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Glycosylation modification of human prion protein provokes apoptosis in HeLa cells *in vitro*

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We investigate the correlation between the glycosylation modified prion proteins and apoptosis. The wild-type PRNP gene and four PRNP gene glycosylated mutants were transiently expressed in HeLa cells. The effect of apoptosis induced by PrP mutants was confirmed by MTT assay, Hochest staining, Annexin-V staining and PI staining. ROS test detected ROS generation within the cells. The mitochondrial membrane potential was analyzed by the flow cytometry. The expression levels of Bcl-xL, Bax, cleaved Caspase-9 proteins were analyzed by Western Blot. The results indicated that the expressed non-glycosylated PrP in HeLa cells obviously induced apoptosis, inhibited the growth of cells and reduced the mitochondrial membrane potential, and more ROS generation and low levels of the apoptosis-related proteins Bcl-xL, the activated the cleaved Caspase-9 proteins were found. The apoptosis induced by non-glycosylated PrP demonstrates that its underlying mechanism correlates with the mitochondria-mediated signal transduction pathway. [BMB reports 2009; 42(6): 331-337]

INTRODUCTION

Prion diseases are a group of transmissible neurodegenerative disorders, including Creutzfeldt-Jakob disease, Kuru, and Fatal Familial Insomnia in human beings (1). The conformational change of the prion protein from cellular type (PrP^C) to PrP^{Sc} is regarded as the crucial step in triggering the pathogenesis of the prion diseases (2-6). Human PrP^c is a protein of 253 amino acids, which contains two consensus sites for N-linked glycosylation. The first is located at codon 181 and the second at codon 197 (7). In the fully matured protein both *N*-glycosylation sites are occupied and typically four variants of prion protein co-exist: the double-glycosylated, two mono-glycosylated and the unglycosylated. It has been reported that lack of sugars would induce the transition of PrP^C to PrP^{Sc} *in vitro*, suggesting that the modification in glycosylation may contribute to the development of the disease (8). We thus investigated the correlation of apoptosis and glycosylations of PrP^{Sc} in the brain tissues of the hamsters infected with scrapie stain 263K (9). In addition, purified PrP^{Sc} from mouse scrapie brain induced apoptosis in N_2A neuroblastoma cells, GT1 cells, as well as in primary cerebella cultures (10-12). Also the inhibition of N-linked glycosylation using tunicamycin (TM) induced cell apoptosis in cultured cells (13). These findings suggest that the absence of N-linked glycosylation is associated with apoptosis.

In golden hamsters, PrP gene was reported to express in the brain and peripheral organs such as heart, liver, kidney and lung (14). In our work, we observed apoptosis induced by the expressed PrP glycosylation mutants in epithelial-derived (HeLa) cell lines *in vitro*. In this study, with HeLa cells as the comparison to SF126, we particularly explored whether the PrP mutants could expressed in a non-neural cell lines. The results on PrP mutant presented in HeLa cells offer valuable basic data that detection of the glycosylation levels of PrP, particularly in peripheral tissues could be an indirect yet effective indicator for the damage of the central nerve system. Additionally, we observed the difference between expression of the non-glycosylated modified PrP and the mono-glycosylated modified ones. We further studied of the possible mechanism of apoptosis induced by the expression of PrP mutants in HeLa cells. It offers some primary data for further studies of prion disease mechanism in this cell model.

RESULTS

Wild type PrP is exclusively presented on the cell surface, whereas non-glycosylated PrP is mainly intracellularly located

The immunofluorescence staining experiments showed that cells expressing wild type PrP were mainly transported to the plasma membrane (Fig. 1B). In contrast, those expressing two non-glycosylated mutants, PRNP N181Q N197Q and T183A T199A mainly located in cytoplasma (Fig. 1C, D) and apparently failed to reach the cell surface after synthesis, which indicated that surface transport of two non-glycosylated mutants is inefficient.

