European Review for Medical and Pharmacological Sciences 2019; 23: 10453-10458

PTEN expression in U251 glioma cells enhances their sensitivity to ionizing radiation by suppressing DNA repair capacity

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Abstract. – OBJECTIVE: Mutations in phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a tumor suppressor gene, lead to altered sensitivity to drugs and radiation in various types of cancer. Restoring PTEN expression in tumor cells can increase radiosensitivity by inhibiting the repair of DNA double-strand breaks (DSBs). Thus, determining the mechanism of action of this protein may lead to novel therapeutic strategies.

MATERIALS AND METHODS: In this study, we transduced U251 cells with a lentiviral vector expressing *PTEN* to examine the mechanism of radiosensitization. Specifically, we examined the formation of radiation-induced DNA DSBs and apoptosis, as well as the expression of several proteins involved in repairing DSBs (p53, ataxia-telangiectasia mutated, DNA-dependent protein kinase C, Ku70-80).

RESULTS: Our results showed that PTEN transduction sensitized U251 cells to X-rays, increasing the number of DSBs per cell and fraction of cells undergoing apoptosis. Additionally, the average size of γH2AX nuclear foci was increased following irradiation. These findings were accompanied by a PTEN-dependent irradiation-independent increase in p53 levels and decrease in phosphorylated Ku70/80 levels.

CONCLUSIONS: Our results suggest that PTEN affects radiosensitivity by reducing DSB repair and by enhancing the p53 pathway, leading to increased apoptosis.

Key Words:
PTEN, Radiosensitivity, DNA double-strand breaks, p53, Ataxia-telangiectasia mutated, U251 cells, Glioma.

Introduction

Radiotherapy is known to be correlated with the radiosensitivity of tumor cells[1,2]. However, the factors controlling radiosensitivity at the cellular level are not well-understood. In a series of recent studies, Pappas et al[3] demonstrated that reintroduction of some genes frequently mutated in cancer can cause radiosensitivity in tumor cells[3]. One such gene was phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a tumor suppressor gene estimated to be inactive in 50-80% of solid tumors including gliomas, prostate carcinomas, melanomas, endometrial carcinomas, and lung carcinomas[4]. Thus, understanding the mechanism of action of this protein is of great interest in cancer research.

It has been proposed that PTEN can increase radiosensitivity by preventing DNA repair[5]. The major cellular target of ionizing radiation is genomic DNA, which frequently results in the production of DNA double-strand breaks (DSBs) that can cause cell death. To prevent DSBs, cells have evolved two complex repair pathways, homologous recombination (HR; mainly active during S phase) and non-homologous end-joining (NHEJ; active throughout the cell cycle)[6]. Major proteins in these pathways include the ataxia-telangiectasia mutated (ATM) protein kinase, the phosphorylation of which plays a pivotal role in the early stages of HR[7], and DNA-PKc and Ku70-80, which are important for NHEJ. PTEN may alter these proteins, affecting the cell's ability to repair DNA damage.

Alternatively, PTEN may affect radiosensitivity *via* p53 and suppress the phosphatidylinositol

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3-kinase/protein kinase B (Akt) pathway[8,9]. Specifically, catalytically inactive PTEN binds to and promotes the stabilization, acetylation, and tetramerization of p53 in the cell nucleus through phosphatase-independent and murine double minute 2-independent mechanisms[10-12]. Thus, in this study, we investigated the relationship between PTEN and radiosensitivity in U251 glioma cells, as well as the effect of PTEN on the expression of p53 and of other proteins involved in HR and NHEJ.

Materials and Methods

Plasmid Construction

The plasmid pCDH-CMV-MCS-EF1-copGFP (http://www.systembio.com/downloads/Manual_pCDH_Vector_v5.pdf) was kindly provided by Dr. Yanbo Li at the Department of Public Health at Jilin University in China (Jilin, China). This plasmid was used as a vector for cloning of PTEN. *Xho*I- and *Bam*HI-tagged primers (P1 and P2, respectively) were used to amplify wild-type *PTEN* cDNA. The primer sequences used for cloning were as follows: P1: 5'-CGGAATTCAATGCCAAAGAAGAAGAGAAAGGTACTCGAGATGACAGCCATCATCAAA-3'; AND P2: 5'-CGGGATCCAAAAGATCTAGTATCGGTTGGCTTTGT-3'.

