Glucose-regulated protein 75 overexpression attenuates ionizing radiation-mediated injury in PC12 cells by inducing the expression of topoisomerase IIα

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Abstract. Glucose-regulated protein 75 (Grp75), also known as mortalin/mthsp70/PBP74, is a member of the heat-shock protein 70 (HSP70) family. Grp75 is known to protect cells from stress-induced injury. Previous studies have shown that the expression of Grp75 is upregulated by a low dose of ionizing radiation (IR). To evaluate the protective role of Grp75 on cell proliferation in response to IR, Grp75 was overexpressed in a rat adrenal pheochromocytoma cell line (PC12). It was revealed that Grp75 overexpression desensitized PC12 cells to IR-mediated cell injury. In addition, the expression of topoisomerase IIα (Topo IIα) was downregulated in PC12 cells following γ-ray IR. The effect of Grp75 overexpression on Topo IIα expression was examined. It was revealed that Grp75 overexpression reversed the inhibitory effect of IR on Topo IIα expression. In conclusion, the data indicated that Grp75 overexpression attenuates IR-induced injury in PC12 cells by maintaining the expression of Topo IIα.

Introduction

Ionizing radiation (IR) induces a variety of cellular responses at clinically relevant doses. MCF-7 cells were arrested at the G1 and G2 phases of the cell cycle and MDA-MB231 cells were arrested at the G2 phase in response to IR (1,2). It has been demonstrated that X-rays at extremely low doses, between 1-5 cGy, stimulate cell proliferation, whereas at doses >1 Gy, IR is lethal to cells (3). Topoisomerase IIα (Topo IIα), a nuclear enzyme involved in a number of cellular processes, is necessary for eukaryotic survival. Topo IIα is significant in the replication and repair of DNA and takes part in chromosome condensation and segregation during mitosis (4). Previous studies have demonstrated that DNA Topo IIα expression levels correlate with chromatid radiosensitivity (5). Topo IIα has also been shown to play a significant role in male mouse meiosis and its activity is required at the metaphase-anaphase transition of the two meiotic divisions for proper chromosome disjunction (6,7). The expression of Topo IIα changes periodically during cell growth and therefore, Topo IIα is thought to be a marker of cell proliferation (8-10). Topo IIα expression is affected by numerous environmental factors, including heat-shock and IR. It has been reported that, if the activity of Topo IIα is restrained or the distribution of Topo IIα in cells changes as the result of genetic mutations, the aneuploid ratio of the cells increases (11,12).

Grp75, a member of the HSP70 family, is involved in multiple functions that are required to maintain cell metabolism, including the stress response, cell proliferation and differentiation (13). Previous studies have demonstrated that Grp75 levels appear to correlate with mitochondrial activity and biogenesis (14) and that Grp75 expression is induced by cerebral ischemia (15), glucose deprivation (16) and low doses of IR (17). The gene expression of Grp75 in HT29 and MCF-7 cells was upregulated following exposure to IR (17,18). To investigate the potential link between the expression of Grp75 and Topo IIα and how they affect cell proliferation in response to low dose IR, the IR-induced proliferation and Topo IIα expression levels in regular and Grp75-overexpressing PC12 cells were analyzed.

Materials and methods

Cell culture. The rat adrenal pheochromocytoma cell line PC12 used in this study was obtained from the Chinese Academy of Sciences, Beijing, China. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml; Gibco) and streptomycin (100 mg/ml; Gibco), in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

