Iodine-131 induces apoptosis in HTori-3 human thyrocyte cell line and G2/M phase arrest in a p53-independent pathway

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Abstract. Iodine-131 is known to destroy residual thyroid tissue following surgical resection of differentiated thyroid carcinoma and is widely used to treat hyperthyroidism. However, the mechanism by which iodine-131 induces apoptosis and cell cycle arrest in the human thyrocyte cell line, Htori-3, remains to be elucidated. In the present study, the cytotoxic effect of iodine-131 on the HTori-3 cell line and the underlying mechanism of iodine-131-induced cell apoptosis were investigated. Cell viability was analyzed using an MTT assay, while cell apoptosis and cell cycle arrest were determined using flow cytometry. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were performed to determine the changes in the expression levels of p53, B-cell lymphoma 2 (Bcl-2), Fas and growth arrest and DNA damage-inducible 45 (GADD45), following iodine-131 treatment. The results demonstrated that iodine-131 may inhibit HTori-3 cell growth via cell apoptosis and G2/M phase arrest in a time- and dose-dependent manner. The iodine-131 dose required for 50% growth inhibition of HTori-3 cell viability 48 h after treatment was 27.75±2.22 MBq/ml. Upregulation of Fas and downregulation of Bcl-2 expression levels were observed following iodine-131 treatment. The results of RT-qPCR revealed an increase in the GADD45 mRNA expression following HTori-3 cell exposure to iodine-131. Notably, the mRNA and protein expression levels of p53 were not altered following iodine-131 treatment. In conclusion, iodine-131 may induce apoptosis in HTori-3 cells by downregulating the expression of Bcl-2 and upregulating the expression of Fas. In addition, iodine-131 may upregulate GADD45 mRNA expression in HTori-3 cells, resulting in G2/M phase arrest in a p53-independent pathway.

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Introduction

Numerous radionuclides used in the diagnosis and therapy of disease are β particle emitters (^{153}Sm-EDTMP, Na^{131}I, ^{186}Re-HEDP and ^{89}SrCl) and demonstrate promising therapeutic results. To date, a large number of preclinical and clinical studies have been performed on emitting radionuclides (1,2). Iodine-131 is known to destroy residual thyroid tissue following surgical resection of differentiated thyroid carcinoma (3) and is widely used to treat hyperthyroidism (4). In addition, iodine-131 has been conjugated to antibody derivatives and used to treat other malignant diseases by radioimmunotherapy (RIT). Cell death induced by RIT has been demonstrated to trigger the apoptosis of tumor cells (5). The cytotoxic effect of iodine-131 on the thyroid cancer cell line, BCPAP, has been investigated and low iodine-131 doses have been found to result in cell apoptosis (6). However, the mechanism through which iodine-131 induces cell death in the thyrocyte cell line, HTori-3, remains to be fully elucidated.

Radiation-induced DNA damage promotes the expression of the p53 gene at the post-translational level (7). Activation of p53 leads to cell growth arrest at G1 and/or G2 phase, DNA repair, senescence or apoptosis (8,9). The B-cell lymphoma 2 (Bcl-2) family is divided into proapoptotic proteins, including Bcl-2 associated X protein (Bax), and antiapoptotic proteins, including Bcl-2 (10). Whether a cell undergoes apoptosis depends, to a certain extent, on the ratio of Bcl-2 to Bax (11). The cell-surface receptor, Fas [also known as CD95 or apoptosis antigen-1 (Apo-1)], is a key component of the extrinsic death pathway and is activated through the binding of its ligand, FasL (12). Radiation-induced cell cycle arrest at the G1 and G2 restriction points allows cells to repair DNA damage prior to DNA synthesis and cell division (13,14).

A number of thyroid cancer cell lines have been demonstrated to lose the expression of thyroid-specific genes and have altered karyotypes, including inactivation of the tumor suppressor gene, p53 (15). HTori-3 cell line is a nontumorigenic, immortalized human thyrocyte cell line and preserves the most specialized functions of differentiated thyroid cells (16). In the present study, the HTori-3 cell line was used as a model and the cytotoxic effect of iodine-131 on the human thyrocyte cell line, HTori-3, was investigated. In addition, the possible

underlying mechanisms of iodine-131-induced cell apoptosis, cell cycle arrest and the effect of iodine-131 therapy in hyperthyroidism were assessed, including the p53 pathway.

