Overexpression of TOB1 confers radioprotection to bronchial epithelial cells through the MAPK/ERK pathway

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Abstract. The aim of this study was to investigate the effects and mechanisms of antiproliferative transducer of erbB2, 1 (TOB1) on the radiosensitivity of the normal human bronchial epithelial cell line HBE. After exposure to different doses of irradiation or a certain dose for different time intervals, the expression of TOB1 mRNA and protein in HBE cells was determined by semi-quantitative RT-PCR and western blot analysis. Liposome-induced recombinant plasmid transfection and G418 selection were performed to establish a stably trans-fected TOB1-overexpressing HBE cell line. A clonogenic assay was used to determine the radiosensitivity of the HBE cells with different TOB1 expression statuses. The cell cycle distribution was detected by flow cytometry. The ionizing radiation (IR)-induced γ-H2AX foci formation was detected by immunofluorescence assay. The related mechanism was explored by western blot analysis. TOB1 expression in the HBE cells was not induced by IR, neither dose-dependently nor time-dependently. Compared to the parental or ‘mock’ transfected HBE cells, the radiosensitivity of HBE cells overexpressing TOB1 was significantly decreased (P<0.05). Exogenous TOB1 prevented HBE cells from apoptosis after IR, in contrast to the control cells (P<0.05), and significantly decreased the IR-induced γ-H2AX foci formation. After IR, the expression of DNA damage repair proteins such as XRCC1, MRE11, FEN1 and ATM was increased in the TOB1-overexpressing HBE cells when compared with the expression levels in the control cells. HBE/TOB1 cells presented a much higher phosphorylated ERK1/2 and phosphorylated p53 when compared with the levels in the control cell lines when receiving 6 Gy of X-rays. Notably, the increased expression of phosphorylated p53 in HBE/TOB1 cells after IR was sufficiently blocked by U0126, a specific inhibitor of MEK1/2. Different from its functions in several lung cancer cell lines, TOB1 demonstrated a radioprotective function in the immortalized normal human bronchial epithelial cell line HBE via the MAPK/ERK signaling pathway.

Introduction

In thoracic tumor radiotherapy, radiation-induced pulmonary injury (RIPI) is the main dose restrictive and common complication which seriously impairs the local control of thoracic tumors, particularly non-small cell lung cancer that is unsuitable for surgery (1,2). Moreover, RIPI affects the prognosis of lymphoma or breast cancer patients who were confirmed to have extended survival following radiotherapy (2,3). Thus, it is necessary to overcome the restraint of RIPI, in order to improve the efficacy of thoracic radiotherapy in the future.

The transducer of erbB2, 1 (TOB1) gene is located on chromosome 17q21, and was originally discovered as a member of the antiproliferative protein family TOB/BTG (4). Numerous studies have revealed the tumor-suppressor properties of TOB1 in different types of human malignancies (5-9). Recently, our laboratory also confirmed that TOB1 may radiosensitize human cancer cell lines such as cervical cancer cell line HeLa, breast cancer cell lines MCF-7 and MDA-MB-231 (10,11), and human lung cancer cell line A549 (unpublished data). However, few studies have investigated the influence of TOB1 on the radiosensitivity of normal tissues or cell lines.

In the present study, parental normal human epithelial cell line HBE and HBE-overexpressing TOB1 were used as cell models. RT-PCR, western blot analysis, clonogenic assay and immunofluorescence assay were used to reveal the effects of TOB1 in regards to the radiation protective effects on normal cells. Flow cytometric assay, western blot analysis and pathway-specific inhibitors were applied to elucidate the related mechanisms. Together with previous studies, we report the theoretical and experimental data on clinical lung cancer radiotherapy and radiation protection.

Materials and methods

Cell culture, transfection and irradiation. Human bronchial epithelial cells were originally obtained from the American
Type Culture Collection (ATCC, Manassas, VA, USA), maintained in our laboratory and cultured with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Invitrogen), at 37°C in an atmosphere of 5% CO₂. Medium was replaced every 2 days, and cells were subcultured every 3 days. Recombinant plasmid pcDNA3.0/TOB1 was constructed by our laboratory as previously described (10). HBE cells were transfected with the vector and pcDNA3.0/TOB1 using Lipofectamine (Invitrogen) and maintained in medium containing 500 µg/ml G418 (Sigma Aldrich, MO, USA) until resistant cell clones were formed. Irradiation was performed with 6-MeV X-ray linear accelerator (Siemens KD2, Germany) with a dose rate of 200 cGy/min, with an irradiating area of 20x20 cm and 100-cm source-skin distance.