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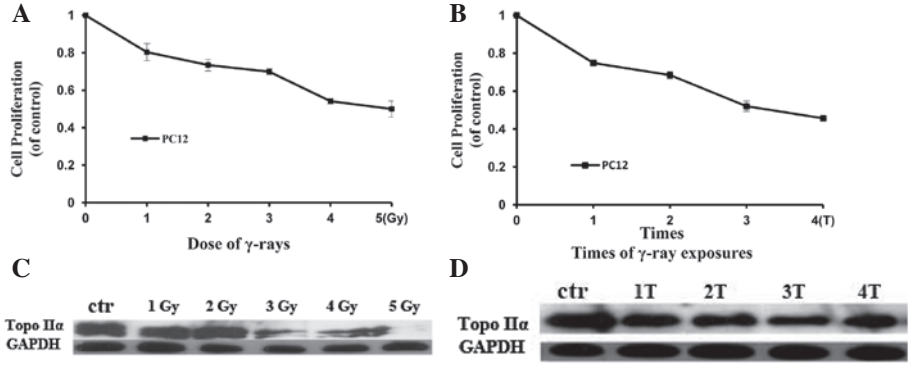

Figure 1. Inhibitory effects of IR on PC12 cell proliferation and the expression of Topo IIα in PC12 cells. (A) Increasing doses of IR inhibit PC12 cell proliferation. Cells were exposed to 1-5 Gy γ-rays. Following treatment, cells were cultured for 24 h and proliferation was determined by MTT. (B) Repeated IR exposure inhibited proliferation of PC12 cells. Cells were exposed to 3 Gy γ-ray IR from 1-4 times at an interval of 24 h. Following treatment, cells were cultured for 24 h and proliferation was determined by MTT. (C) Dose-dependent effects of IR on Topo IIα expression. PC12 cells were treated with 1-5 Gy, respectively. The treated cells were harvested after incubating for 24 h. Topo IIα expression was determined by western blotting. GAPDH was used as a loading control. Representative results of three independent experiments are shown. (D) Accumulative effects of IR on the expression of Topo IIα. PC12 cells were exposed to 3 Gy IR from 1-4 times at an interval of 24 h. The treated cells were harvested after incubation for 24 h. The determination of the expression of Topo IIα was the same as (C). IR, ionizing radiation; Topo IIα, topoisomerase IIα.

Cell transfection. The coding sequence of the Grp75 gene (NM_001100658) was cloned and inserted into pcDNA3 vectors. The plasmids of the pcDNA3-Grp75 and pcDNA3 vectors were separately transfected into PC12 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Transfected cells stably expressing Grp75 were selected using 800 μg/ml of G418 after 48 h. Control cells were transfected with pcDNA3 alone. Grp75 expression in transfected PC12 cells was determined by western blot analysis.

IR treatment. Cells were exposed to various doses of IR or the same dose of IR at a regular intervals (Gammatron-3; dose rate, 0.0244 Gy/min) at room temperature. To measure radiation sensitivity, the cells were seeded on four 100-mm culture dishes at a density of $5x10^5$ cells per dish and 24 h later they were irradiated with various doses of IR, ranging from 1 to 5 Gy or with 3 Gy of γ-rays 1-4 times at regular intervals of 24 h. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) 24 h after IR treatment and harvested for western blotting. Experiments were repeated at least three times.

Cell proliferation assay. Cells were seeded in 96-well tissue culture plates at a density of $5x10^3$ cells per well. The following day, cells were treated with IR at the indicated doses and were incubated for 24 h. Cell proliferation was determined using an MTT assay. Fresh medium containing 0.5 mg/ml MTT (Amresco, Solon, OH, USA) was added to each well. Following incubation at 37˚C for 4 h, the MTT solution was removed and replaced with approximately 150 μl of dimethylsulfoxide. The cells were then incubated for another 10 minutes at 37˚C with gentle agitation. The absorbance was measured using a microplate reader (Thermo Scientific, Anaheim, CA, USA) at a wavelength of 492 nm. Experiments were performed in quadruplicate and repeated at least three times.