Materials and methods

Cell culture. The HTori-3 cell line was provided by Professor Peng Hou (Endocrinology Laboratory, The First Affiliated Hospital of Xi'an Jiaotong University College of Medicine, Xi'an, China). The cells were cultured in RMPI-1640 medium (Hyclone, Logan, UT, USA) and supplemented with 10% heat-inactivated fetal bovine serum (Minhai, Lanzhou, China) and 1% penicillin/streptomycin (Hyclone) in a 5% CO_2 humidified atmosphere at 37˚C. The study was approved by the ethics committee of the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China.

Iodine-131 uptake assay. The cells were seeded at $1x10^5$/well in 6-well plates for 24 h. Subsequently, the cells were cultured for 24 h with 2 ml culture medium per well containing 14.8 MBq/ml iodine-131. Next, the iodine-131-containing medium was decanted, and the cells were washed twice with phosphate-buffered saline (PBS) and trypsinized (Hyclone) to count the total cell number. The radioactivity of the cells was measured with a radioactivity counter (Sim-max LSA3000; Sim-max Technology Co., Ltd, Shanghai, China).

Cell viability assay. Cell viability was determined using an MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MP Biomedicals, Santa Ana, CA, USA]. The cells were seeded at $1.5x10^4$/ml in 96-well plates for 24 h. Subsequently, iodine-131 was added to achieve final iodine-131 activity concentrations of 7.4, 14.8 and 29.6 MBq/ml. Next, the cells were exposed to iodine-131 treatment for 12, 24, 48, 72 and 96 h with six replicates for each concentration value, while identical volumes of PBS were added to the control wells. Following addition of 40 μl/well MTT, the samples were incubated for a further 4 h. The medium was discarded and the formazan crystals were solubilized in 150 μl/well DMSO. The sample absorbance at 570 nm/630 nm was measured using a Multiskan plate reader (BioTek Instruments, Inc., Winooski, VT, USA). The absorbance was directly associated with the viable cell number. The experiment was performed at least in triplicate.

Cell apoptosis assay. A cell apoptosis assay was performed using an Annexin-V-fluorescein isothiocyanate (FLOUS) staining kit (Roche Diagnostics GmbH, Mannheim, Germany). The cells were seeded at $5x10^4$/ml in 6-well plates for 24 h and then treated with various doses of iodine-131 (7.4, 14.8 or 22.2 MBq/ml) (2) for 48 h or 14.8 MBq/ml iodine-131 for different time periods (24, 48 or 72 h). A dose of 14.8 MBq/ml iodine-131 was selected for evaluation over various time-points following preliminary experiments which revealed low apoptotic levels with a dose of 7.4 MBq/ml and almost total apoptosis with a dose of 22.2 MBq/ml. At the designated time-points, the medium and the trypsinized cells were collected in a centrifuge tube. Subsequently, the cells were washed with PBS and centrifuged at 200 x g for 5 min. Finally, the cells were resuspended in 100 μl of Annexin-V-FLOUS labeling solution and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Cell cycle assay. The cells were seeded at $1x10^5$/well in 6-well plates for 24 h and then exposed to various iodine-131 doses (7.4, 14.8 or 22.2 MBq/ml) for a further 24 h or the same dose (14.8 MBq/ml) for different time periods (12, 24 and 48 h). At the various time points, the cells were trypsinized and fixed in ice-cold 70% ethanol, followed by storing at 4˚C overnight. Following centrifugation at 1,000 rpm for 5 min, the samples were resuspended in a solution containing 50 g/ml propidium iodide (MP Biomedicals) and 100 U/ml RNase (MP Biomedicals). The DNA content of the nuclei was measured using flow cytometry (BD Biosciences), counting 15,000 gated events.

