Combined antitumor activity of the nitroreductase/CB1954 suicide gene system and γ-rays in HeLa cells *in vitro*

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Abstract. *Escherichia coli* nitroreductase (NTR) may convert the prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) into a bifunctional alkylating agent, which may lead to DNA crosslinks and the apoptosis of cancer cells. NTR/CB1954 has been demonstrated to be an effective gene therapy in cancer cells. The present study examined whether the NTR/CB1954 suicide gene system had cytotoxic effects on HeLa cells and may improve the radiosensitivity of HeLa cells to γ-rays. It was observed that the NTR/CB1954 suicide gene system exerted marked cytotoxic effects on HeLa cells. The combined therapeutic effects of NTR/CB1954 and γ-rays on HeLa cells demonstrated a synergistic effect. CB1954 at concentrations of 12.5 and 25 μmol/l increased the sensitization enhancement ratio of HeLa cells to 1.54 and 1.66, respectively. Therefore, when compared with monotherapy, the combined therapy of NTR/CB1954 and γ-rays may increase the apoptotic rate and enhance the radiosensitivity of HeLa cells. The combined therapy of γ-ray radiation and the NTR/CB1954 suicide gene system may be a novel and potent therapeutic method for the treatment of cervical carcinoma.

Introduction

Gene-directed enzyme-prodrug therapy (GDEPT), or suicide gene therapy, is a promising treatment strategy, which acts by tumor-targeted delivery of an exogenous genes that may express an enzyme capable of converting a non-toxic prodrug into an activated cytotoxic agent, which may then result in apoptosis of tumor cells (1-3). Various suicide gene therapy systems have been previously investigated, including the herpes simplex thymidine kinase/prodrug GCV (HSV1-tk-GCV) and cytosine deaminase and 5-fluorocytosine (CD/5-FC) (4-6).

Nitroreductase/(5-(aziridin-1-yl)-2,4-dinitrobenzamide) (NTR/CB1954) is another GDEPT strategy that has been previously investigated in clinical trials (7). NTR is responsible for the conversion of CB1954, a weak monofunctional alkylating agent, into 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a DNA inter-strand cross-linking agent that may trigger apoptosis of cancer cells that express the enzyme NTR. Tumor cells are radiation resistant when they are in the S phase of the cell cycle. Previous studies have confirmed that compared with the HSV1-tk-GCV system, the advantages of the NTR/CB1954 suicide gene system are as follows: i) Independence from the cell cycle; ii) ability to target both dividing and growth-arrested cancer cells; and iii) induction of a potent bystander effect on the cell cycle (8-11).

Radiotherapy has been used due to its efficacy against various tumor types, including head and neck, lung and gastrointestinal tumors. Radiotherapy is usually used alone or in combination with surgery and chemotherapy, and is important for the successful clinical treatment of patients with cancer. However, the modality of this treatment is associated with serious side-effects, including damage to normal tissues, thus requiring restriction of the doses used. A radiosensitizing agent or system may provide the opportunity to circumvent these issues (12,13).

Previous studies have confirmed that gene-directed enzyme prodrug therapy may sensitize tumor cells to the effects of ionizing radiation, and have demonstrated excellent results, including enhanced radiosensitivity of numerous tumor cells *in vitro* and *in vivo* (14-17). These observations have promoted the use of cancer gene therapy to enhance the effect of ionizing radiation.

The present study investigated whether the NTR/CB1954 suicide gene system and γ-rays have a combined effect and whether NTR/CB1954 may enhance the cytotoxic effect of γ-rays on cervical carcinoma cells *in vitro*.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) with high glucose and 10% fetal calf serum (FCS) was

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purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) assay kits were obtained from Beijing Modern Gold Biotechnology Co., Ltd. (Beijing, China). The endonucleases and ligase enzymes used for molecular cloning technology were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Lipofectamine 2000, G418, CB1954 and MTT were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany).