Western blot analysis. The cells were washed and lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% Doc, 0.1% sodium dodecyl sulfate, 1 μg/ml of aprotinin and 100 μg/ml of phenylmethylsulfonyl fluoride supplemented with phosphatase inhibitor (Sigma, St. Louis, MO, USA) cocktails. Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to 0.45-μm polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Amersham, UK) in a Bio-Rad Trans-Blotting apparatus (100 V, 90 min). The membranes were blocked for at least 1 h in 5% (W/V) non-fat milk in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.05% Tween-20; pH 7.0). The membranes were incubated with primary antibodies in blocking buffer at 4˚C overnight. After being washed with 0.2% TBS-T for 20 min, the membranes were incubated with horseradish peroxidase conjugated secondary antibody for 45 min at room temperature and were visualized with the enhanced chemiluminescent reagents (Pierce Biotechnology Inc., Rockford, IL, USA). Antibodies specific for Grp75 and GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA) and Topo IIα was purchased from BioWorld (Visalia, CA, USA).

Statistical analysis. The data are expressed as the mean ± SEM from at least three replicates of the experiment. The comparison of groups was performed using a Student's t-test or ANOVA. $P<0.05$ was considered to indicate statistically significant differences.

Results

Inhibitory effect of IR on PC12 cell proliferation. To determine whether the inhibitory effect of IR on cell proliferation acted in a dose-dependent manner or whether it correlated with treatment frequency, PC12 cells were either treated with IR for various doses from 1-5 Gy or repeatedly treated with IR (3 Gy of γ-rays) from 1-4 times at regular intervals of 24 h. Cell proliferation was analyzed by an MTT assay 24 h after treatment. As shown in Fig. 1A and B, the cells demonstrated a gradual decrease in proliferative ability when compared with control cells with increasing doses or frequency of IR treatment. These results suggest that IR exposure not only inhibits

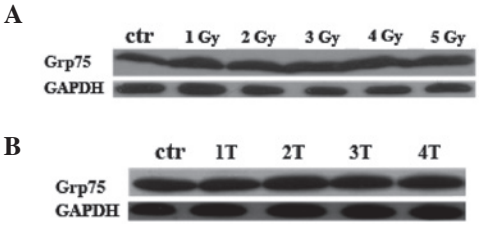

Figure 2. IR induces Grp75 expression in PC12 cells. (A) Dose-dependent effects of γ-ray IR on Grp75 expression. PC12 cells were treated with IR at various doses from 1-5 Gy. The treated cells were harvested 24 h after IR. Grp75 level was determined by western blot analysis. GAPDH was used as an equal loading control. Representative results of three independent experiments are shown. (B) Accumulative effects of IR on the expression of Grp75. PC12 cells were exposed to 3 Gy IR from 1-4 times at an interval of 24 h. The treated cells were harvested after incubating 24 h. The determination of the expression of Grp75 was the same as (A). IR, ionizing radiation.

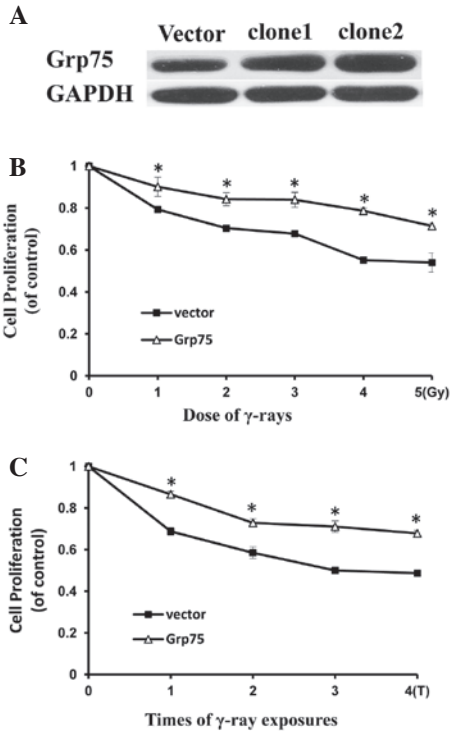

Figure 3. Grp75 overexpression protected against IR-induced cell injury in PC12 cells. (A) Western blot analysis of Grp75 protein expression in untransfected controls (endogenous), empty vector-transfected (vector) and pcDNA3-Grp75 transfected (clone) PC12 cells. GAPDH was used as an equal loading control. Representative results of three independent experiments are shown. (B and C) The effects of Grp75 overexpression on the IR-induced decrease of PC12 cell proliferation. Empty vector-transfected cells and Grp75-overexpressing cells were treated with 1-5 Gy γ-ray (B) and treated with 3 Gy IR from 1-4 times at an interval of 24 h (C). The proliferation of the treated cells was determined by MTT after incubating for 24 h. Data are expressed as the percentage of the control and presented as mean ± SD for four independent experiments. *P<0.05, versus control. IR, ionizing radiation.