Western blot analysis. HTori-3 cells were treated with 14.8 MBq/ml iodine-131 for different time periods (12, 24 and 48 h). At the indicated times following irradiation, total protein was extracted using the radioimmunoprecipitation assay buffer (Wolsen, Xi'an, China). Total protein (~150 μg) was denatured in loading buffer at 100˚C for 10 min and electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lysates containing 150 μg proteins were subjected to SDS-PAGE, followed by transfer of proteins to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin (MP Biomedicals LLC, Santa Ana, CA, USA) for 1 h at room temperature and incubated overnight at 4˚C with the primary rabbit anti-human p53, mouse anti-human Bcl-2 and mouse anti-human Fas monoclonal antibodies (1:1,000; Epitomics, Burlingame, CA, USA). Subsequently, the membranes were washed with Tris-buffered saline containing 0.1% Tween-20 for 20-30 min, followed by incubation with the secondary antibody (1:20). The secondary antibodies used were horseradish peroxidase-labeled anti-rabbit or -mouse immunoglobulin (Ig)G (Santa Cruz Biotechnology, Heidelberg, Germany). Membranes reprobed with mouse anti-GAPDH (Abmart, Arlington, MA, USA) were used as the internal control for equal protein loading.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The RNA was extracted from cells with TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The concentration of total RNA was determined using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) and the 260/280 absorbance ratio, used to investigate the sample purity, was between 1.8 and 2.0. cDNA synthesis was performed using 500 ng total RNA in a reaction volume of 10 μl. The thermal cycling conditions were as follows: 15 min at 37˚C and 5 sec at 85˚C. Copy number quantification was performed with RT-qPCR using β-actin as the internal reference. The primer sequences of p53, Bcl-2, Fas, growth arrest and DNA damage-inducible 45 (GADD45) and β-actin are shown in Table I. Following reverse transcription, the conditions were as follows: 95˚C for 2 min, 40 cycles of 15 sec at 95˚C and 60 sec at 60˚C for annealing and extension were run on a CFX96 Thermal Cycler Dice™ real-time PCR system (Bio-Rad Laboroatories, Inc., Hercules, CA, USA).

Table I. Primers sequences of p53, Bcl-2, Fas, GADD45 and β-actin.

Gene	Forward	Reverse	Length (bp)
p53	CTCCTCAGCATCTTACCGAGT	GCTGTTCCGTCCCAGTAGATTA	239
Bcl-2	ATGTGTTGGAGAGCGTCAAC	AGAGACAGCCAGGAGAAATCAAAC	182
Fas	TGGCTTTGTCTTCTTCTTTTGC	CTTGGTTTTCCTTTCTGTGCTT	97
GADD45	CCCAGTGGACAGCGAGCCAGC	ACTGCAGGCTTCCTGTGGGC	137
β-actin	CTGGGACATGGAGAAA	AAGGAAGGAAGAGTGC	285

Bcl-2, B-cell lymphoma 2; GADD45, growth arrest and DNA damage-inducible 45.

The RT-qPCR reaction was conducted with 100 ng cDNA in a 20 μl reaction volume containing 10 μl SYBR® Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.), 0.4 μM forward primer, 0.4 μM reverse primer and 6.4 μl dH_2O. The relative representation of the target gene copy number in the irradiated cells with respect to the non-irradiated control was obtained using the formula $2^{-\Delta\Delta Ct}$, which is a simplified calculation tool derived from the ΔΔCt method for gene expression analysis. Each sample was analyzed twice and the mean values were calculated for statistical analysis.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS 16.0 statistical software for Windows (SPSS, Inc., Chicago, IL, USA). The statistical significance of differences between the groups were investigated using Student's unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

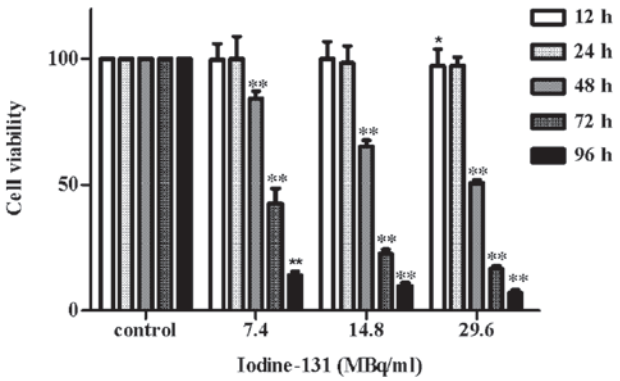

Figure 1. Effect of iodine-131 on cell viability of HTori-3 cells, which were treated with various doses of iodine-131 (7.4, 14.8 and 29.6 MBq/ml) for 12, 24, 48, 72 or 96 h. Cell viability was determined by MTT assay. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 versus the control group.