Plasmid vector construction. The *nfsB* sequence was amplified by PCR from the *Escherichia coli k12* genome using the following primers: 5'-CGGGATCCATGGATATCATTT CTGTCG-3' and 5'-CGGAATTCTTACACTTCGGTTAAG GTG-3'. This sequence contained *BamH*I and *EcoR*I sites for insertion into the pcDNA3 plasmid. Following endonuclease digestion and DNA sequencing, the successful insertion of the *nfsB* gene was confirmed and was re-cloned into the pcDNA3 plasmid. The following the following thermocycling conditions: 94˚C for 3 min, 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min and 72˚C for 5 min, 35 cycles. The resulting plasmid was termed pcDNA3-*nfsB*.

Cell culture and transfection. HeLa cells were maintained in DMEM, supplemented with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin and were incubated at 37˚C in a 5% CO_2 atmosphere. All of the cultures were demonstrated to be free of mycoplasma. The HeLa cells were transfected with the pcDNA3 plasmid and pcDNA3-*nfsB* with G418 selection at a concentration of 400 μg/ml. Following 1 month of selection, several independent clones were selected and the NTR mRNA and protein expression levels were determined. Subsequently, the concentration of G418 was reduced to 200 μg/ml.

RT-PCR and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total RNA was extracted from the pcDAN3-*nfsB*-HeLa, pcDNA3-HeLa and non-transfected control HeLa cells using TRIzol reagent. RT was performed according to the manufacturer's protocol (Transgen Biotech Co., Ltd. (Beijing, China). The reaction mixture was placed in a 42˚C water bath for 50 min, the temperature was increased to 70˚C for 15 min to inactivate TransScript RT and synthesise cDNA. For *nfsB*, the following primers were used: Sense, 5'-ATGGACGATGTCTGGCTGAA-3' and antisense 5'-AAC GCTGTGATGACCTACCG-3'. Endogenously expressed human β-actin mRNA was used as an internal control with the following primers: Sense 5'-GGCATCCTCACCCTGAAG TA-3' and antisense 5'-GGGGTGTTGAAGGTCTCAAA-3'. A DNA product of 208 base pairs (bp) was then amplified. A total of 500 ng cDNA were used. The following the following thermocycling conditions: 94˚C for 3 min, 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min and 72˚C for 5 min, 35 cycles. The PCR products of *nfsB* and β-actin were separated by electrophoresis on 1.5% agarose gel. The DNA bands were visualized and analyzed by staining with ethidium bromide. The proteins were collected from the three cell lines and SDS-PAGE was performed with a 10% acrylamide separating gel and 4% acrylamide, 10 μg protein were loaded per lane. The samples were prepared in a Tris-glycine buffer with 1% SDS at pH 8.8. Electrophoresis was conducted at a current of 10 mA for 5 h in electrophoretic Tris-glycine buffer with 0.1% SDS. Following electrophoresis, the gel sheets were stained for proteins with 0.25% coomassie brilliant blue-R250 and then were destained with 10% acetic acid and 20% methanol. Next, the protein bands were visualized and analyzed.

Cell growth curve and the determination of the cell survival fraction and survival curves following transfection of HeLa cells. The pcDNA3-*nfsB*-HeLa, pcDNA3-HeLa and HeLa cells were incubated for 96 h and counted every 12 h. The number of cells was determined using trypan blue staining. Subsequently, a cell growth curve was generated and the differences between the three cell lines were compared. pcDNA3-*nfsB*-HeLa, pcDNA3-HeLa and HeLa cells of different concentrations were irradiated with different doses of γ-rays. The cells were subsequently incubated for 12 days and the survival fraction of the cells was detected by a colony-formation assay. The cell survival curve was generated using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Analysis of apoptosis by Hoechst 33258/propidium iodide (PI) fluorescent vital staining. Hoechst 33258/PI fluorescent vital stain was used to quantitatively determine the percentage of apoptotic cells. Briefly, the cells were washed with PBS, and Hoechst 33258 (15 μg/ml) was added. Following a 50 min incubation at ambient temperature, PI (10 μg/ml) was added, followed by another incubation for 10 min. The samples were analyzed using fluorescence microscopy. The nuclei of apoptotic cells appeared bright fluorescent blue, whereas the nuclei of necrotic cells were bright fluorescent red. The nuclei of normal cells appeared only weakly fluorescent blue (Fig. 1). This method was used to analyze the effects of the NTR/CB1954 suicide gene system in combination with γ-ray treatment on HeLa cells. Bradford assay (Leagene Biotech Co., Ltd., Beijing, China) was used for quantification.

