17β-estradiol induces up-regulation of PTEN and PPARγ in leiomyoma cells, but not in normal cells

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Abstract. The tumor suppressor protein, PTEN (phosphatase and tensin homologue), has been reported to play a crucial role in a variety of tumor cells. Recent studies indicate that the transcription factor, PPARγ (peroxisome proliferator-activated receptor γ) up-regulates PTEN expression transcriptionally through the binding of two response elements in the genomic sequence upstream of PTEN in tumor cells. Here, we determined the PTEN and PPARγ expression in human leiomyomas, and whether estrogen can change their protein expressions in cultured leiomyoma cells. Immuno-histochemical staining and Western blot analysis revealed that PTEN protein levels in leiomyomas were higher than that the adjacent normal myometrial tissues, which was paralleled with decrease in phospho-Akt (ser-473) and phosphatidylinositol 3,4,5-tris phosphate (PIP3) level. Interestingly, leiomyomas exhibit high PPARγ expression compared to the adjacent normal myometrial tissues. In addition, we found that 17β-estradiol (E2) significantly stimulated PTEN and PPARγ expression in cultured leiomyoma cells, but did not change both protein levels in normal cells. This report shows the first evidence that PTEN and PPARγ are up-regulated in leiomyoma tissues, and estrogen stimulates expression of PTEN and PPARγ in leiomyoma cells, but not in normal cells.

Introduction

PTEN on chromosome 10q23 has been recognized as one of the major tumor suppressor genes in human cancers. PTEN is also called MMAC1 (mutated in multiple advanced cancers), and TEP1 (TGF-β-regulated and epithelial cell-enriched phosphatase) (1,2). Mutations and/or deletions in PTEN are found in a variety of cancer cells, including endometrial, breast, ovarian, and lung neoplasia (3-7). Germline mutations in PTEN are also found in inherited harmatoma tumor syndromes such as Cowden's syndrome, which has a high risk of breast, thyroid and other tumors (8). Mechanisms by which the expression of PTEN is regulated are, however, unclear. Recently it has been known that PPARγ is able to bind two response elements in the genomic sequence upstream of PTEN (9), indicating that PPARγ can control PTEN expression transcriptionally in tumor cells.

Uterine leiomyomas, also called fibroids, are the most common reproductive tract neoplasm and the leading indication for hysterectomy in premenopausal women. These tumors are the leading indication for hysterectomy in the world (10,11). Currently, there is no effective medical treatment option for this condition. Uterine leiomyoma growth is indicated by the fact that most of these tumors are diagnosed during the reproductive years, increase in size during pregnancy,
and regress after the onset of menopause (10), and events that all coincide with changes in hormonal milieu. Other evidence supporting estrogen dependence of uterine leiomyomas are the increased estrogen receptor (ER) expression and decreased E2 metabolism observed in these tumors (23). Consequently, the growth of uterine leiomyomas is thought to be modulated by the ovarian hormones estrogen (E2) and progesterone.

The progression of the neoplastic state is influenced by deregulation of oncogenes or inactivation of tumor suppressors (24). It is known that endometrial expression of PTEN is not constant throughout the menstrual cycle, but changes in response to the hormonal environment (25,26). Although sex steroid hormones have been shown to regulate PTEN, little is known concerning PTEN expression in uterine leiomyoma. The present study therefore was conducted to elucidate the PTEN expression in human leiomyomas and its regulation by estrogen.

Materials and methods

Materials. Antibodies against PTEN, p53, ß-actin, myosin, and ERa were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-Akt (ser-473), p-PTEN (ser-385), and Akt were purchased from Cell Signaling (Beverly, MD, USA). Fetal bovine serum (FBS) and charcoal-dextran treated FBS were obtained from Gibco BRL (Life Technologies, Inc., Grand Island, NY, USA). HBSS (Hanks balanced salt solution), 17-ß-estradiol (E2), DMEM, phenol red-free DMEM obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Tissue collection. Twenty uterine leiomyomas and the adjacent normal myometrial tissues were collected from different patients. The patients ranged in age from 30 to 45 years and underwent hysterectomy for medically indicated reasons at Chonbuk National University Hospital. Histological diagnosis of each uterine specimen was determined by a pathologist. This study had local ethics committee approval obtained from Chonbuk National University Hospital. Histological diagnosis of each uterine specimen was determined by a pathologist. This study had local ethics committee approval obtained from Chonbuk National University Hospital's institutional review board. Informed consent was provided according to the Declaration of Helsinki.