Results

Human thyrocyte HTori-3 cells exhibit iodine uptake activity. To determine whether the HTori-3 cells possessed iodine uptake activity, the iodine uptake was assessed with or without iodine-131 treatment. Following treatment with 14.8 MBq/ml iodine-131 for 24 h, the radioactivity count was found to be 51128±6.02 cpm, while the radioactivity in the control group was 366.5±4.13 cpm, indicating that HTori-3 cells possessed iodine uptake activity.

Iodine-131 inhibits cell growth in a time- and dose-dependent manner. In order to investigate the effect of iodine-131 on human thyrocyte cells, HTori-3 cells were incubated with various concentrations of iodine-131 (7.4, 14.8 or 29.6 MBq/ml) for designated time periods (12, 24, 48, 72 and 96 h) and the cell viability was determined using an MTT assay. The results (Fig. 1) revealed that iodine-131 produced a significant inhibition of cell growth compared with the control group. Notably, 15.7% inhibition of cell growth was observed at a dose of 7.4 MBq/ml (P<0.01) and 49.5% inhibition was observed at a dose of 29.6 MBq/ml (P<0.01) after 48 h. Furthermore, treatment with 14.8 MBq/ml iodine-131 induced a significant reduction in cell viability (34.8%) after 48 h (P<0.01), which was maintained to 93.2% after 96 h (P<0.01). The experiments revealed that iodine-131 exerted a cytotoxic effect on the human thyrocyte cell line. Growth inhibition was induced in a time- and dose-dependent manner. The iodine-131 dose required for 50% growth inhibition of HTori-3 cells after 48 h was 27.75±2.22 MBq/ml.

Iodine-131 induces growth inhibition of HTori-3 cells via cell cycle arrest and apoptosis. To determine whether iodine-131 affected the HTori-3 cell apoptotic response, HTori-3 cells were treated with different doses of iodine-131 (7.4, 14.8 or 22.2 MBq/ml) for 48 h or with 14.8 MBq/ml iodine-131 for various time periods (24, 48 or 72 h). Apoptosis was analyzed using flow cytometry. The negative control was treated with PBS. Apoptosis was observed in the HTori-3 cells upon iodine-131 administration in a time- and dose-dependent manner. The apoptotic cell percentage 48 h after iodine-131 treatment was 20.3% at 7.4 MBq/ml, 35.4% at 14.8 MBq/ml (P<0.01) and 53.6% at 22.2 MBq/ml (P<0.05), while only 5.8% cell death via apoptosis was observed in the control group (Fig. 2A). Evident apoptosis was induced at 48 h after treatment with 14.8 MBq/ml iodine-131 (39.9%; P<0.01), while a higher number of apoptotic cells was observed at 72 h (50.6%; P<0.01; Fig. 2B).

The capacity of iodine-131 to inhibit cell cycle progression was evaluated via flow cytometry. A representative example depicting the effect of iodine-131 treatment on cell cycle phase distribution in the HTori-3 cell line is shown in Fig. 3. At 24 after iodine-131 treatment, the percentages of cells in the G2/M phase were 24.4% at 7.4 MBq/ml (P<0.05), 38.6% at 14.8 MBq/ml (P<0.01) and 72.4% at 22.2 MBq/ml (P<0.05), while the percentage of the control group was 13.5%.