Growth suppression and viability assays

MTT data indicated that transfection of pcDNA3.1 group showed

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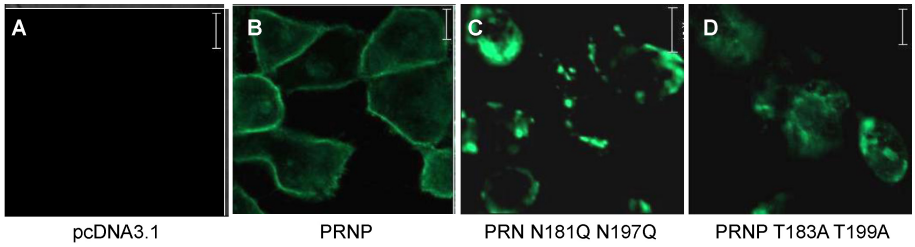

Fig. 1. Localization of wild type and mutated PrP in HeLa cell by immunofluorescence staining using confocal analysis. HeLa cells expressed wild type and mutated PrP after transfection for 24 h with Lipofectamine 2000. HeLa cells were labeled with anti-PrP antibody 3F4, and stained with a fluorescein-conjugated goat anti-mouse IgG antibody. All panels were photographed at the same exposure.

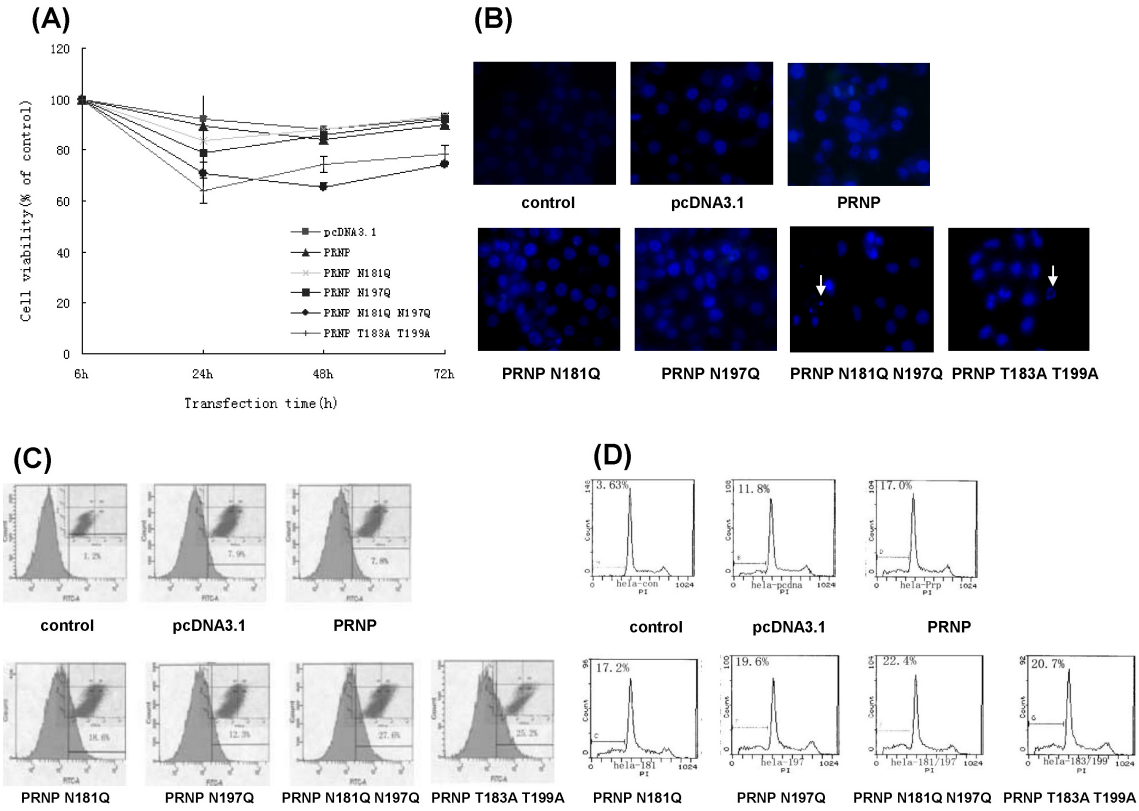

Fig. 2. Expressing PrPs mutants inhibiting cell growth and inducing apoptosis in HeLa cells. (A) Mutated PrPs expression caused inhibition of growth of HeLa cells *in vitro* by MTT assay. Data are means ± SD (n = 3) (B) Hochest staining assays of HeLa cells 30 h after transfected with PRNP gene mutants (×400). (C) Flow cytometry analysis of HeLa cells early apoptosis after transfected with PRNP gene mutants. (D) Flow cytometry analysis of HeLa cells apoptosis after transfected with PRNP gene mutants.

somewhat arrest effect of cell proliferation on the growth (Fig. 2A). No significant difference was observed in PRNP, PRNP N181Q and PRNP N197Q groups, compared to pcDNA3.1 group. However, the viability of cells in the groups transfected with two mutants of non-N-glycosylated plasmids PRNP N181Q N197Q and PRNP T183A T199A was significantly reduced compared to those of pcDNA3.1 group.

