Endoplasmic reticulum stress protects human thyroid carcinoma cell lines against ionizing radiation-induced apoptosis

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Abstract. Radiotherapy is one of the most effective forms of cancer treatment, used in the treatment of a number of malignant tumors. However, the resistance of tumor cells to ionizing radiation remains a major therapeutic problem and the critical mechanisms determining radiation resistance are poorly defined. In the present study, a cellular endoplasmic reticulum (ER) stress microenvironment was established through the pretreatment of cultured thyroid cancer cells with tunicamycin (TM) and thapsigargin (TG), in order to mimic the ER stress response in a tumor microenvironment. This microenviroment was confirmed through the X-box binding protein 1 splice process, glucose-regulated protein 78 kD and ER degradation-enhancing α-mannosidase-like mRNA expression. A clonogenic assay was used to measure cancer cell resistance to ^{60}Co-γ following TM pretreatment; in addition, human C/EBP homologous protein (CHOP) mRNA expression was determined and apoptosis assays were performed. The results showed that TM or TG pretreatment inhibited CHOP expression and reduced the apoptotic rate of cells. Furthermore, the results demonstrated that the induced ER stress response rendered cancer cells more resistant to ionizing radiation-induced apoptosis. Therefore, the ER stress pathway may be a potential therapeutic target in order to improve the clinical efficiency of radiotherapy.

Introduction

Radiotherapy is commonly accepted as one of the most effective forms of cancer treatment, used on a wide range of malignant tumors (1-4). The sensitivity of tumor cells to ionizing radiation (IR) is a critical determinant of the treatment effectiveness in patients undergoing radiotherapy cancer treatment. However, the resistance of thyroid cancer cells to IR remains a major therapeutic problem and the critical mechanisms determining the radiation resistance are poorly defined.

A number of physiological and pathological conditions, including depletion of energy supply to the endoplasmic reticulum (ER; resulting from hypoxia and/or nutrient deprivation), viral or bacterial infection and Ca^{2+} depletion in the ER, can result in the accumulation of unfolded and misfolded proteins and lead to ER stress response (5). ER stress triggers a unique signaling cascade, known as the unfolded protein response (UPR), which restores ER homeostasis by enhancing the protein folding and degradation capacity (5,6). Glucose-regulated protein-78 kDa (GRP78), a well-characterized ER chaperone, is released by ER transmembrane stress sensors, including the pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), leading to the activation of the UPR signaling pathways (7).

Previous studies have indicated that the metabolic environment of the majority of solid human tumors is often hypoxic, nutrient deprived and acidic, with reduced levels of amino acids and glucose (7-9). These conditions induce ER stress and activate the UPR stress response pathways, reducing the effectiveness of cancer therapy (7-9). An increased expression of GRP78 protein has been reported in various cancers, including astrocytoma (10) and breast (11), gastric (12), lung (13), and prostate cancers (14). Further studies have revealed that the overexpression of GRP78 contributes to apoptosis inhibition, cancer progression, metastasis and angiogenesis, and confers a resistance to antitumor therapies (15-17). In addition, numerous studies have indicated that the IRE1/X-box binding protein 1 (XBP1) and PERK/eIF2α/ATF4 pathways are important in the promotion of tumor survival and growth under stress conditions (18,19). Although the ER stress response has been reported in various tumors, the correlation between radiation resistance and ER stress response remains unclear. Therefore, endogenous ER stress from the thyroid tumor microenvironment may lead to cancer cell resistance to radiation .

The aim of the current study was to investigate the effect of ER stress on the radiation-induced apoptosis of human thyroid carcinoma cell lines. A cellular ER stressing microenvironment was established by pretreating cultured follicular thyroid

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carcinoma, FTC-133, cells with tunicamycin (TM; inhibitor of N-glycosylation) and thapsigargin [TG; inhibitor of endoplasmic reticular Ca^{2+}-adenosine triphosphatase (ATPase)], in order to mimic the ER stress response in a tumor microenvironment. The mimicked ER stress response was used to investigate whether the TM-induced ER stress was involved in the resistance of cultured FTC-133 cells to IR and provide an explanation for the intrinsic resistance of certain solid tumors to radiotherapy.

Materials and methods

Materials. Trypsin, dimethyl sulfoxide (DMSO), TM, TG, propidium iodide (PI) and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TM and TG were dissolved in DMSO as stock-solutions (50 μg/ml and 5 μm, respectively), RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM) were obtained from Macgene Biotechnology (Beijing, China). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA).