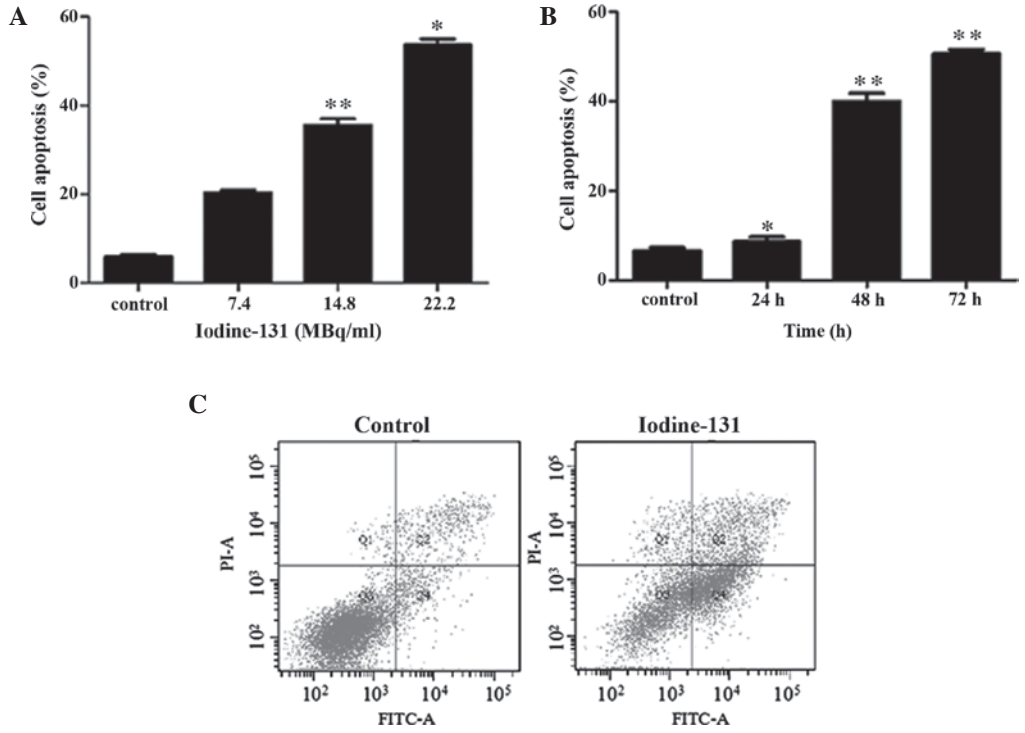

Figure 2. Iodine-131 induces apoptosis. HTori-3 cells were treated with (A) various concentrations of radioactive iodine-131 (7.4, 14.8 and 22.2 MBq/ml) for 48 h, or (B) 14.8 MBq/ml iodine-131 for 24, 48 or 72 h. (C) Cell apoptosis was determined by flow cytometry. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group. PI, propidium iodide; FITC, fluorescein isothiocyanate.