Expression of PrP mutants induced morphological change in transiently transfected HeLa cells

As shown in Fig. 2B, we observed that the transfection with two mutants of non-N-glycosylated plasmids induced a significantly higher apoptotic rate in HeLa cell line compared to those transfected with mono-N-glycosylated plasmids and the control group, whereas no apparent changes were observed between the groups transfected with two mono-N-glycosylated plasmids.

PS inverting analysis cell apoptosis in transiently transfected HeLa cells

Annexin-V and PI staining results are the indicators of early phase of apoptosis. As shown in Fig. 2C, the results indicated

that the percentage of early apoptosis in HeLa cells transfected with non-glycosylated plasmids increased remarkably compared to those of the control cells, transfection with PRNP N181Q N197Q and PRNP T183A T199A induced more apoptosis (27.6% and 25.2%). In contrast, transfection with mono-glycosylated plasmids did not show significant apoptosis (18.6% and 12.3%, respectively).

PI staining analysis cell apoptosis in transiently transfected HeLa cells

As shown in Fig. 2D, the precentages of apoptosis (peak of hypodiploid DNA before G1 phase) in HeLa cells transfected with PRNP N181Q N197Q and PRNP T183A T199A were 22.4% and 20.7%, respectively. The percentages of the apoptosis were 17.2% and 19.6% in two mono-glycosylated plasmids groups. Cells expressing wild type PrP showed the percentage of apoptosis at 17%.

ROS detection

We monitored ROS production with the specific fluorescent probe DCFH-DA (Fig. 3A). In comparison with wild-type PrP, ROS production in cells transfected with PRNP N181Q N197Q and PRNP T183A T199A was significantly increased at 6 h, 12 h, 24 h, 30 h, 36 h and 48 h in a time-dependent manner. And a peak of ROS production appeared at 12 h after transfection. Cells transfected with either of the two mono-glycosylated plasmids PRNP N181Q and PRNP N197Q generated more ROS compared to the control.

The mitochondrial membrane potential assay by flow cytometry

As shown in Fig. 3B, the mitochondrial membrane potential of Hela cells transfected with PRNP N181Q and PRNP N197Q was 75% and 83.2% at 48 h, compared to 83.8% of PNRP plasmid transfected cells. Furthermore, the mitochondrial membrane potential of cells transfected with PRNP N181Q N197Q and PRNP T183A T199A showed a more remarkable decrease to 67.2% and 75.7% respectively.

PrP mutants induced decreased expression of Bcl-xL and activation of cleavage of Caspase-9 by Western Blotting

As shown in Fig. 4, Bcl-xL expression was decreased at 48 h after transfection with various plasmids whereas Bax was not affected. Activation of cleaved caspase-9 was highest at 48 h after transfection with various plasmids.

DISCUSSION

Several evidences have shown that some pathogenic mutants (point and insertion mutants within PRNP gene) relate to familial prion diseases and might play an important role in TSEs. For example, one of the HuPrP mutants, HuPrP E200K[h] is closely related to familial Creutzfeldt-Jakob disease (fCJD) (15). A valine to isoleucine mutation at residue 180 was detected in a French patient with Creutzfeldt-Jakob disease (CJD) (16). We modified two glycosylation sites at amino acid residues Asn181, Thr183, Asn197 and Thr199 in human PrP to explore the effect of glycosylation on PrP biochemical properties and its cellular biological traits.