Hypodiploid HeLa cell formation detected by flow cytometry following NTR/CB1954 treatment. The three cell lines were plated at a density of $1.0x10^6$ cells/well in a 6-well plate. Following incubation with CB1954 at various concentrations (0, 12.5, 25, 37.5 and 50 μmol/l) for 36 h, the cells were collected, washed twice with ice-cold PBS (pH 7.4), fixed with 70% alcohol for a minimum of 18 h and then stained with PI (50 μg/ml) in the presence of 20 μg/ml RNAse A for a minimum of 30 min prior to flow cytometric analysis. The data were analyzed with ModFit version 3.2 (Verity Software House, Inc., Topsham, ME, USA) and CellQuest version 7.5.3 (BD Biosciences, Franklin Lakes, NJ, USA).

Detection of the combined effect of γ-rays and the NTR/CB1954 suicide gene system via Hoechst 33258/PI fluorescent vital staining. pcDNA3-*nfsB*-HeLa cells in the exponential growth phase were incubated for 24 h at 37˚C and CB1954 was added at concentrations of 0, 12.5 and 25 μmol/l. After 24 h the cells were irradiated at doses of 0, 2, 4 and 6 Gy with Co^{60} γ-rays. Subsequently, the cells were incubated at 37˚C with an atmosphere of 5% CO_2 for 48 h. Detection of apoptosis was performed using Hoechst/PI fluorescent vital staining.

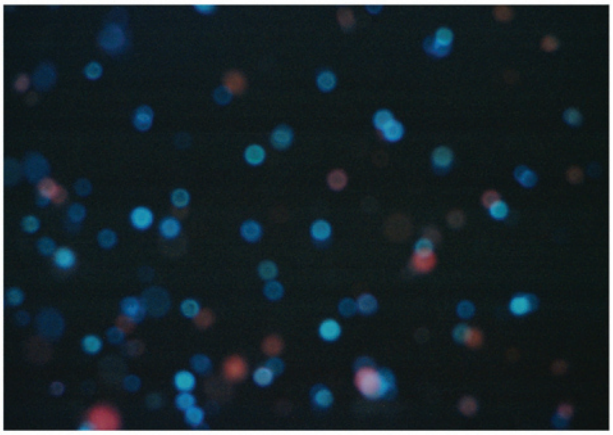

Figure 1. Apoptosis of HeLa cells was observed using Hoechst 33258/propidium iodide staining. The cell nuclei of apoptotic cells was bright fluorescent blue, whereas the nuclei of necrotic cells were bright fluorescent red. The nuclei of the normal cells appeared only weakly fluorescent blue.

Determination of cell survival via colony-formation assay following treatment with NTR/CB1954 combined with γ-rays. Cells were cultured in 25 cm^2 flasks. Single suspension was obtained, the cells were diluted to $1x10^4$/ml and $1x10^3$/ml. Next, 0.2, 0.4, 0.6, 0.8 and 1 ml from the concentration of $1x10^3$/ml, and a cell suspension of 0.3, 0.8 and 1 ml from the concentration of $1x10^4$/ml, all of which were cultured in 50 cm^2 flasks, and the number of cells were 200, 400, 600, 800, 1,000, 5,000, 8,000 and 10,000 from low to high. Following the addition of CB1954 at concentrations of 0, 12.5 or 25 μmol/l, the cells were irradiated at doses of 0, 0.5, 1, 1.5, 2, 4, 6 and 8 Gy with Co^{60} γ-rays, according to the number of cells from low to high. Next, the cells were incubated at 37˚C with 5% CO_2 atmosphere for 2-15 days. Following this, the medium was removed, the cell culture plate was washed with PBS, and the cells were fixed in methanol for 15 min. The colonies were then stained with a solution of crystal violet for 20 min. Colonies that were visible to the naked eye were counted and the cell survival fraction (SF) was calculated using the following formulas: Plating efficiency (PE) = cell colonies formed / cells inoculated and SF = cell colonies formed / cells innoculated x PE.