The amplification product was then inserted into the corresponding *Xho*I and *Bam*HI sites of the *pCDH-CMV-MCS-EF1-copGFP* vector, which was used to overexpress PTEN in U251 cells.

Cell Culture, Transduction, and Clonogenic Survival Assay

The human glioma cell line U251 was generously provided by the Public Health Institute of Jilin University in China. Cells were grown under standard conditions in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin. U251 cells were transduced with the lentiviral vector at a multiplicity of infection (MOI) of 15 in the presence of 4 μg/mL polybrene. Briefly, at 24 h after plating, the cells were rinsed in phosphate-buffered saline (PBS) and incubated for 1 h at 37°C in serum-free media in the presence of the plasmid. The cultures were rocked cultures gently every 10 min. Fresh media supplemented with fetal bovine serum (FBS) was added to the cultures, and the cells were grown for additional 48 h before adding 3 μg/mL puromycin as a selection agent. The radiosensitivity of U251 cells was evaluated before and after PTEN transduction. Specifically, at 48 h after transduction, the cells were irradiated with a dose of 4.0 Gy and plated on 10-cm culture plates. The plates were then placed in an incubator and allowed to grow colonies for 14 days. Colonies were counted as positive only if they contained at least 50 cells. The survival rate of the transduced cells was calculated relative to the number of colonies of non-irradiated cells.

Detection of Apoptotic Cells and DSB Quantification

Transduced and non-transduced cells were evaluated for their sensitivity to radiation by assessing the fraction of apoptotic cells and number of DSBs/cell present, on average, in each population. Specifically, the fraction of apoptotic cells was determined by flow cytometry to count the cells stained positive with Annexin V-fluorescein isothiocyanate (Dingguo Biotechnology, Beijing, China). In contrast, the number of DSBs was estimated from the number of nuclear foci containing histone H2AX phosphorylated at serine 139 (γH2AX). H2AX phosphorylation and foci formation are among the earliest cellular responses to DSBs[13-16], and the rate at which they occur can be used to predict cell sensitivity to radiation[17]. In our experiments, the presence of nuclear foci was determined by immunofluorescence in paraformaldehyde-fixed cells prepared using rabbit anti-γH2AX antibodies (Trevigen, Inc., Gaithersburg, MD, USA; 1:300 dilution) and anti-rabbit fluorescein isothiocyanate-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA)[3].

Quantification of DNA Damage Response Proteins

To detect variations in the expression of various DNA damage response (DDR) proteins, lysates were prepared from transduced and non-transduced cells[3]. Briefly, lysates were prepared at 48 h after transduction using approximately 5×10^6 trypsinized U251 cells. Homogenates were collected by centrifugation at $1000 \times g$ for 10 min at 4°C, and the protein content in the supernatants was measured spectrophotometrically to ensure equal loading of each sample for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Next, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Protein detection was carried

out using primary antibodies against PTEN, p53, H2AX, ATM, DNA-PKc, or Ku70/80 diluted by 1:1000 (Signalway Biotechnology, Houston, TX, USA). A horseradish peroxidase(HRP)-conjugated secondary antibody diluted 1:2000 (Signalway Biotechnology, Houston, TX, USA) was added and signals were quantified by chemiluminescence using an enhanced chemiluminescence plus Western Blotting Detection System (Pierce, Rockford, IL, USA) and Storm 860 scanner (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical Analysis

Statistical analysis was performed using SPSS version 22.0 software (IBM, Armonk, NY, USA). Data were represented as the mean ± standard deviation. The *t*-test was used to analyze measurement data. $p < 0.05$ indicated a significant difference.