Inhibition of N-linked glycosylation by tunicamycin (TM) induces cell apoptosis such as the appearance of oligonucleosomally fragmented DNA in SV40-transformed cells (13). We had observed the apoptosis and changes in glycosylation ratio of PrP[Sc] in brain tissues of the hamsters infected with scrapie stain 263K (9). In this study, MTT test indicated that expression of mono-glycosylated and non-glycosylated PrP showed certain effect on the cell growth. This result is consistent with the previous findings in other systems (17). Both on the early and late stages of apoptosis, non-glycosylated modified PrP was

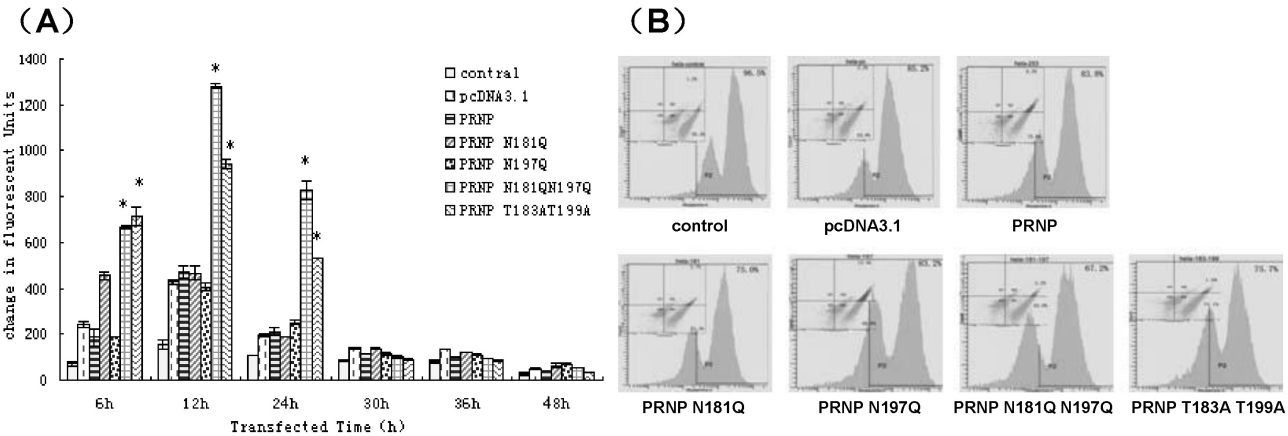

Fig. 3. (A) Generation of ROS in HeLa after the transfection with PRNP gene mutants (*represents $P < 0.05$). Values represented as mean ± S.D. *$P < 0.05$, $n = 3$. (B) Flow cytometry analysis of mitochondrial membrane potential collapse of HeLa cells after 48 h transfection with PRNP gene mutants using Rhodamine 123.