Statistical analysis. Differences between treatment groups were determined using Student's t-test and one-way analysis of variance. Statistical analysis was performed using Statistics Analysis System version 8.0 (SAS Institute, Inc., Cary, NC, USA). Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NTR mRNA expression in HeLa cells. NTR mRNA expression level was determined using RT-PCR. In the pcDNA3-*nfsB*-HeLa cells, a 381 bp DNA fragment of *nfsB* was amplified, whereas no mRNA was detected in either the control pcDNA3-HeLa cells or in the non-transfected HeLa cells. A 208 bp human β-actin mRNA fragment, which was used as an internal control, was amplified in the three different cell lines (Fig. 2A). NTR protein expression level was determined using SDS-PAGE. In the pcDNA3-*nfsB*-HeLa cells, a 24 kDa protein was identified, which coincided with the

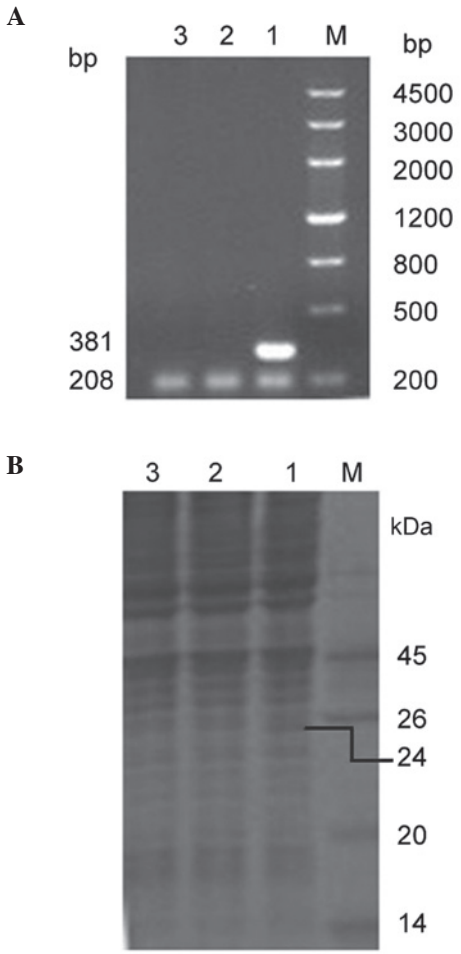

Figure 2. Protein and mRNA expression levels of NTR in HeLa cells. (A) DNA product of *nfsB* 381 base pairs in length was present in the pcDNA3-nfsB-HeLa cells; however, it was not identified in the control cells. Lane 1, pcDNA-nfsB-HeLa cells; lane 2, pcDNA-HeLa cells; lane 3, HeLa cells. (B) Protein expression level of NTR. A protein of 24 kDa was present in the pcDNA3-nfsB-HeLa cells; however, it was not identified in the control cells. Lane 1, pcDNA-nfsB-HeLa cells; lane 2, pcDNA-HeLa cells; lane 3, HeLa cells. NTR, nitroreductase.

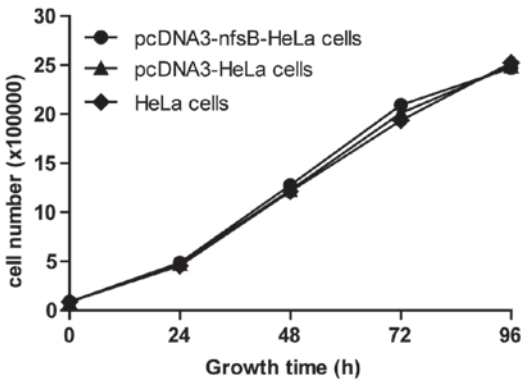

Figure 3. Cell proliferation following *nfsB* transfection. No significant difference was identified in terms of proliferation among pcDNA3-nfsB-HeLa, pcDNA3-HeLa and HeLa cells, as determined by cell counting analysis.