Clonogenic assay. The exponentially growing cells were plated at different cell densities and irradiated with 0, 0.5, 1, 2, 4, 6 Gy of X-rays. After 12-14 days of incubation at 37°C, cells were fixed in methanol followed by Giemsa staining. The number of colonies per dish was counted, and the surviving fractions were calculated as the ratio of plating efficiencies for irradiated and unirradiated cells. Plating efficiency is defined as the colony number divided by the number of cells plated for unirradiated controls. Experiments were conducted in triplicate, and data are presented as means ± standard deviation (SD) from three independent experiments. All surviving fractions were fitted into the linear quadratic model using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Flow cytometric assay. Cells were removed with trypsin and collected into centrifuge tubes together with the culture medium. The detailed methods for flow cytometry and Annexin V-FITC apoptosis analysis have been previously described (10). The cell cycle distribution and apoptotic rate were calculated from 10,000 cells using ModFit LT software (Becton-Dickinson, San Jose, CA, USA) using FACSCalibur (Becton-Dickinson).

Immunofluorescence detection for phosphorylated γ-H2AX. Cells were grown on sterile glass chambers (Becton-Dickinson and Company, Franklin Lakes, NJ, USA). After a single dose of X-ray exposure, all cells were fixed in 4% formaldehyde before being permeabilized in 0.25% Triton X-100. The chamber was blocked with 1% BSA for at least 1 h. The primary anti-phosphorylation monoclonal antibody γ-H2AX (1:200, Epitomics, Burlingame, CA, USA) was added and incubated for 2 h at room temperature. Specific staining was visualized with a secondary antibody conjugated to FITC 488. Hoechst 33342 (Sigma Aldrich, St. Louis, MO, USA) was utilized for nuclear staining. Fluorescence was observed using a laser scanning confocal microscope at a wavelength of 488 nm within 30 min. The number of foci in 20 cells in each sample was counted randomly.

Western blot analysis. Western blot analysis was performed as previously described (10). Briefly, the following primary antibodies were used for immunoblotting: β-actin (C-4), TOB1 (N-20), XRCC 1 (33-2-5), FEN1 (B-4), ATM (C-20), MRE11 (C-16) (1:1000 dilution; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands were visualized using an enhanced chemiluminescence system (Hangzhou Youither Bioscience Co., Ltd., Hangzhou, China) with prestained markers as molecular size standards.

RT-PCR assay. TOB1 mRNA expression was determined using semi-quantitative RT-PCR assays. The PCR reaction conditions and cycle numbers were rigorously adjusted so that each reaction occurred within the linear range of amplification. The detailed methods for RNA isolation, cDNA synthesis and RT-PCR analyses have been previously described (10,12). For specific intent genes, the PCR primers were as follows: GAPDH sense, 5'-CAA CTA CAT GGT CTA CAT GTT CC-3' and antisense, 5'-CAA CCT GGT CCT CAG TGT AG-3'; TOB1 sense, 5'-GGA TCG ACC CAT TTG AGG TTT CT-3' and antisense-5'-CTA CCC AAG CCA AGC CCA TAC AG-3'. The PCR products were analyzed via electrophoresis through 1% agarose gels containing 0.1 mg/ml ethidium bromide (EB). The gels were photographed under ultraviolet light.

Statistical comparisons. The data are presented as means and standard deviations. Statistical comparisons of the experimental results between the treated group and the control group were carried out using the two-tailed Student's t-test. All statistical tests were performed using the SPSS version 17.0. P-value <0.05 between groups was considered statistically significant.

Results

TOB1 expression in the normal human bronchial epithelial cell line HBE is not induced by ionizing radiation (IR). HBE cells were respectively irradiated with 0, 2, 4 and 8 Gy of X-rays generated by a linear accelerator and harvested after 24 h. The cell samples were also collected 2, 12 and 24 h after exposure to 6-Gy irradiation. The relevant expression level of TOB1 mRNA and protein was determined by semi-quantitative RT-PCR and western blot analysis. As shown in Fig. 1, IR did not increase the expression level of TOB1, neither in a dose-dependent nor in a time-dependent manner.