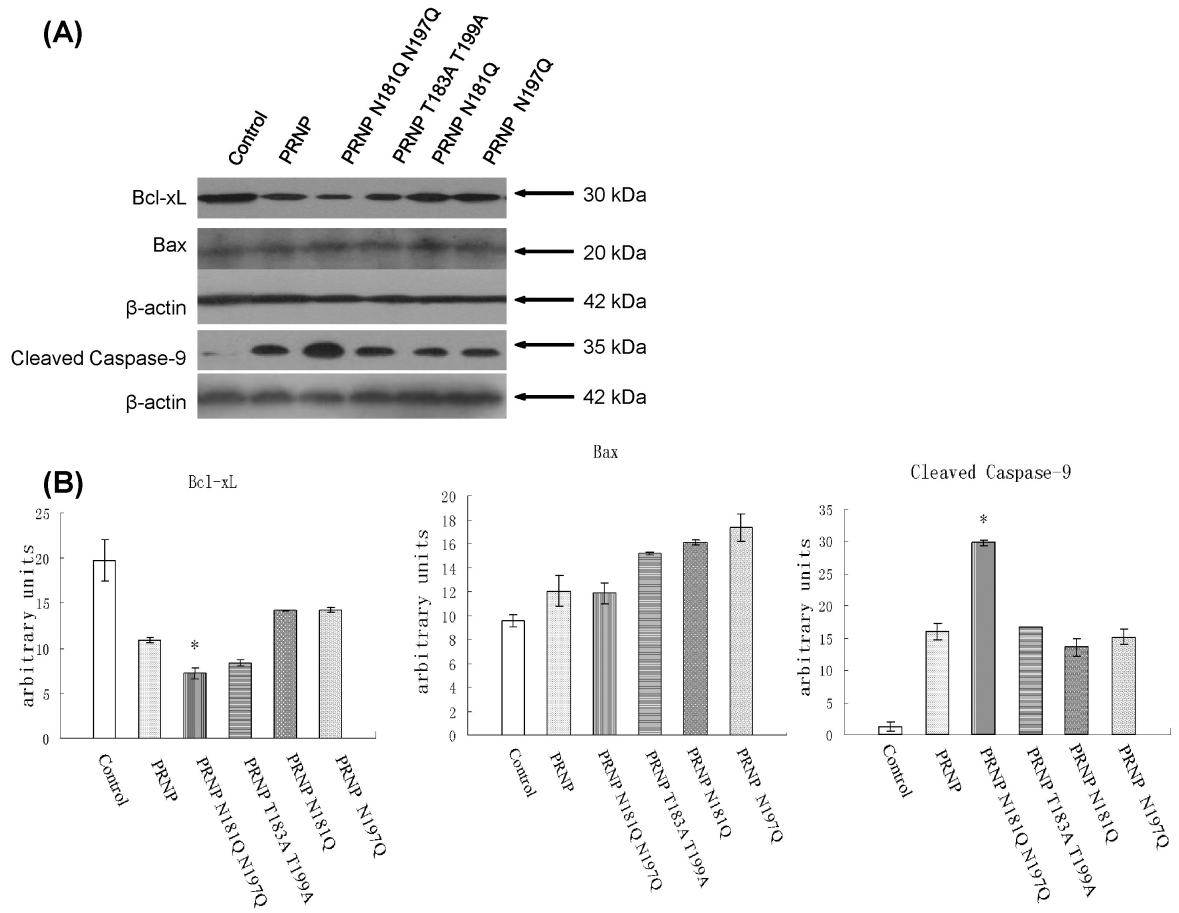

Fig. 4. Western Blot analysis of Bcl-xL Bax and Cleaved Caspase-9 in HeLa cells transfected with PRNP gene mutants (A) The expression levels of Bcl-xL, Bax, Cleaved Caspase-9 were compared by Western Blotting among total protein extract from transfected HeLa cells. (B) The intensities of Western Blot signals were quantified and plotted as described. The expression levels of Bcl-xL, Bax, Cleaved Caspase-9 were compared among PRNP gene mutants by using arbitrary units (mean ± SD). ANOVA, *P < 0.05, n = 3).

found to be much easier in triggering the apoptosis than the mono-glycosylated modified ones in PS inverting and PI staining analysis, which strongly suggests that the absence of N-linked glycosylation modification is associated with apoptosis. The degree of apoptosis was negatively proportional to the number of N-glycosylation chains. Some experiments suggested that the unglycosylated PrP located intracellularly and did not possess the traits of the PrP^{Sc} in the transgenic mice brains expressing differentially glycosylated PrP (18, 19). Misfolded PrP is transported to the cytosol which is strongly neurotoxic and included in the pathogenesis of prion diseases in cultured cells and transgenic mice (20, 21). The immunofluorescence result shown in Fig. 1 suggested that PrP glycosylation could significantly affect the PrP transportation. Our opinion is, most likely, other unmodified glycosylation site is capable of linking a glycan chain even only one glycosylation site is modified. Thus, to some degree, the mono-glycosylation does not affect PrP conformation as much as the deglycosylation ones.

Many reports suggest that oxidative stress might play an important role in the pathogenesis of some neurodegenerative disorders (22). N.T. Watt, *et al*. demonstrated that the PrP^{C} conformational conversion was correlated with the increased amounts of intracellular oxygen radicals which contribute to a pathway involved in oxidative stress (23). In our study, we demonstrated that ROS may be one of the apoptosis inducers, but not the product of the apoptosis which adds weight to the suggestion that ROS plays an important role in the early stages of apoptosis. We noticed that, as shown in Fig. 2, 3, overexpression of wild-type PrPc induced minor apoptosis and increased ROS level in HeLa cells. PrP normal function is known to be neuroprotection which is performed by cell surface signaling, antioxidant activity, or anti-Bax function. It was reported when cytosolic PrP was expressed from a cDNA lack-

ing both the N and the C-terminal signal peptides, accumulation of cytosolic PrP was cytotoxic (24). Therefore, the data in this study suggest that overexpression of wild-type PrP may result in the accumulation of cytosolic PrP which makes cells to be cytotoxic and induces ROS production. It was also known that mitochondrion was essential during the apoptosis of cell and the change of its function relates tightly to apoptosis (25). Some experiments in cell culture models have shown that PrP^C can prevent neuronal cell apoptosis and mitochondrial dysfunction (26, 27). In this work, expression of PrP on which N-linked glycosylation sites were removed induced a great reduction of the membrane potential of mitochondria compared to the wild-type PrP.