Immunohistochemical staining for PTEN protein. Uterine tissue specimens were fixed in 4% buffered neutral formaldehyde solution, dehydrated, and embedded in paraffin. Sections, 5-6 μm thick, were deparaffinized and followed by standard histological techniques. The avidin/biotin immunoperoxidase staining method was performed with the use of a polyvalent immunoperoxidase kit (Omnitags, Lipshaw, MI). A mouse monoclonal antibody to human PTEN protein was used at a dilution of 1:200 as the primary antibody in this study. To assure the specificity of the immunological reaction, control sections were subjected to the same immunoperoxidase method, except that the primary antibody was replaced by non-immune murine IgG (Miles, Elkhart, IN) at the same dilution as the specific primary antibody.

Cell culture. Uterine leiomyoma tissues were cut into small pieces and digested in 0.2% collagenase (wt/vol) at 37°C for 3-5 h as previously described (24). The leiomyoma cells were collected by centrifugation at 460 x g for 5 min and washed three times with DMEM containing 1% antibiotic solution. Cell viability was determined by trypan blue exclusion test. The isolated leiomyoma cells were plated at density of ~105 cells/dish in 10-cm2 culture dishes and then subcultured for 120 h at 37°C in a humidified atmosphere of 5% CO2-95% air in phenol red-free DMEM supplemented with 10% FBS (vol/vol; Life Technologies, Inc.).

Protein extraction and Western immunoblotting. Leiomyomas and the adjacent normal myometrial tissues for protein extraction were collected immediately after hysterectomy. These tissue samples were homogenized at 4°C in the presence of lysis buffer. Homogenates were subsequently centrifuged at 13,000 x g for 30 min, and the supernatants were collected. After washing with phosphate buffered saline (PBS), cultured cells were lysed with the lysis buffer (20 mM HEPES pH 7.2, 1% Triton X-100, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 1 μg/ml aprotinin). The protein concentration was determined using protein assay kits (Bio-Rad Laboratories, USA). Samples (50 μg) were prepared with four volumes of 0.5 M Tris buffer (pH 6.8) containing 4% SDS, 20% glycerol and 0.05% bromophenol blue at 95°C for 5 min. SDS-PAGE was performed in 10% slabs. Proteins were then transferred to nitrocellulose paper. Blots were incubated with the primary antibodies against protein followed by peroxidase-conjugated secondary antibody. The antigen-antibody complexes were detected with the enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech).

Reverse transcription polymerase chain reaction (RT-PCR) for PTEN. RT-PCR was performed using RNA PCR kits (GeneAmp, Applied Biosystems, USA). Total RNA was isolated from cells using TRIzol reagent following the manufacturer's instructions. Five micrograms of total RNA was transcribed into cDNA in a 20 μl final volume of reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, and 2.5 U M-MLV RNase H-reverse transcriptase manufacturer's instructions). Five micrograms of total RNA was transcribed into cDNA in a 20 μl final volume of reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, and 2.5 U M-MLV RNase H-reverse transcriptase). cDNA synthesis was initiated with 3 min of denaturation at 94°C followed by 26 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and 72°C for 3 min. After the last cycle of amplification, the samples were incubated for 5 min at 72°C. ß-actin PCR was performed with 2.5 μl aliquots of the synthesized cDNA using a 45-μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, and 2.0 μl Taq DNA polymerase, and 0.4 μM of each PCR primer: sense primer, human PTEN (5'-CCGGAAATTCTAGCAGGCAATCATCAAAGA-3'), and antisense primer, human PTEN (5'-CCGGGTATCCAGACCTTGGTGGTGTG-3'). Amplification for PTEN was initiated with 3 min of denaturation at 94°C followed by 26 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. After the last cycle of amplification, the samples were incubated for 5 min at 72°C. ß-actin PCR was performed with 2.5 μl aliquots of the synthesized cDNA using primers at a concentration of 0.15 μM: sense primer, human ß-actin (5'-CCACCGAAACTAATTTTTCAC-3'), and antisense primer (5'-TCACTCTTCCTGTCCTGCTGA-3'). The obtained PCR products were analyzed on ethidium bromide-stained agarose gels (2%).
Real-time quantitative reverse transcription-polymerase chain reaction for PTEN. Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd., Lewes, UK) according to the manufacturer's instructions. Total RNA was isolated from tissues using TRIzol reagent (Life Technologies Ltd., Paisley, UK). RNA was isopropanol...
precipitated, dissolved in DEPC-treated distilled water and kept at -80°C until use. RNA (1 µg) was reverse transcribed with Superscript II reverse transcriptase (Life Technologies Ltd.). The cDNA was diluted 10-fold prior to PCR amplification. The oligonucleotide primers used for PCR are as follows: PTEN upstream 5’-CCACAGCTAGAACCTATCAAACC-3’, downstream 5’-ATGAACTTGTCTTCCGGT-3’, E 2F upstream 5’-TGGGAAAGAGGAGGACCTCTA-3’, E 2F downstream 5’-TCATCACCAGCATGTGG-3’. Reactions were performed in a 20-µl volume with 0.5 µM primers and MgCl₂ concentration optimized between 2-5 mM. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler-DNA Master SYBR Green I mix (Roche Diagnostics Ltd.). PCR amplification began with a 10-min per-incubation step at 95°C, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 56°C (PTEN) or 55°C (E 2F) for 5 sec, and elongation at 72°C for 10 sec. The relative concentration of PCR product derived from the target gene (PTEN) was calculated using software of the LightCycler System. A standard curve for each run was constructed by plotting the crossover point against the log (number of starting molecules). The number of target molecules in each sample was then calculated automatically by reference to this curve. Results were expressed relative to the number of E 2F transcripts used as an internal control. All experiments were performed in triplicate.

