regulate telomerase activity by a novel mechanism in which inhibition of Akt activation by PTEN leads to decreased hTERT mRNA levels. Thus, loss of PTEN may allow endometrial cells to continue to express high levels of telomerase activity, facilitating the neoplastic transformation of the endometrium.
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Keywords: Endometrial cancer; PTEN; Telomerase

Introduction

The PTEN tumor suppressor gene is inactivated by mutation in approximately 30–60% of early stage endometrial carcinomas [1]. Wild-type PTEN downregulates the phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway, which transduces extracellular growth regulatory signals to intracellular mediators of growth and cell survival [2]. Loss of PTEN results in constitutive activation of Akt and subsequently leads to promotion of cellular proliferation and resistance to apoptosis [3,4]. Unlike most other tumor types, loss of PTEN expression is observed in premalignant lesions of the endometrium, suggesting that PTEN loss may be a potential initiator of endometrial cancer development [1].

Telomerase is a ribonucleoprotein that attempts to solve the end-replication problem by adding DNA sequences back to the ends of chromosome before each cell division. Telomerase is comprised of an RNA template (hTR) and the catalytic protein hTERT which has reverse transcriptase activity. hTERT is considered to be the most important factor in the formation of functional telomerase [5]. In most normal somatic cell types, telomerase activity is usually undetectable; however, the endometrium is one exception [6]. Telomerase activity is dynamic throughout the menstrual cycle and is high during the proliferative phase under the

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influence of estrogen and falls during the secretory phase under the influence of progesterone [7].

PTEN expression in normal endometrium is inversely correlated with telomerase expression, suggesting a possible relationship between PTEN and telomerase in control of proliferation [8]. Approximately 90% of endometrial carcinomas exhibit telomerase expression [9–12]; and therefore, its activation is thought to be important in endometrial carcinogenesis. Given that PTEN is often absent and telomerase is usually overexpressed by endometrial cancers, we hypothesize that the PTEN/Akt signaling pathway may play a vital role in the regulation of telomerase in endometrial cells. In this work, we show that reconstitution of PTEN expression in endometrial cancer cells leads to loss of telomerase activity.

Materials and methods

Cell culture and reagents

The regulation of telomerase expression was investigated in the PTEN-null Ishikawa endometrial cancer cell line. This cell line was kindly provided by Dr. Bruce Lessey (Center for Women's Medicine, Greenville, SC). Ishikawa cells were grown in EMEM supplemented with 5% fetal bovine serum (FBS), 5 μg/ml of bovine insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence 5% CO_2 at 37°C. LY294002 was purchased from Sigma (St. Louis, MO). Polyclonal anti-phospho-Akt, anti-pan Akt, and anti-PTEN were from Cell Signaling Technology (Beverly, MA). Anti-β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence Western blotting detection reagents were from Amersham (Arlington Heights, IL). ^{32}P-labeled ATP (3.000 ci/mmol) was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). All other chemicals were from Sigma (St. Louis, MO).

PTEN adenovirus infection

Adenoviruses Av-CMV-PTEN, Av-CMV PTEN GE mutant, Av-CMV, and Av-CMV-GFP have been previously described [3]. Av-CMV-PTEN contains the wild-type PTEN cDNA under the control of the CMV promoter. Av-CMV-PTEN GE mutant expresses the G129 mutant form of PTEN which has lost its lipid phosphatase activity and is unable to inhibit Akt activation [3]. Adenovirus Av-CMV contains the CMV promoter with no cDNAs subcloned downstream to it. Adenovirus Av-CMV-GFP expresses enhanced green fluorescence protein under the CMV promoter. Two days before adenoviral infection, 2×10^5 cells were seeded in each well of a six-well plate. On the day of infection, cells from one well were infected with Av-CMV-GFP, detached with trypsin, and counted by flow cytometric analysis. This information was used to calculate the adenovirus dose of multiplicity (MOI). It was found that an MOI of 45 could infect 99.9% of Ishikawa cells. Infections were carried out with 500 μl of infection media (the same culture media used for this cell line with 2% FBS and 1% P and S) in a 5% CO_2 incubator at 37°C for 1 h on a rocker.