Bcl-2 family proteins, the important apoptosis-associated factors, were reported to interact with PrP in the yeast two-hybrid system (28). In the brains of 263K scrapie-infected hamsters the expression level of Bcl-2 mRNA and the protein were significantly decreased, whereas the expression levels of Bax mRNA and the protein were increased (29). Recent studies suggest that in aged transgenic mice overexpressing wild-type PrP could invoke mitochondria-mediated neuronal apoptosis which was accompanied by cytochrome c release, caspase-3 activity and DNA fragmentation (30). Our conclusion is that apoptosis induced by glycosylation mutant PrP related to the Bcl-xL/Bax network and the downstream factor cleaved caspase-9. The results of our current study indicate that the expression of PrP with N-linked glycosylation sites modified would induce apoptosis in HeLa cells, and di-glycosylated PrP is comparatively easier to facilitate the apoptosis than the wild-type PrP. However, PrP with only one glycosylation site mutated had an apoptosis-inducing effects similar to that of the wild-type. The most important conclusion based on our study is that the apoptosis correlates with the mitochondria-mediated signal transduction pathway, including ROS production, down-regulation of apoptosis-related proteins Bcl-xL, upregulation of Bax and the activation of cleaved Caspase-9 proteins.

It was found that PrP gene expression was prevalently presented in the brain and peripheral organs. In peripheral organs examined, inguinal lymph node showed a high level of expression similar to that of the overall brain; spleen and lung showed moderate levels of the expression (14). In our study, we had observed similar cell apoptosis induced by PrP mutants in both neuron- (SF126) (31) and epithelial-derived cell lines (HeLa), what is interesting is that non-glycosylated modified PrP was found to be much easier in triggering the apoptosis than the mono-glycosylated modified ones in PS inverting and PI staining analysis in both cells. Furthermore, the apoptosis correlated with the mitochondria-mediated signal transduction pathway, including down-regulation of apoptosis-related proteins Bcl-2 and Bcl-xL in both cells. This finding not only explaines various glycosylation levels at different stages of the prion diseases, but also suggests that detection of the glycosylation levels of PrP, particularly PrP in peripheral tissues could be an indirect yet effective indicator for the damage of the central nerve system because the similar modifications in glycosylation were observed in both central and peripheral nerve systems and the glycosylation levels correlated with the disease severity. Our results on PrP mutant presented here offer valuable baseline data for further studies of prion disease mechanisms in this cell model.

MATERIALS AND METHODS

Recombinant plasmids, cell lines and transient transfection

In our previous work, we had constructed the full-length human *PRNP* gene encoding the polypeptide consisting of the amino acid residues 1 to 253, PRNP N181Q, PRNP N197Q, PRNP T183A or *PRNP* T199A were genes in which one potential glycosylation sites at the 181^{st},197^{th},183^{rd} or 199^{th} codon were removed, respectively and two non-N-glycosylated human *PRNP* genes were named *PRNP* N181Q N197Q and PRNP T183A T199A. All constructs were cloned into the expression plasmid pcDNA3.1 and transfected into Human cervical carcinoma cell line (HeLa). According to the manufacturer's instruction, 2 μg recombinant plasmids and the mock plasmid pcDNA3.1 transfected the cultured cells using Lipofectamine 2000 transfection reagent (Invitrogen, USA).

Immunofluorescence staining

HeLa cells grown on glass coverslips were fixed at room temperature in PBS containing 4% paraformaldehyde for 30 min, and then washed twice. The cells were permeabilized in 0.5% Triton X-100 in PBS at room temperature (RT) for 30 min. The cells were blocked with 3% bovine serum albumin in PBS (blocking buffer) for 30 min at 37°C. After that, the cells were incubated at 4°C with primary antibodies 3F4 (1 : 50) in blocking buffer overnight. The cells were incubated with FITC conjugated secondary antibodies (1 : 200) in PBS at 37°C for 30 min. Coverslips were visualized by laser-scanning confocal microscopy with a Zeiss microscope.