the proliferation of PC12 cells but such inhibition is dependent on either dose or time of treatment.

Previous studies have demonstrated that Topo IIα plays a crucial role in DNA replication and therefore is significant in cell proliferation (19). To explore a potential link between the inhibition of cell proliferation induced by IR and Topo IIα expression, the level of Topo IIα protein was analyzed in PC12

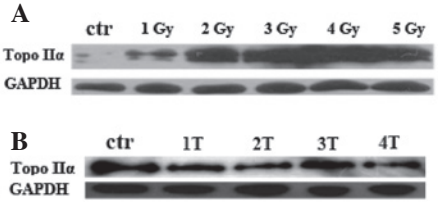

Figure 4. The overexpression of Grp75 increased the expression of Topo IIα in response to IR treatment, determined by western blot analysis of Grp75-overexpressing PC12 cells treated with IR. (A) Dose-dependent effects of IR on Topo IIα expression in Grp75-overexpressing PC12 cells. The cells overexpressing Grp75 were treated with 1-5 Gy. The treated cells were harvested after incubating for 24 h. Topo IIα was determined by western blotting. GAPDH was used as a loading control. Representative results of three independent experiments are shown. (B) Accumulative effects of IR on the expression of Topo IIα in Grp75-overexpressing PC12 cells. The stable cells were exposed to 3 Gy IR from 1-4 times at a regular interval of 24 h. The treated cells were harvested after incubation for 24 h. The determination of the expression of Topo IIα was essentially the same as described in Fig. 2. IR, ionizing radiation; Topo IIα, topoisomerase IIα.

cells. These cells were either treated with IR at various doses from 1 to 5 Gy or repeatedly treated with IR (3 Gy) from 1 to 4 times at regular intervals of 24 h, and the level of Topo IIα protein was measured 24 h after treatment by anti-Topo IIα western blot analysis. As shown in Fig. 1C and D, Topo IIα protein was downregulated by IR treatment. Inhibition of Topo IIα expression was observed at 1 Gy and following the first treatment of IR, Topo IIα protein level decreased as the dose of IR and frequency of IR treatment increased. In IR dose-dependent or frequency of treatment-dependent experiments, the observed decreased cell proliferation correlated with the reduced expression level of Topo IIα protein. These data suggest that IR inhibits PC12 cell proliferation by repressing the expression of Topo IIα protein.

The expression of Grp75 was induced by IR in PC12 cells. Previous studies have reported that the expression of Grp75 is inducible in response to IR treatment (17,18). However, there is no evidence concerning whether the induction of the Grp75 gene depends on the amount of IR or the frequency of IR. In this study, the regulation of Grp75 expression in PC12 cells by various doses or frequencies of IR treatment was examined. The level of endogenous Grp75 protein was determined by western blot analysis 24 h after treatment. As shown in Fig. 2, increasing doses/frequency of IR treatment gradually led to the upregulation of Grp75 protein. These data demonstrate that the expression of Grp75 in PC12 cells is induced by IR in a dose- and frequency-dependent manner.