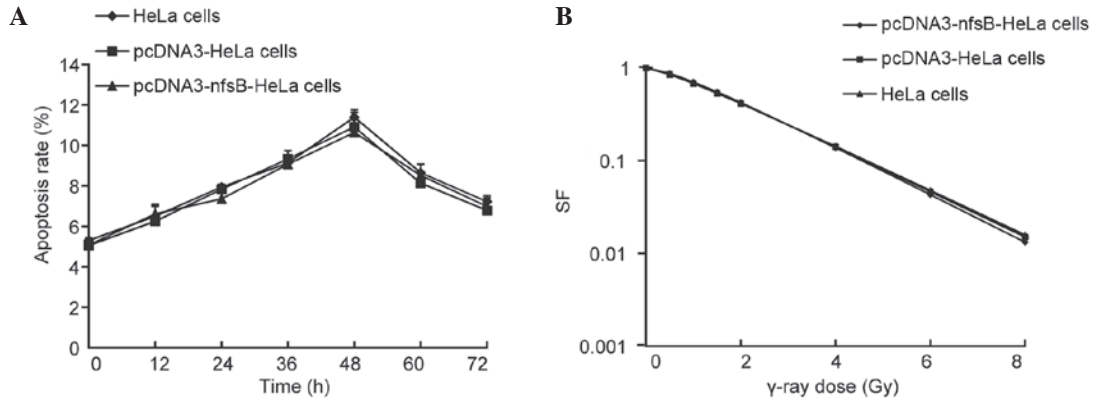

Figure 4. Analysis of apoptotic rate in γ-irradiated cells. No significant difference was identified among the three cell types. (A) pcDNA3-nfsB-HeLa, pcDNA3-HeLa and HeLa cells were irradiated by different doses of γ-rays, and subsequent to an incubation of 12 days. (B) Fraction of surviving cells was observed by colony-formation assay. No significant difference of the cell-survival fraction between the different cell lines was identified. SF, surviving fraction.

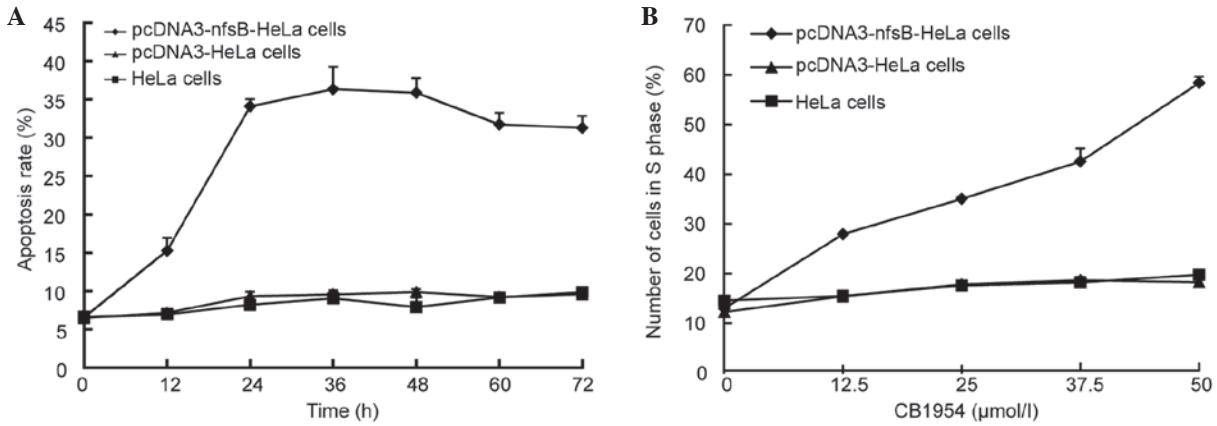

Figure 5. pcDNA3-nfsB-HeLa had greater sensitivity to the CB1954 prodrug at a concentration of 30 μmol/l. (A) Cells were incubated for 36 h and the effects peaked, the rate of apoptosis was >35%. No obvious changes were observed between the two control groups. (B) pcDNA3-nfsB-HeLa, pcDNA3-HeLa and HeLa cells were treated with various concentrations of CB1954. S-phase arrest of pcDNA3-nfsB-HeLa cells occurred; however, for the pcDNA3-HeLa and HeLa cells it was observed 36 h later. At CB1954 concentration 50 μmol/l 70.51% pcDNA3-nfsB-HeLa cells were in the S phase.

size of NTR. However, in the control pcDNA3-HeLa cells and in the non-transfected HeLa cells, no corresponding protein band was detected (Fig. 2B). These observations indicated that NTR was stably and correctly expressed in the transfected HeLa cells.