MTT assay

Cells were harvested at 6 h, 24 h, 48 h and 72 h after transfection and seeded in 96-well tissue culture plates (10^4 cells/well). At each indicated time point, fresh medium containing 0.5 mg/mL MTT (Sigma, US) was added to each well. The cells were incubated at 37°C for 4 h. Absorption at 490 nm was read on a microplate reader following the addition of 150 μL solubilization of Dimethyl sulfoxide to each well for 30 min with gentle shaking.

Hoechst staining test

After 30 h of transfection, cells were washed once with PBS and stained for 15 min in the dark at RT with 10 ug/ml Hoechst 33258 and 10 ug/ml PI (Sigma USA). After cells had been washed twice with PBS, Nuclear fragmentation was visualized using a fluorescence microscope equipped with a UV-2A filter.

Annexin V assay

After 30 h of transfection, cells were collected and washed twice with PBS. Cells were re-suspended in reaction buffer (96 μL Hepes buffer, 2 μL Annexin-V-FITC, 2 μL 50 μg/mL Propidium iodide) and then incubated in dark for 15 min at RT. Cells were centrifuged at 1,500 g, 5 min and the pellet was re-suspended with 0.5 ml Hepes buffer (10 mM Hepes/ NaOH, 140 mM NaCl, 5 mM $CaCl_2$, pH = 7.4). For each experiment, 10,000 cells were sorted and the percentage of cell apoptosis was analyzed with Flow Cytometry (Becton Dickinson).

Cell apoptosis analysis with PI staining by flow cytometry

After 48 h of transfection, 10^6 cells were harvested and fixed in 5 ml 70% pre-colded ethanol for at least 12 h at -20°C. For the detection of low molecular mass fragments of DNA, the cells pellets were incubated for 5 min in 0.5 ml PCB buffer (192 ml 0.2 M Na_2HPO_4, 8 ml 0.1 mM citric acid, pH 7.8). After twice washing the cells with PBS, 25 μl 1 mg/ml RNase was added and incubated for 30 min at 37°C. The cells were stained with 25 μl 1 mg/ml propidium iodide (PI) in the dark for 30 min at 37°C. The DNA content was analyzed by flow cytometry (Becton Dickinson) using a laser with 488 nm excitation and 560 nm emission. The peak of hypodiploid DNA below G1 was regarded as the apoptosic peak.

ROS detection

ROS generated within cells were detected by the fluorescent probe dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma, USA). Briefly, cells were incubated for 20 min at 37°C with 10 μM DCFH-DA. Afterwards, the suspension was centrifuged to remove the non-incorporated probe. The cell pellet was re-suspended in PBS. The DCF fluorescence was recorded continuously in a 96-well plate with a fluorescence multi-well plate reader of excitation and emission wavelengths respectively at 485 nm and 525 nm.

The mitochondrial membrane potential assay

After 48 h of transfection, cells were collected and washed twice with PBS. Cells were re-suspended in 1 mL of 10 μg/mL Rhodamine 123 (Rh123), and incubated in dark for 1 h at 37°C. Cells were washed once with PBS and re-suspended with 0.5 ml PBS comprising 2.5 μL of 1 mg/mL PI and 2.5 μL of 1 mg/mL RNAase and incubated in dark for 30 min. Percentage of Rh123 positive cells was analyzed with Becton Dickinson Flow Cytometer.

Western blot analysis

20～50 μg of cell protein samples were mixed with Laemmli sample buffer and separated by SDS-PAGE gel electrophoresis (15% acrylamide), then transferred to nitrocellulose membranes (GE) according to standard procedures. Membranes were blocked in 5% non-fat dried milk and Tris-buffered saline with 0.1% Tween 20 for 1 h at RT. The primary antibodies used in Western blot assays include monoclonal antibody 3F4 (DAKO, Denmark), anti-Bcl-xL antibody (Cell Signaling Technology, USA), anti-Bax antibody (Cell Signaling Technology, USA), anti-Cleaved Caspase-9 Antibody (Cell Signaling Technology, USA) and anti-β-actin antibody (Sigma, USA). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse or anti- rabbit IgG (Santa Cruz, Cell Signaling Technology, USA). The blot was revealed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, USA).

Statistical analysis

Data of the flow cytometry were presented as percentages. Some experimental data were expressed as mean ± SE. Statistical comparisons were made using an unpaired two-tailed Student's t test. $P < 0.05$ was considered statistically significant.

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