Cell culture. FTC-133 (follicular thyroid carcinoma), TT (medullary thyroid cancer), SW579 (poorly differentiated carcinoma with nuclear features of papillary carcinoma and squamous differentiation) and ARO (anaplastic thyroid carcinoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). FTC-133, SW579 and TT cells were cultured in DMEM with 10% FBS, while ARO cells were cultured in RPMI-1640 medium, supplemented with 10% FBS. The cells were maintained in a humidified atmosphere with 5% CO_2 at 37˚C. The present study was approved by the ethics committees of the Henan Provincial People's Hospital and the People's Hospital of Zhengzhou University (Henan, China).

Cell irradiation. Cells were irradiated at room temperature using a ^{60}Co laboratory irradiator (Gamma Cell 220 irradiator; MDS Nordion, Inc., Ontario, Canada) at a dose rate of 1 Gy/min for the time required to generate a dose curve of 0, 0.5, 1, 2, and 4 Gy. Cells were pre-incubated with TM or TG for 8 h before exposure to radiation.

SRB assay. An SRB assay was performed according to the method described by Kaur *et al* (20). Briefly, the cells were collected using a trypsinization kit (Amresco Inc., Solon, OH, USA), counted and plated at a density of 10,000 cells/well in 96-well plates. Following incubation for 24 h, the cells were exposed to different concentrations of TM (0, 0.625, 1.25 and 2.5 μg/ml) and TG (0, 100, 250 and 500 nM). Next, the cells were fixed with 10% (w/v) trichloroacetic acid (TCA; Beijing Chemical Works, Beijing, China) at 4˚C for 1 h and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. The protein-bound dye was subsequently dissolved in 10 mmol/l Tris. The optical density (OD) of the cells was determined at a wavelength of 540 nm using a colorimetric plate reader (iMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Clonogenic assays. A total of 600-10,000 cells were plated on 25-cm^2 flasks in triplicate for each experiment. The cells were cultured for 16 h and treated with TM (1.25 μg/ml) or TG (100 nM) for 8 h prior to irradiation. The control cells (0 Gy) were sham irradiated. FTC-133 cells were pretreated with radiosensitizer [bromodeoxyuridine (Brdu); Aladdin-reagent, Inc., Shanghai, China] and radioprotector [mercaptoethylamine (MEA)] drugs for 48 h and 30 min, respectively, prior to exposure to 0, 0.5, 1, 2 and 4 Gy radiation as the positive control. Following the irradiation, the cells were cultured in a humidified atmosphere with 5% CO_2 at 37˚C for an additional nine-day incubation period, then fixed with 100% carbinol (Beijing Chemical Works) and stained with 0.5% crystal violet. Only colonies containing >50 cells were manually counted, colonies containing <50 cells were excluded from the experiment. The cell surviving fraction (SF) was calculated based on plating efficiency (PE) as follows:

$$PE = (\text{colony number/inoculating cell number}) \times 100.$$

$$SF = PE_{\text{tested group}}/PE_{\text{0 Gy group}} \times 100$$

The cell survival rates were used in the single-hit multitarget formula, $SF=1-(1-e^{-D/D0})^N$ (21), in order to calculate the radiobiological parameters of cellular radiosensitivity (mean lethal dose, D_0), the capacity of sublethal damage repair (quasi-threshold dose, D_q) and the extrapolation number (N). These values were then used to calculate the SF following irradiation at a dose of 2 Gy (SF2) and the sensitization enhancement ratio (SER).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and XBP1 splicing assay. RT-qPCR and XBP1 splicing assays were performed on the following treatment groups: Control, 0 Gy; 1Gy, 2Gy, 4Gy; 0.625 μg/ml TG; 0.625 μg/ml TM + 2 Gy; 0.625 μg/ml TG + 2 Gy. Total RNA was extracted using a RNAiso Plus kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. Aliquots (5.0 μg) of total RNA from each sample were treated with the ReverTra Ace kit (Takara Bio, Inc., Otsu, Japan) to produce first-strand cDNA.

qPCR was performed using a SYBR® Green I Master Mix kit (Takara Biotechnology Co., Ltd.) on a Bio-Rad IQ5

Table I. Survival fraction of 2 Gy-irradiated cells in the various treatment groups.