Overexpression of TOB1 reduces the sensitivity of HBE cells to IR. To investigate whether TOB1 overexpression affects the radiosensitivity of HBE cells, the TOB1 stable transfectant (HBE/TOB1) was used. The TOB1 expression was determined by western blot analysis (Fig. 2A). As shown in Fig. 2B, the parental HBE cell line and 'mock' transfectant HBE/PC3 both showed a typical clonogenic survival curve with a shoulder representing the potential DNA damage repair, while exogenous TOB1 significantly protected HBE cells from the IR-induced damage.

Flow cytometry assay was also performed 24 h after a single-dose irradiation of 6-Gy of X-rays. As shown in Fig. 2C, all cell lines exhibited typical IR-induced cell cycle G2/M arrest without significant difference (P>0.05). However, the apoptotic cell portion of HBE/TOB1 (24.1%) was obviously less than HBE (32.98%) and HBE/PC3 (34.39%) after IR (P<0.05) (Fig. 2D).

Exogenous TOB1 increases DNA damage repair in HBE cells. In order to gain insight into the molecular mechanisms...
of the radioprotective effect of TOB1 and to investigate its effect on the initial DNA damage response to IR, the induction of DNA double-strand breaks (DSBs), as analyzed by the formation of phospho-γ-H2AX foci, was measured 2 h after irradiation of the HBE cells, HBE/PC3, and HBE/TOB1 cells. The results showed that IR-induced γ-H2AX foci were

Figure 1. TOB1 expression is not induced by ionizing radiation (IR) in HBE cells. (A) To assess the dose response, HBE cells were treated with different doses of IR and then harvested for mRNA and protein analysis 24 h later, using GAPDH and β-actin as loading controls, respectively. (B) For assessment of the time dependence, after exposed to 6 Gy of X-ray, TOB1 mRNA and protein expression in HBE cells was examined at different time points. All the experiments were performed independently at least three times.

Figure 2. Overexpression of TOB1 reduces HBE cell sensitivity to ionizing radiation (IR). (A) Exponentially growing HBE, HBE/PC3, and HBE/TOB1 cells were collected by trypsin and centrifuge. Western blot analysis was then performed to detect TOB1 expression, β-actin was used as a loading control. (B) Cells transfected with indicated plasmids were irradiated with a single dose of 0.5, 1, 2, 4 or 6 Gy. Parental and ‘mock’ transfected cells (0 Gy) were used as control. Colonies >50 cells were counted and determined by Giemsa staining at least 14 days after IR. Results are represented as means ± SD. *P<0.05 vs. the control group. (C) Cells were treated with a single dose of 6 Gy IR and incubated for an additional 24 h. After fixation with 70% ethanol followed by PI staining, the DNA contents were measured by flow cytometric analysis. (D) Flow cytometric analysis was performed to detect the binding of Annexin V to externalized phosphatidylserine in conjunction with PI in viable cells (Annexin V/PI−), late apoptotic cells (Annexin V+/PI+); necrotic cells (Annexin V+/PI+); and apoptotic cells (Annexin V+/PI+). Twenty four percent of HBE/TOB1 cells were apoptotic (Annexin V+/PI+) after 6 Gy IR, while >30% of the parental or HBE/pcDNA3.0 cells were apoptotic. The result was representative of three individual experiments.
decreased in HBE/TOB1 cells 2 h post-radiation, in contrast to the control cells (Fig. 3A and B) suggesting facilitation of DNA damage repair.

Furthermore, we explored the underlying molecular mechanisms related to different IR-induced DNA damage responses due to TOB1 status. As shown in Fig. 3C and D,
compared with the HBE/PC3 and parental cells, the expression levels of important DNA repair proteins such as XRCC1, MRE11, FEN1 and ATM were increased in the HBE/TOB1 cells after IR.