Results

Clonogenic Cell Survival as a Function of Radiation Dose

The radiosensitivity of U251 cells was first measured by conducting a clonogenic survival assay. As shown in Figure 1, after administering 4 Gy of radiation, the survival rate of cells decreased from 30-33% (cells that were not transduced or were transduced with empty vector) to 20% (for cells transduced with the wild-type-PTEN vector). These results indicate that increased PTEN expression significantly increased the radiosensitivity of U251 cells ($p < 0.05$, n = 3).

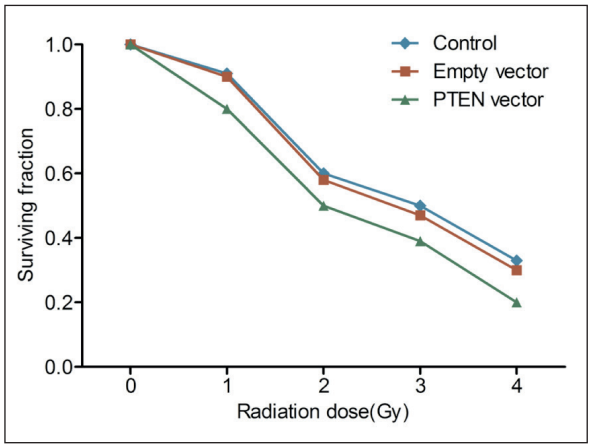

Figure 1. Clonogenic cell survival as a function of radiation dose.

Fraction of Apoptotic Cells Present Before and After Irradiation (4 Gy)

These findings were detected by evaluating radiation-induced apoptosis at 24 and 48 h after irradiation (Figure 2). After 24 h, the fraction of apoptotic cells in the PTEN group was 5% compared to 2.8% in non-irradiated cells and 3.4% in cells transduced with empty vector. These values increased to 6.7%, 4.7%, and 4.6%, respectively, at the 48-h time point. Thus, the expression of PTEN sensitized U251 cells to radiation by significantly inducing apoptosis ($p < 0.05$, n = 5).

Detection of γH2AX Foci at Various Times Following Irradiation

Ionizing radiation induces the formation of DSBs, which can be lethal if not promptly repaired. Therefore, we determined whether the increased radiosensitivity induced by PTEN occurred because of alterations in the repair of DSBs. Specifically, we observed the average number of DSBs per cell based on the presence of γH2AX foci (Figure 3). Our results indicated that PTEN caused a significant increase in the number of DSBs present at 15 min after cell irradiation with 4 Gy (107.8 DSBs per cell compared to 41.6 in non-transduced and mock-transduced cells). However, the number of DSBs decreased dramatically within 12 h following irradiation both in absolute and relative terms compared to non-transduced and mock-transduced cells (8.6 DSBs *vs.*

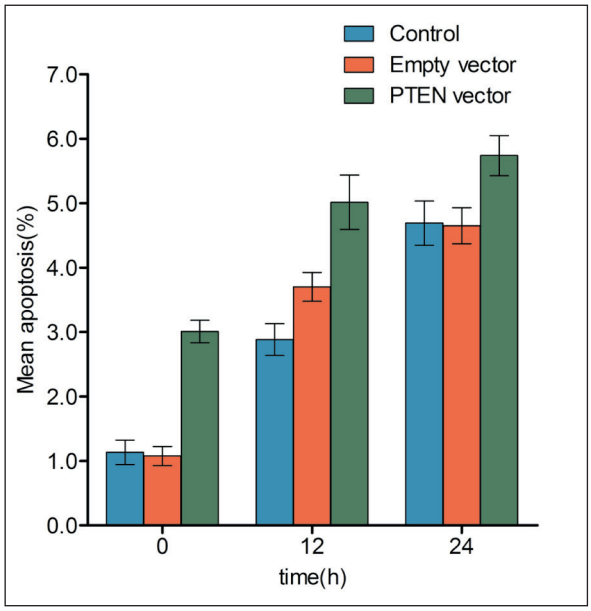

Figure 2. Fraction of apoptotic cells present before and after irradiation (4 Gy) as a function of PTEN expression.