Transfection of the nfsB gene had no effect on cell growth or on the reaction of HeLa cells to γ-ray irradiation. The cell counting method was used following the transfection with the *nfsB* gene to observe the growth and proliferation of the three groups of cells. No significant differences were observed between the pcDNA3-*nfsB*-HeLa cells and the control cells (Fig. 3), which indicated that the *nfsB* gene did not affect the growth and proliferation of HeLa cells. Additionally, the apoptotic activity of the pcDNA3-*nfsB*-HeLa, pcDNA3-HeLa and non-transfected HeLa cells was examined following γ-ray irradiation (6 Gy) using Hoechst 33258/PI fluorescent vital staining. No significant differences were observed among the three different groups of cells in terms of apoptotic rate over time (0-72 h) (Fig. 4A). The cell survival fraction in the three groups of cells was also quantified. Following treatment with various doses of γ-ray irradiation, the cells were incubated for 12 days. No significant differences were identified among the three groups of cells in terms of the cell survival fraction (Fig. 4B). These observations demonstrated that transfection with the *nfsB* gene alone did not have an influence on the reaction of the cells to γ-rays.

CB1954 increased the cytotoxicity in NTR-expressing cells and led to an extended S phase. The rate of apoptosis of pcDNA3-*nfsB*-HeLa, pcDNA3-HeLa and HeLa cells treated with CB1954 was determined using Hoechst 33258/PI fluorescent staining. Apoptosis was observed in the pcDNA3-*nfsB*-HeLa cells 12 h following treatment with CB1954 (Fig. 5A). No apoptosis was observed in the pcDNA3-HeLa cells or in the wild-type HeLa cells. The rate of apoptosis of the pcDNA3-*nfsB*-HeLa cells peaked at 36 h of treatment (Fig. 5A). Additionally, an MTT assay and flow cytometry were used to determine cell viability and apoptotic rates, with the observations consistent with the previous experiments (data not shown). The pcDNA3-*nfsB*-HeLa, pcDNA3-HeLa and non-transfected HeLa cells were treated with various concentrations of CB1954 (0, 12.5, 25 and 50 μmol/l) for 36 h. Flow cytometry was then used to analyze the number of cells in the different cell cycle phases, and the results demonstrated that the NTR/CB1954 suicide