**TOB1 regulates the radiosensitivity of HBE via the MAPK/ERK signaling pathway.** Phosphorylation and subsequent activation of p53, as well as its transcription induction, are key initial responses to stress responses (13). Therefore, western blot analysis was used to investigate the related mechanisms through which TOB1 is involved in radioprotection of normal cells. In the present study, our results revealed that overexpression of TOB1 significantly increased IR-induced phosphorylation of p53 and ERK1/2 (Fig. 4).

We then explored the specific signaling cascade involved in this response by using the MEK1/2 inhibitor (U0126) specific to the MAPK/ERK signaling pathway. The increased phosphorylation of p53 increased by TOB1 overexpression after IR was blocked by U0126 (Fig. 4B).

**Discussion**

At present, although 70% of cancer patients require radiotherapy, it is still a difficult task to protect adjacent normal tissue and to increase the precision of dose delivery to the target tumor for radiotherapy (14,15). Gene therapy targeted by radiation may be a promising solution in which the complementary DNA for a cytotoxic gene is ligated downstream of IR-inducible promoters, which involve the expression of immediate early genes (16). In our previous study, it was demonstrated that TOB1 was IR-inducible in lung cancer cell lines A549 and NCI-H1975, and that TOB1 radiosensitized lung cancer cells through certain signaling pathways (unpublished data). However, for the first time, we revealed the different biological functions of TOB1 in the immortalized normal cell line HBE. The expression of TOB1 was neither induced by X-rays, nor did it increase the radiosensitivity of HBE cells (Figs. 1 and 2). On the contrary, TOB1 overexpression obviously decreased the sensitivity of HBE to X-ray irradiation (Fig. 2B). All of these results together provide us with clues to the potential gene therapy application of TOB1 in clinical radiotherapy.

A double-strand break (DSB) of DNA, the main biological target of IR, determines mutation or cell death after IR (17-19). The ability to repair DSBs determines the sensitivity of mammalian cells to IR (18,20). The recruitment of several proteins to the damage site is the initial cellular response to DSBs, through which γ-H2AX focus formation is one of the earliest events (19,21). In our study, exogenous TOB1 reduced the amount of γ-H2AX foci induced by IR in the HBE cells (Fig. 3A and B), and decreased the percentage of apoptotic HBE cells after IR (Fig. 2D), which indicated either a decrease in DNA damage or an increase in DNA repair.

In response to IR-induced DSBs, DNA damage signals are transmitted through several pathways, from which ataxia telangiectasia-mutated (ATM) signaling plays perhaps the most important role (13,22,23). IR-induced ATM activation leads to the phosphorylation not only of γ-H2AX, but also of other important downstream effectors of DDR (apoptosis-related p53 and MDM2; cell cycle checkpoint-related CHK1/2; DNA repair-related MRE11) (13,24-26). In our study, exogenous TOB1 obviously increased the expression of ATM and its downstream DNA repair effectors XRCC1, MRE11, and FEN1 (Fig. 3C and D). These data suggest that TOB1 overexpression promotes IR-induced DNA repair, thus, further confirming the feasibility of TOB1 application for thoracic radiotherapy.

Our previous studies demonstrated that the MAPK/ERK pathway plays a significant role in cell survival after IR regulated by TOB1 (unpublished data). We demonstrated in this study that TOB1 overexpression enhanced the phosphorylation status of ERK1/2 in HBE cells, which was mainly associated with MAPK activation (Fig. 4A). After exposure to IR the activated MAPK pathway leads to the phosphorylation of p53 at multiple sites, which are key initial responses to cell cycle distribution and DNA damage (27,28). Our results revealed that exogenous TOB1 strongly enhanced IR-induced p53 phosphorylation in normal HBE cells, suggesting these cells have a more robust DNA damage surveillance and repair mechanism helping them either adapt to or overcome critical IR-induced DNA damage (Fig. 4A). Furthermore, the MAPK/ERK pathway-specific inhibitor U0126 was used just following IR. As shown in Fig. 4B, the serine 15 phosphorylation of p53 after IR was significantly inhibited by U0126 in the HBE/TOB1 cells.

In conclusion, although the mechanisms of how TOB1 is involved in IR-induced DNA damage are still to be fully elucidated, the results of the present study together with previous results in lung cancer cell lines, suggest that gene therapeutic approaches enhancing TOB1 expression may alleviate the side effects of radiotherapy as well as enhance radiotherapeutic benefit.

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**References**