Grp75 overexpression protects PC12 cells against IR-induced injury. The expression of Grp75 has been shown to be adaptively increased in response to IR (Fig. 2). This study investigated whether the overexpression of Grp75 protects the cells from IR-induced injury. PC12 cells were transfected with a pcDNA3.1-Grp75 vector to enhance the expression of Grp75 protein and determine the expression of Grp75 using western blotting. As shown in Fig. 3A, enhancement of Grp75 expression in positive clone2 was evident compared with control cells transfected with an empty vector. The effect of IR on the proliferation of PC12 cells overexpressing Grp75 was

then examined. As shown in Fig. 3B and C, PC12 cells overexpressing Grp75 demonstrated a moderate decrease in cell proliferation upon IR treatment when compared with controls. These results indicate that Grp75 overexpression reduced IR injury in PC12 cells and thus overexpressing Grp75 protected PC12 cells against IR injury.

The level of Topo IIα protein in Grp75-overexpressing PC12 cells treated with IR was examined (Fig. 3A and B). Western blot analysis demonstrated a dose-dependent expression of Topo IIα protein in response to increasing IR, whereas the level of Grp75 protein was almost constant during treatments 1-4 (Fig. 4A and B). These data suggest that the protective role of Grp75 against IR-induced cell proliferation inhibition acts through either enhancing or stabilizing the expression of Topo IIα protein, which plays a crucial role in cell proliferation.

Discussion

IR, which causes DNA damage and inhibits cell proliferation, has become a predominant therapeutic tool to treat tumors clinically. Consistent with previous studies, cell proliferation was inhibited after PC12 cells were exposed to IR. Notably, a dose/time-dependent inhibition of cell proliferation in IR-treated PC12 cells was demonstrated. It has been shown that Topo IIα is significantly involved in chromosome condensation during DNA replication and repair to confer cell survival and multiplication (20). Previous studies have reported that the repression of Topo IIα mRNA is induced by IR in 25 cell lines (21). However, the action of Topo IIα protein in response to various doses/frequencies of IR treatment has not been previously recognized. In keeping with previous studies, the expression of Topo IIα was downregulated in PC12 cells which were treated with IR (Fig. 1C and D). Significantly, IR inhibited the expression of Topo IIα protein in a dose- or frequency of treatment-dependent manner which was correlated with the observed inhibition of cell proliferation under the same treatment conditions. The data suggests that IR-induced Topo IIα protein repression may be responsible for IR-induced cell proliferation inhibition.

Grp75 is a member of the heat-shock protein family that is not heat-inducible and is mainly located at multiple subcellular sites, including in mitochondria. Grp75 has been implicated in various biological processes of human cells (13) and has been shown to respond to numerous forms of stress, including ischemia (15), glucose deprivation (16), oxidative injury (22) and drug resistance (23). In addition, data from previous studies have shown that the treatment of IR led to the upregulation of the Grp75 protein (17,18). In this study, the expression of Grp75 induced by IR was highlighted and a dose/time-dependent induction of Grp75 expression in response to IR treatment was identified (Fig. 2A and B). The level of Topo IIα protein in cells treated by IR and whether the overexpression of Grp75 could affect cell proliferation was then evaluated.

Grp75 overexpression markedly attenuated IR-induced cell injury as demonstrated by higher levels of cell proliferation and hence increased expression of Topo IIα (Fig. 3A and B). These results suggest that the stable expression of Grp75 desensitizes the cells to IR injury. Thus, it is reasonable to speculate that changes in Grp75 reflect a protective response in cells exposed to IR and maintaining the endogenous expression of Topo IIα might be one of the mechanisms by which Grp75 reduces cell injury induced by IR.

The expression of Grp75 gradually increased as the dose of IR increased, whereas the level of Topo IIα decreased. Although the expression of Grp75 protein is enhanced in response to increasing doses of IR treatment, the level of Grp75 protein remains too low to restore the expression of Topo IIα protein.

In conclusion, the current study reveals a protective effect of Grp75 against IR-induced cell injury which may be associated with the increased expression of Topo IIα, a marker of cell proliferation. This study focused on a cellular model of IR injury and further *in vivo* studies are required to confirm the protective effects of Grp75 against radiation damage. This is likely to aid in developing novel strategies to improve radiotherapy based on the manipulation of Grp75.

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