**Immunohistochemical methods for PIP₃ analysis.** The intracellular PIP₃ level was directly determined by an immunohistochemical method using a recent developed monoclonal antibody to PIP₃, as described by Niswender et al (27). Slide glasses containing tissue specimens were equilibrated in PBS at room temperature. The tissues were fixed for 5-10 min (room temperature) in 4% paraformaldehyde. After blocking in 5% normal goat serum (NGS) and 2% bovine serum albumin (BSA), samples were incubated with mouse anti-PIP₃, monoclonal antibody (Echelon) at a 1:100 dilution overnight at 4°C. The negative immune control for antibody was an equivalent concentration of non-immune mouse IgM. Primary antibodies were detected with goat anti-mouse IgM-TRITC at a 1:200 dilution 1 h at 4°C. Samples were mounted in aqueous mounting medium with anti-fading agents. Images were acquired with an Axiovert S100 fluorescence microscope (Zeiss) equipped with a DP70 digital camera (Olympus).

**Statistical analysis.** The t-test was used to determine the statistical significance of the data obtained and to compare the means of the two groups. One-way ANOVA and Student-Newman-Keuls test was used for multiple comparisons. P<0.05 was set as a statistically significant difference between the values of two group means.

**Results and Discussion**

**Human uterine leiomyomas exhibit high PTEN expression compared to the adjacent normal myometrial tissues.** We firstly measured PTEN expression by using immunohistochemical analysis in leiomyoma. The tumor suppressor PTEN was immunolocalized to the cytosol of leiomyoma cells. PTEN expression of leiomyoma tissue was higher than that of the adjacent normal myometrial tissue (Fig. 1Aa). PTEN expression level was the highest in the cytosols of leiomyoma cells than other organelles (Fig. 1Ac). Western immunoblot analysis also revealed that PTEN expression of leiomyoma tissue was higher than that of the adjacent normal myometrial tissue (Fig. 1B). However, RT-PCR and real-time PCR showed that PTEN mRNA level of leiomyoma was lower than that of the adjacent normal myometrial tissue (Fig. 1C and D). Consequently, these findings suggest that PTEN level of leiomyoma is dependent on protein stability rather than protein synthesis.

**Human uterine leiomyomas exhibit high p-PTEN expression compared to the adjacent normal myometrial tissues.** The phosphorylation level of PTEN protein regulates its stability (28). In particular, serine phosphorylation (ser-370 and ser-385) of PTEN assumes a closed conformation with enhanced stability (29), suggesting that high phosphorylation level of PTEN (p-PTEN) cause an increase in its stability. Thus, we measured p-PTEN (ser-370 and ser-385) level using Western blotting in leiomyoma. As we expected, there was a clear difference in p-PTEN protein content between leiomyoma tissues and the adjacent normal myometrial tissues. P-PTEN level was significantly increased in leiomyoma tissues compared to the adjacent normal myometrial tissues (Fig. 2).
Figure 3. Immunolocalization of PIP3 in human uterine leiomyoma. Confocal laser scanning microscopy was performed for analysis of intracellular PIP3 level in leiomyoma. PIP3 level of leiomyoma was less than in the normal tissues. Tissues were visualized by confocal microscopy with a ×400 objective.