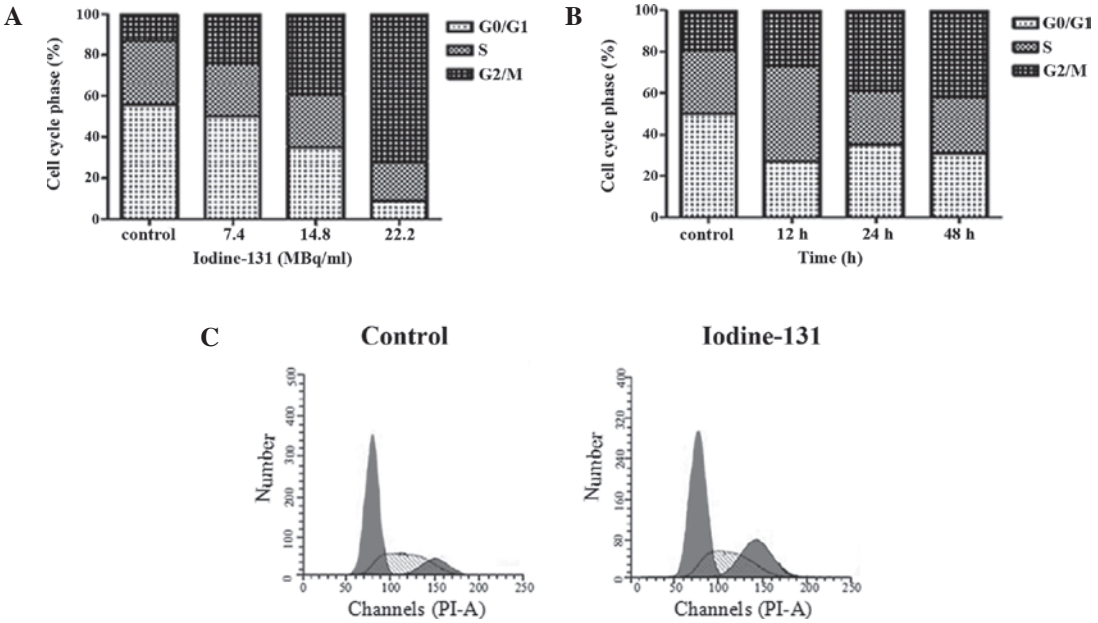

Figure 3. Iodine-131 induces accumulation of cells in G2/M. HTori-3 cells were harvested following exposure to (A) different concentrations of iodine-131 (7.4, 14.8 and 22.2 MBq/ml) for 24 h, or (B) (B) 14.8 MBq/ml iodine-131 for 12, 24 or 48 h. (C) The cells were fixed and stained with PI for flow cytometry. The data are presented as the mean ± standard deviation of three independent experiments. PI, propidium iodide.

Treatment with 14.8 MBq/ml iodine-131 for 24 h resulted in an accumulation of 39.2% cells in the G2/M phase compared with the control (19.1%; P<0.01). The G2/M accumulation was more pronounced in cells treated with iodine-131 for 48 h (42.3%; P<0.01).

Mitochondrial and death receptor pathways are involved in iodine-131-induced apoptosis. To assess the effect of iodine-131 treatment on the expression of apoptosis-associated genes in HTori-3 cells, the expression levels of Bcl-2 and Fas were determined using western blot analysis and RT-qPCR. Fas was found to be transcriptionally increased, while Bcl-2 mRNA expression decreased following iodine-131 treatment (Figs. 4A and B), determined by RT-qPCR. The increased Fas and decreased Bcl-2 expression levels, following treatment with iodine-131, were also observed by western blot analysis (Fig. 4C) .

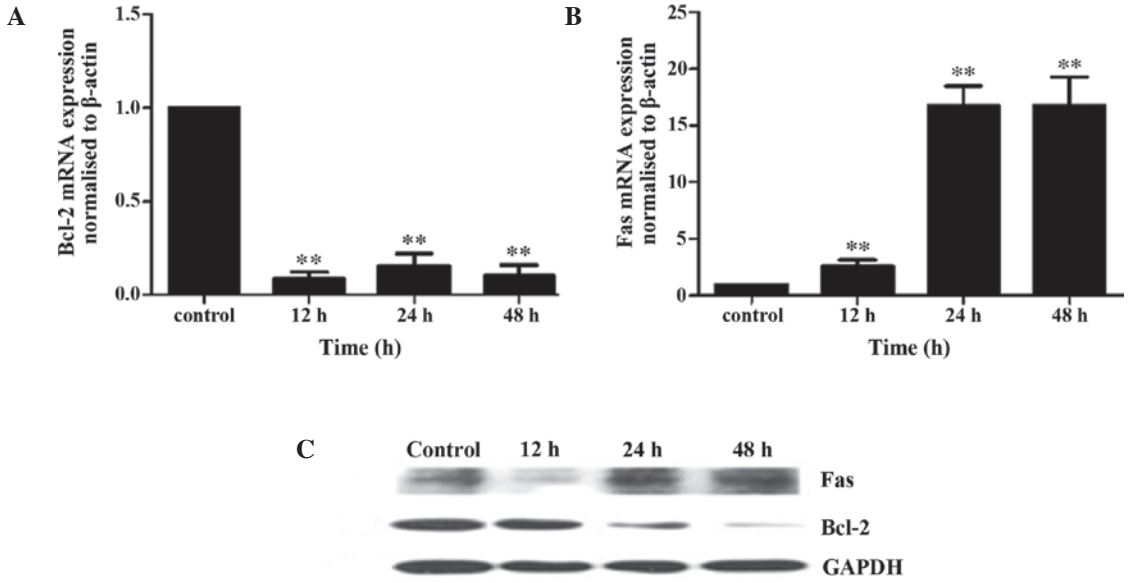

Figure 4. Activation of apoptotic molecules following iodine-131 treatment. HTori-3 cells were treated with 14.8 MBq/ml iodine-131 for 12, 24 or 48 h and the mRNA expression levels of (A) Bcl-2 and (B) Fas were determined by reverse transcription-quantitative polymerase chain reaction. (C) HTori-3 cells were treated with 14.8 MBq/ml iodine-131 for 12, 24 or 48 h and the protein expression levels of Bcl-2 and Fas were determined by western blot analysis. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group. Bcl-2, B-cell lymphoma 2.