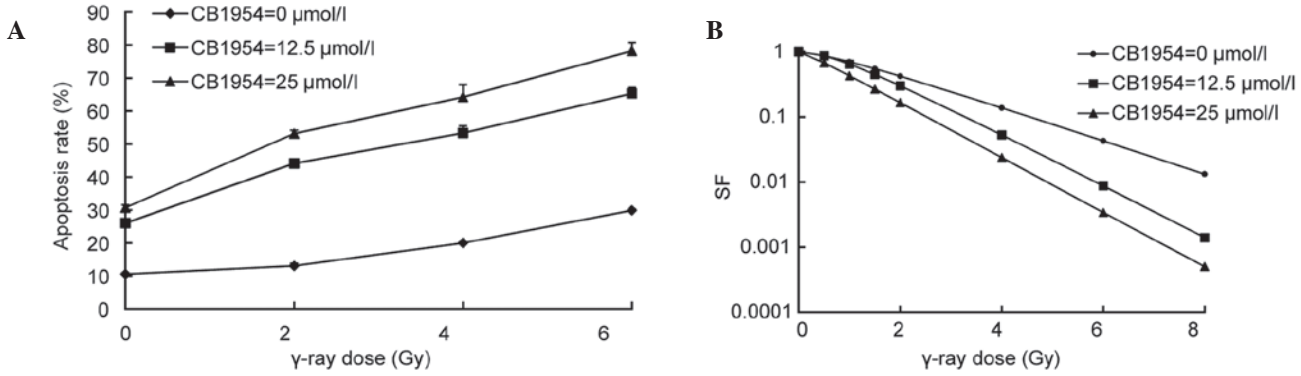

Figure 6. pcDNA3-nfsB-HeLa cells exhibited enhanced cytotoxicity in response to CB1954 combined with irradiation. (A) CB1954 concentrations of 12.5 and 25 μmol/l radiation and dose-dependent cytotoxicity in pcDNA3-nfsB-HeLa cells were evident. Cytotoxicity in pcDNA3-nfsB-HeLa cells was both radiation and drug dose dependent. (B) pcDNA3-nfsB-HeLa cells exhibited a reduced fraction of surviving cells in response to CB1954 plus irradiation. When the concentration of CB1954 was 12.5 and 25 μmol/l, the sensitization enhancement ratio was 1.54 and 1.66, respectively. SF, surviving fraction.

gene system primarily affected HeLa cells in S phase. When the concentration of CB1954 was 50 μmol/l, the percentage of cells in S phase was 70.51%, whereas no clear alterations were observed in the control cell groups, the pcDNA3-HeLa and HeLa cells (Fig. 5B).

CB1954 combined with γ-ray irradiation increased cytotoxicity and reduced the cell survival fraction in pcDNA-nfsB-HeLa cells. pcDNA-*nfsB*-HeLa cells were treated with CB1954 at various concentrations (0, 12.5 and 25 μmol/l) for 16 h prior to γ-ray irradiation (0, 2, 4 and 6 Gy), and the rate of apoptosis was determined using Hoechst 33258/PI fluorescent vital staining. The cytotoxicity was proportional to the radiation dose in pcDNA-*nfsB*-HeLa cells and the CB1954 concentration used. Cytotoxicity increased with the combination of radiation and CB1954 at 12.5 and 25 μmol/l. For example, when the concentration of CB1954 was 12.5 μmol/l, the apoptotic rate of pcDNA3-*nfsB*-HeLa cells was 12.92% and at a dose of γ-ray irradiation of 6 Gy, the rate of apoptosis was 12.84%. However, with the combination of 12.5 μmol/l of CB1954 and 6 Gy of γ-ray irradiation, the rate of apoptosis rate increased to 39.9%, which was increased compared with irradiation or CB1954 alone for pcDNA3-*nfsB*-HeLa cells (Fig. 6A). These observations demonstrated that the cytotoxic effect on HeLa cells was due to the interaction between the suicide gene system NTR/CB1954 and radiation, and was not a simple additive effect. In addition, the present study determined the cell survival fraction with a colony-formation assay. The cells were treated with CB1954 at various concentrations (0, 12.5 and 25 μmol/l) for 16 h prior to the delivery of various doses of γ-rays (0, 0.5, 1, 1.5, 2, 4, 6 and 8 Gy). Next, the cell survival fraction was then determined using a colony-formation assay. Following treatment with 12.5 and 25 μmol/l of CB1954, reduced cell survival fraction was evident with the combination of radiation and CB1954. SER was obtained from the cell survival curve. At concentrations of 12.5 and 25 μmol/l CB1954 radiosensitivity ratios were 1.54 and 1.66, respectively (Fig. 6B). These results indicated that the NTR/CB1954 suicide gene system may significantly enhance the sensitivity of HeLa cells to radiation.

Discussion

The GDEPT treatment approach may sensitize tumor cells to ionizing radiation. Previous studies determined that the HSV1-tk-GCV and CD/5-FC suicide gene therapy systems may improve the sensitivity of tumor cells to radiotherapy, and these systems have demonstrated considerable advantages both *in vitro* and *in vivo* (5,18). The NTR/CB1954 gene therapy system is another form of GDEPT and demonstrated an effective tumor reducing treatment on various cells *in vivo* and *in vitro* (19,20). The NTR/CB1954 system has been previously investigated in clinical trials. The present study aimed to investigate this system and determine whether it may be used as a potential radiosensitizing gene therapy.

The present study cloned the *nfsB* gene from the *E. coli* K12 genome using PCR. This gene was then cloned into the eukaryotic expression vector pcDNA3 to obtain the pcDNA3-*nfsB* vector. Following the transfection of HeLa cells, RT-PCR and SDS-PAGE were performed to determined that the DNA fragment and protein size were consistent with those of the *nfsB* gene and NTR. The transfection was deemed successful as the fragment was missing in the control groups (HeLa and pcDNA3-HeLa cells). The present study demonstrated that the cloned *nfsB* gene was correct and that the eukaryotic expression vector pcDNA3-*nfsB* was stable and functional, which laid a foundation for the observation of the effects of NTR/CB1954 suicide gene therapy in the subsequent experiments.