Figure 4. Western blot analysis for PPARγ content of human uterine leiomyoma and the adjacent normal myometrial tissues. The tissue lysates were analyzed with Western blots for PPARγ. Values were expressed as relative density of PPARγ to actin. The PPARγ content in leiomyoma was higher than in the normal tissues. β-actin was used as a loading control. Error bars, SD; n=20 in each group. *P<0.001 compared with normal.

Figure 5. Effect of E2 on PTEN and PPARγ expression in isolated cells from human uterine leiomyoma and the adjacent normal myometrial tissues. (A) Immunolocalization of myosin in human uterine leiomyoma. Confocal laser scanning microscopy was performed for identification of smooth muscle cells. Cultured cells isolated from leiomyoma and the adjacent normal myometrial tissues showed myosin imaging. Cells were visualized by confocal microscopy with a ×400 objective. (B) Effect of E2 on PTEN expression in human breast cancer MCF-7 cells. ERα-positive MCF-7 human breast cancer cells (5×10⁵) were treated with 10 μM E2 for the indicated times. Cell lysates were analyzed with Western blots for PTEN. β-actin was used as a loading control. Human uterine leiomyoma cells or normal cells (1×10⁵) were treated with 10 nM E2 for 48 h. Cell lysates were analyzed with Western blots for PTEN. β-actin was used as a loading control. (C) Effect of E2 on PPARγ expression in isolated cells from human uterine leiomyoma and the adjacent normal myometrial tissues. Human uterine leiomyoma cells or normal cells (1×10⁵) were treated with 10 nM E2 for 48 h. Cell lysates were analyzed with Western blots for PPARγ. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan). Values were normalized to control (no incubation) and expressed as percent of control. Error bars, SD; n=5 in each group. *P<0.005, compared with control.
Human uterine leiomyomas exhibit high PPARγ expression compared to the adjacent normal myometrial tissues. One emerging therapeutic approach to cancer treatment is the induction of growth arrest of cancer cells through ligand activation of nuclear hormone receptors (NHRs). This family includes estrogen receptors, retinoic acid receptors (RARs), retinoid X receptors (RXRs), and the vitamin D receptor. The latest member of NHR superfamily to be identified is the peroxisome proliferator-activated receptor γ, commonly known as PPARγ (31). This is a transcriptional factor that plays an essential role in mediating the pharmacological actions of PPARγ ligands, and is highly expressed in epithelial malignancies (32), indicating that PPARγ play a vital role in regulating the growth of human leiomyomas. It was reported that PPARγ induces upregulation of PTEN in various cancer cells (9,33). In the next experiments, we therefore determined expression of PPARγ in leiomyoma tissues. As we expected, Western blot analysis showed that human uterine leiomyomas exhibited higher PPARγ expression compared to the adjacent normal myometrial tissues (Fig. 4).

17β-estradiol (E2) induces upregulation of PTEN and PPARγ expression in human uterine leiomyoma cells. Finally, experiments were performed to elucidate PTEN and PPARγ regulation by estrogen. Thus, we first isolated successfully leiomyoma cells from leiomyoma tissues (Fig. 5A). Identification of smooth muscle cells was determined by confocal laser scanning microscopic imaging for myosin. In a previous experiment, we determined the ability of E2 to regulate PTEN and PPARγ expression in leiomyoma cells from leiomyoma tissues (Fig. 5A). Identification of smooth muscle cells was determined by confocal laser scanning microscopic imaging for myosin. In a previous study, we found that E2 increased PTEN expression in the ERE-positive breast cancer cell line, MCF-7. To investigate the ability of E2 to regulate PTEN and PPARγ, leiomyoma cells or the adjacent normal cells were exposed to E2 (10 nM) for 48 h, and levels of PTEN and PPARγ were determined. Western blot analyses revealed an increase of PTEN and PPARγ levels in human uterine leiomyoma cells (Fig. 5B and C). However, E2 did not affect PTEN and PPARγ in normal myometrial cells, indicating that E2 induces tumor-specific upregulation of PTEN and PPARγ in human uterine leiomyoma cells.

In conclusion, we have demonstrated that PTEN tumor suppressor content was remarkably increased in leiomyomas compared to the adjacent normal myometrial tissues. Consistent with these in vivo findings, the PTEN expression in leiomyoma cells cultured under serum-free, phenol red-free conditions was stimulated by E2, but was not affected in the normal cells.

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