Cell proliferation assay

Ishikawa cells were seeded at 1×10^5 cells/well in six-well plates and then infected with adenovirus Av-CMV-PTEN, Av-CMV PTEN GE mutant or control vectors Av-CMV, and Av-CMV-GFP (MOI 45:1) in media with charcoal-stripped serum. Cells were incubated for 12–96 h after adenovirus infection. Cells counts were performed with a hemocytometer.

Western blot analysis

Cells were plated at 2×10^5 cells/well in six-well plates. After 24 h, cells were treated with LY294002 at doses ranging from 1 to 50 μM. DMSO was used as a control. Cell lysates were prepared in RIPA buffer (1% NP40, 50 nm Tris, and 150 mM NaCl). Equal amounts of protein were separated by gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and then incubated with a 1:1000 dilution of primary antibody (polyclonal anti-phospho-Akt, anti-pan Akt or anti-PTEN) overnight at 4°C. The membrane was washed and incubated with a secondary peroxidase-conjugated antibody for 1 h after washing. Antibody binding was detected using an enhanced chemiluminescence detection system according to the manufacturer's recommendations. Western blot films were digitized, and band net intensities were quantified using a Millipore Digital Bioimaging System (Bedford, MA). After developing, the membrane was stripped and reprobed using antibody against β-actin to confirm equal loading.

Telomerase activity assay

Telomerase activity in cultured cells was assayed using a PCR-based telomeric repeat amplification protocol (TRAP) as described previously [7]. The TRAP-eze Telomerase Detection Kit (Intergen, Purchase, NY) was used as recommended by the manufacturer with minor modifications. Briefly, 1×10^5 cell pellets were stored at −80°C until lysis was performed. Samples were homogenized with 1 × CHAPS lysis buffer (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane-sulphonate) at 1×10^5 cells/100 μl, incubated on ice for 30 min, and centrifuged at 13,000 *g* for 21 min at 4°C. The supernatant was transferred into a fresh tube, and the protein concentration was measured by ABC kit (Bio-Rad, Hercules, CA). Between 0.25 and 0.5 μg of protein from the cell extract was mixed with ^{32}P-labeled TS primer in a 50 μl reaction mixture. After 30 min of incubation at 30°C, PCR amplification was then performed with 27 cycles at 94°C for 30 s and 59°C for 30 s. The PCR products were separated by electrophoresis on 10% polyacrylamide nondenaturing gels. Phosphor-Imager and Imagequant software from Molecular Dynamics (Sunnyvale, CA) were used to quantify the band intensities. Telomerase activity is expressed quantitatively as TPG, which reflects a ratio of the TRAP product

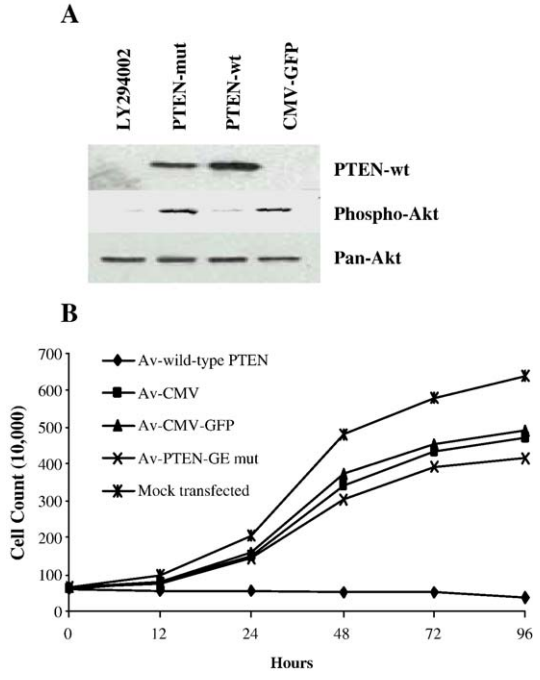

Fig. 1. Reconstitution of PTEN expression suppresses the phosphorylation of Akt and inhibits cell growth in PTEN-null endometrial carcinoma cells. (A) Ishikawa cells were infected with wild-type PTEN, PTEN GE mutant or the control vectors CMV and CMV-GFP. Noninfected Ishikawa cells were treated with LY294002 (20 (M) for comparison. Phospho-Akt and pan-Akt expressions were assessed by Western immunoblotting. (B) Inhibition of cell growth was determined by counting cell numbers 12–96 h after infection. This graph represents one of three experiments.

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