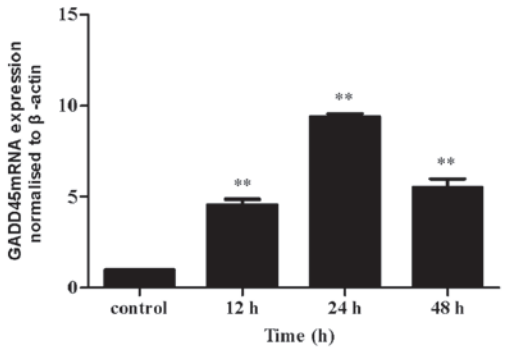

Figure 5. GADD45 mRNA accumulation following radioactive iodine-131 treatment. HTori-3 cells were treated with 14.8 MBq/ml iodine-131 for 12, 24 or 48 h. The mRNA expression of GADD45 was determined by reverse transcription-quantitative polymerase chain reaction. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group. GADD45, growth arrest and DNA damage-inducible 45.

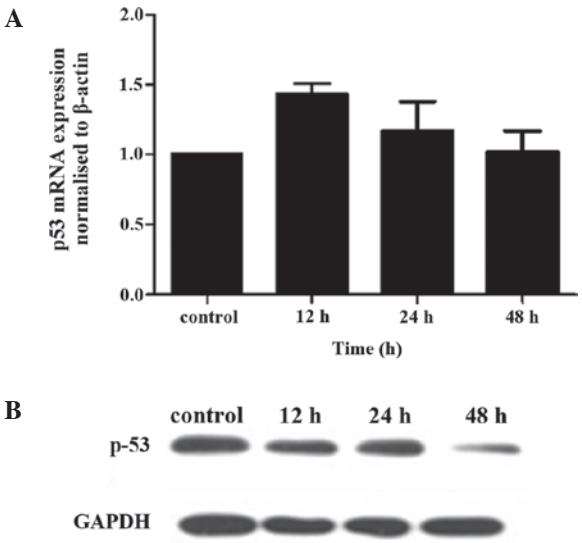

Figure 6. Iodine-131 treatment results in growth inhibition of HTori-3 cells in a p53-independent pathway. HTori-3 cells were treated with 14.8 MBq/ml iodine-131 for 12, 24 or 48 h. (A) The mRNA expression of p53 was determined by reverse transcription-quantitative polymerase chain reaction. (B) The protein expression of p53 was determined by western blot analysis. The data are presented as the mean ± standard deviation of three independent experiments.

Iodine-131 induces GADD45 accumulation in HTori-3 cells. To further examine whether the G2 phase arrest induced by iodine-131 was correlated with GADD45 in the thyroid cell line, the expression of GADD45 was evaluated by RT-qPCR prior to and following iodine-131 exposure. A statistically significant increase was observed in GADD45 transcription compared with the control (Fig. 5).

Iodine-131 results in HTori-3 cell growth inhibition in a p53-independent manner. To investigate whether p53 was involved in the iodine-131-induced inhibition of HTori-3 cell growth, mRNA and protein expression levels of p53 were determined following cellular stress induced by iodine-131. No changes were observed in p53 mRNA expression following exposure to iodine-131 (Fig. 6A). In addition, iodine-131 did not affect p53 protein expression, and even presented a reducing tendency on p53 expression at 48 h after iodine-131 treatment (Fig. 6B).

Discussion

The extent of DNA damage induced by ionizing radiation depends on the type of radiation and the dose applied, as well as other factors. Higher radiation linear energy transfer results in greater complexity of the lesions, as well as more challenging repair of the induced lesions (17,18). The results

of the present study revealed that the inhibitory effect of iodine-131 on HTori-3 cell growth occurred via cell apoptosis and G2/M phase arrest in a time- and dose-dependent manner, indicating that the cytotoxic effect of iodine-131 is associated with the irradiation time and dosage, as well as a number of other factors, including cell type or oxygenation status, which may require further study.