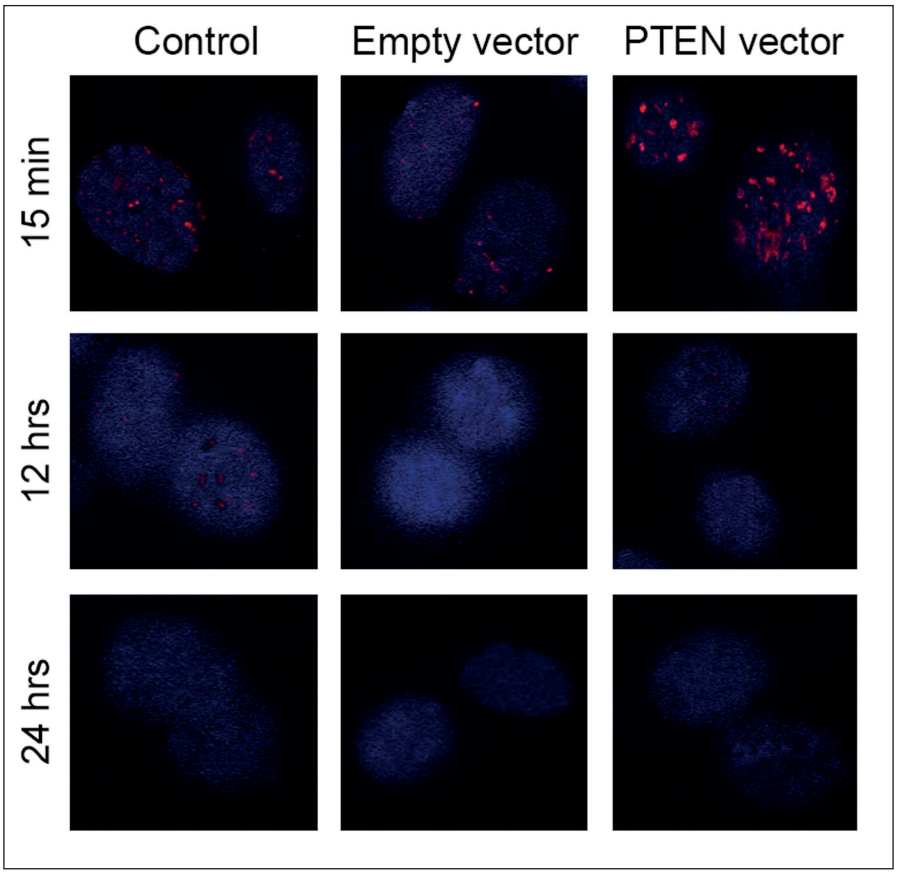

Figure 3. Detection of γH2AX foci at various times following irradiation (15 min, 12 h, and 24 h). PTEN (red fluorescence) and DAPI (blue fluorescence). (magnification: 400×).

9.4 and 8.6, respectively). At 24 h post-irradiation, all experimental groups had completely repaired the DSBs. These findings indicate that the presence of DSBs led to increased radiosensitivity and that increased apoptosis occurred because of abnormalities in the kinetics of DSB repair following DNA damage.

Protein Expression in DDR and DSB Repair Before and After Transduction with PTEN

To identify the pathways involved in PTEN-induced radiosensitivity changes, we performed Western-blotting analyses of several phosphorylated and unphosphorylated proteins associated with DDR and DSB repair (Figure 4). Phosphorylated (p)-DNA-PKc was decreased in transduced cells following irradiation. In contrast, clear differences were observed following PTEN overexpression. Particularly, both irradiated and non-irradiated cells showed a significant increase in the levels of p53. Finally, we detected a modest decrease in Ku70/80 phosphorylation in irradiated cells transduced with *PTEN*. Thus, the increase in apoptosis induced by PTEN was correlated with an irradiation-independent increase in the expression of p53 and irradiation-dependent reduction in the levels of p-DNA-PKc and p-Ku70/80, which were consistent with enhanced radiosensitivity of U251 cells.