The growth and proliferation of HeLa cells following the transfection were determined. No significant differences were identified between the pcDNA3-*nfsB*-HeLa cells and the control groups (pcDNA3-HeLa and HeLa cells). Therefore, the *nfsB* gene itself did not affect the cytotoxicity of NTR/CB1954 and γ-rays. Following the transfection, the rate of apoptosis of the three groups of HeLa cells following γ-ray irradiation was quantified and it was confirmed that transfection of the *nfsB* gene did not affect the fraction of surviving cells. Therefore, these observations indicated that the HeLa cell line and the *nfsB* gene were suitable for the present experiments, ensuring the potential for further cytotoxicity research.

The cytotoxic effects of NTR/CB1954 were also evaluated and it was confirmed that the NTR/CB1954 suicide gene system exerted a specific cytotoxic effect on HeLa cells. CB1954 exerted selective cytotoxicity against pcDNA3-*nfsB*-HeLa cells compared with the controls (pcDNA3-HeLa cells and HeLa cells). The present study determined that the mechanism of cytotoxicity was primarily via apoptosis; however, the peak effect of cytotoxicity was observed after 36 h, which was sooner compared with other suicide gene systems, such as the HSV/TK suicide gene therapy system (21). Cell cycle analysis of pcDNA3-HeLa cells was performed following treatment with CB1954. The results indicated that the NTR/CB1954 suicide gene system led to S-phase arrest. This was not in agreement with a previous study performed by White *et al* (18). White *et al* (18) concluded that the NTR/CB1954 gene therapy system acted independently of the cell cycle, which was the greatest advantage of this system compared with other types of GDEPT (18). However, following multiple replications of the experiments in the present study, the results were still in contradiction with that of White *et al* (18). This may be due to the cell line that was used; therefore, it may be useful for future studies to use different cell lines to verify this.

The present study examined the combined effect of the NTR-CB1954 gene system and γ-ray irradiation in pcDNA3-*nfsB*-HeLa cells. Increased cell apoptosis was observed when CB1954 treatment was combined with γ-ray radiation in pcDNA3-*nfsB*-HeLa cells. Subsequent experiments were performed to demonstrate the reduced level of surviving cells in the pcDNA3-*nfsB*-HeLa cell group that received CB1954 and γ-ray radiation compared with cells that only received CB1954 or γ-ray radiation. Based on the survival curve, the SER was 1.54 and 1.56 when the concentrations of CB1954 were 12.5 and 25 μmol/l. Therefore, this indicated that NTR/CB1954 may enhance the radiosensitivity of HeLa cells and that this was due to a synergistic effect as opposed to an additive effect.

The current study demonstrated that the NTR/CB1954 suicide gene system led to S phase arrest in HeLa cells. As cells in S phase are resistant to radiation, the S phase arrest may be the result of DNA synthesis inhibition, which was not reduced when a combination of NTR/CB1954 and γ-ray radiation was used. However, the result of this combination was cooperative. This may be due to the fact that the cells were treated when they were in the S phase, and they became damaged by NTR/CB1954, which resulted in S phase arrest. It is possible that the cells remained in the S phase in order to undergo repairs of either lethal or sublethal damage as the purpose was to enter the next phase of the cell cycle to enable the cells to proliferate and grow. However, at this time point, as the cells were exposed to γ-ray radiation they became lethally injured whilst in the S phase. Therefore, S-phase arrest may be due to the combined effect of the NTR/CB1954 and the γ-ray radiation. However, additional studies are required to test this hypothesis.

The use of suicide genes in combination with radiation therapy is currently underway and whilst progress has been achieved, it may take a considerable amount of time for this to become a widely used treatment. Efforts should be focused on the identification of a potential means for the development of strategies that aim to use GDEPT to enhance radiotherapy in clinical treatments.

Acknowledgements

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