DNA damage induced by irradiation or drugs used for cancer chemotherapy may lead to apoptotic cell death (19). Irradiated HTori-3 cells presented a dose- and time-dependent increase in apoptosis. The Bcl-2 family is divided into proapoptotic proteins, including Bax, and antiapoptotic proteins, including Bcl-2 (10). Whether cell undergo apoptosis depends on the ratio of Bcl-2 to Bax, to a certain extent (11). Fas is known to be critical in the process of cell apoptosis induced by radiation damage and other DNA damage (20,21). The intrinsic apoptotic pathway is dominated by the Bcl-2 family of proteins (22), while the cell-surface receptor, Fas (CD95/Apo-1) is a key component of the extrinsic death pathway (20,21). The results of the present study (Fig. 4) identified that treatment of thyrocyte cells with iodine-131 resulted in decreased Bcl-2 and significantly increased Fas expression levels, indicating that the Bcl-2 and Fas expression levels are involved in signal transmission in the process of cell apoptosis induced by β-ray radiation. In addition, the apoptosis of HTori-3 cells, which was induced by iodine-131, may occur through the death receptor pathway and the mitochondrial pathway. Friesen *et al* (1) also demonstrated that β radiation-induced and -activated apoptosis pathways in leukemia cells by upregulating the expression levels of CD95 ligand and receptor, as well as activating caspases. In human breast cancer cells, apoptosis induced by strontium-89 is also regulated by the decreased Bcl-2 and increased Fas expression levels (2).

G1/S and G2/M cell cycle checkpoints are essential for the maintenance of genomic stability in response to DNA damage. An evident effect was observed on cell cycle phase distribution in the HTori-3 cell line due to the iodine-131 treatment, exhibiting a positive correlation with the concentration and time of iodine-131 treatment. A number of previous studies have also reported that the tumor cell cycle G2/M retardation was induced by γ-ray radiation (23,24). This process was also identified in the human breast carcinoma cell line, MCF-7, when induced by ^{89}Sr and irradiated with β-rays (2). Eriksson *et al* (5) also demonstrated that HeLa Hep2 cells exhibited a transient G2/M phase arrest following iodine-131 irradiation. GADD45, a DNA damage-inducible protein, has been identified to be critical in the G2/M cell cycle checkpoint (25). GADD45 mRNA expression was found to be significantly increased following iodine-131 treatment of HTori-3 cells (Fig. 5). These results indicate that G2/M phase arrest mediated by GADD45 may be one of the underlying mechanisms of the growth inhibitory effect of iodine-131 on the HTori-3 cells.

The role and mechanism of the p53 tumor suppressor protein on the cellular response to radiation-induced DNA damage is well established. The p53 tumor suppressor gene is activated in the cellular response to various cellular stresses, including radiation-induced DNA damage, oncogene stimulation, hypoxia and nucleotide depletion (26). Activation of p53 results in cell growth arrest at G1 and/or G2 phase, DNA repair, senescence or apoptosis (8,9). In the present study, to verify whether iodine-131 induced HTori-3 cell apoptosis in a p53-dependent pathway, the p53 mRNA and protein expression levels were evaluated following iodine-131 treatment. The results (Fig. 6) revealed that the mRNA and protein expression levels of p53 were not altered following iodine-131 exposure, indicating that apoptosis induced by iodine-131 is independent of p53.

In conclusion, the current study demonstrated that iodine-131 may induce apoptosis in HTori-3 cells by downregulating the expression levels of the Bcl-2 antiapoptotic protein and upregulating the mRNA and protein expression levels of Fas. Furthermore, iodine-131 treatment may upregulate the expression of GADD45 in the G2/M phase arrest in a p53-independent pathway. Therefore, the results of the present study suggest that cell apoptosis and cell cycle arrest in a p53-independent pathway may be the underlying mechanism in iodine-131 therapy of hyperthyroidism. Further investigation of the effects of β radiation at the cellular level is required in order to enable its direct application in nuclear medicine.

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