Discussion

PTEN is frequently inactivated in many tumors and is considered the most important tumor suppressor gene after p53 in cancer[18]. The complete loss of PTEN is most frequently observed in endometrial cancers and glioblastomas and is generally associated with advanced cancer stages and metastases[4,19]. Pappas et al[3] showed that restoring PTEN function in PTEN-defective cells can radiosensitize human glioma and prostate carcinoma cells[3]. In this study, we found that PTEN transduction sensitized U251 cells to radiation. Moreover, we showed that PTEN transduction resulted in a significant increase in the number of γH2AX foci, which appeared within minutes following irradiation. These results suggest that PTEN affects the process of DNA repair. Moreover, we detected a reduction in the levels of DNA-PKc and Ku70/80 phosphorylation following irradiation.

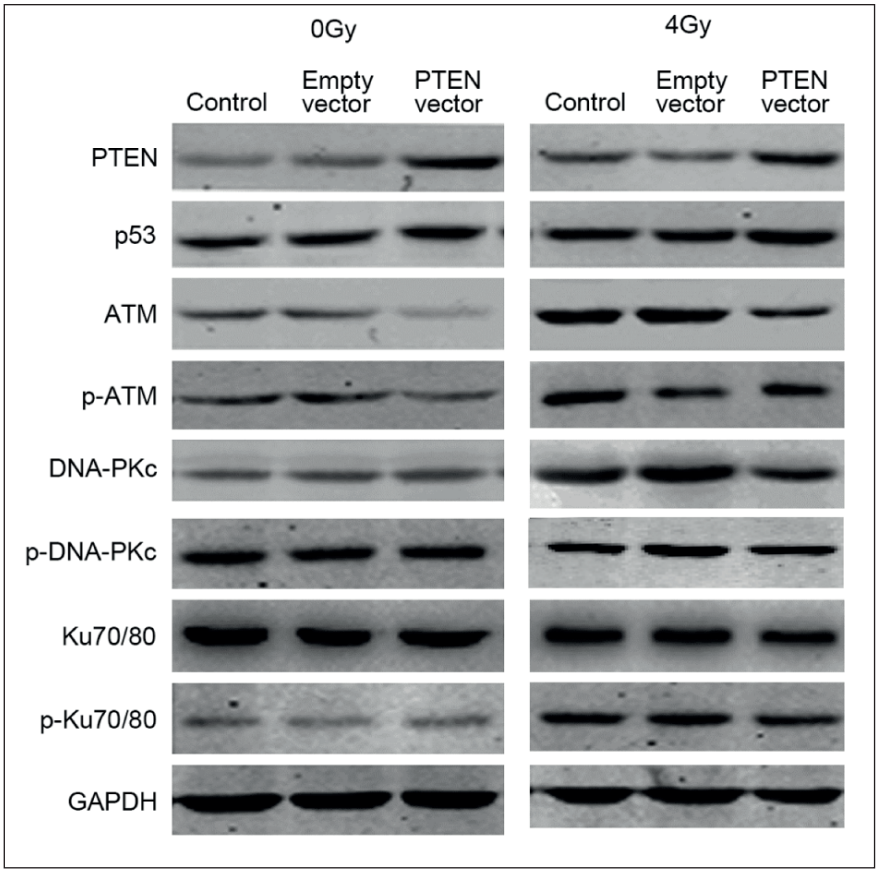

Figure 4. Proteins that participate in DDR and DSB repair before and after transduction with PTEN.

Thus, PTEN may reduce DNA-PKc and Ku70/80 phosphorylation, as well as inhibit DSB repair. Consistent with these findings, we pointed out that the enhanced radiosensitivity induced by PTEN was correlated with an increase in the expression of p53. While these results do not reveal the mechanisms utilized by PTEN to enhance radiosensitivity, they provide insight for future researches. Our results indicate that radiosensitivity is linked to a reduced cellular ability to repair DSBs and activate the p53 pathway, promoting apoptosis.

Conclusions

In the present study PTEN affects radiosensitivity by reducing DSB repair and enhancing the p53 pathway, leading to increased apoptosis.

Statement of funding interests

This study was funded by the National Natural Science Foundation of China [Grant No. 81171887].

Conflict of Interests

The authors declare no conflict of interest.

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