Cell line	Treatment	SF 2 Gy (mean ± SEM[a])	P-value[b]
FTC-133	Control	0.455±0.024	
	TM pretreatment	0.522±0.017	0.017
	TG pretreatment	0.595±0.036	0.005
TT	Control	0.552±0.023	
	TM pretreatment	0.608±0.015	0.024
SW579	Control	0.593±0.027	
	TM pretreatment	0.672±0.023	0.018
ARO	Control	0.618±0.052	
	TM pretreatment	0.739±0.024	0.022

[a]n=3; [b]P-values were derived from the independent-sample t-test. SF 2 Gy, survival fraction following 2 Gy irradiation; SEM, standard error of mean; TM, tunicamycin; TG, thapsigargin.

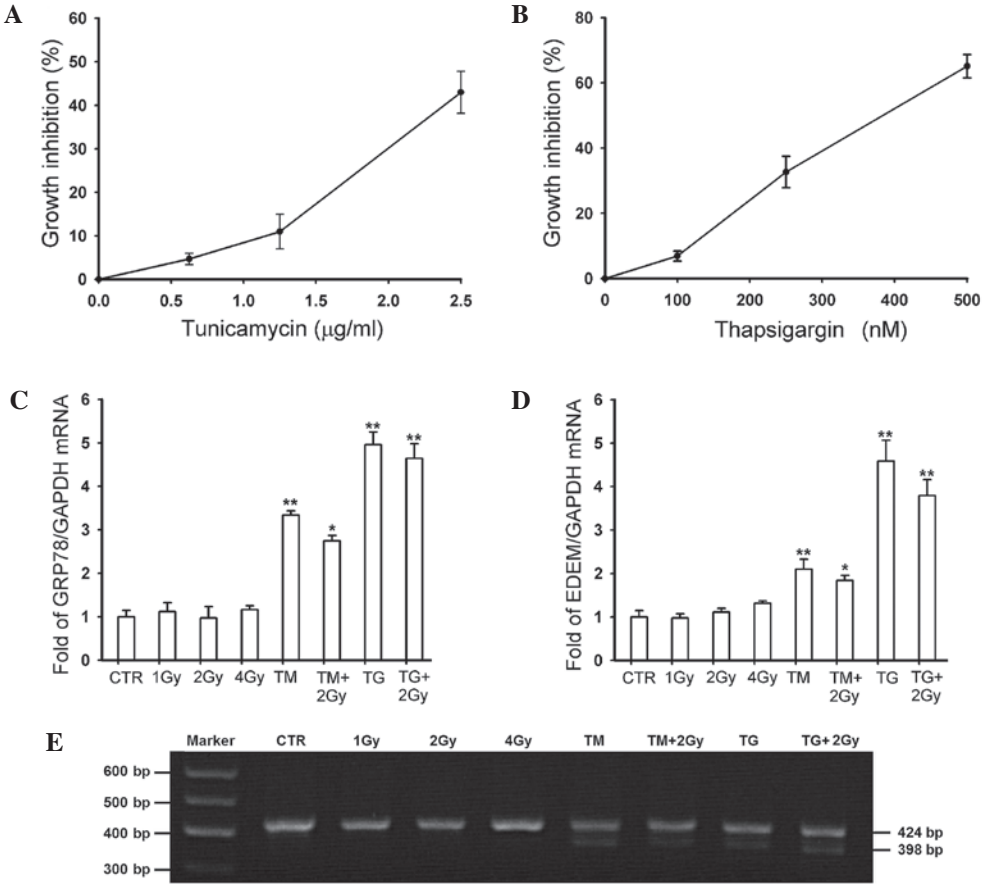

Figure 1. ER stress was induced by tunicamycin (TM) or thapsigargin (TG), but not by ionizing radiation, in cultured FTC-133 cells. Growth inhibition of FTC-133 cells following treatment with various concentrations of (A) TM (0, 0.625, 1.25 and 2.5 μg/ml) and (B) TG (0, 100, 250 and 500 nM) for 8 h, was determined by sulforhodamine B assay. Relative (C) GRP78 and (D) EDEM mRNA ratios of FTC-133 cells in the different study groups, determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), using GAPDH mRNA as reference. All the data are expressed as the mean ± standard error of mean of the experiments in triplicate. *P<0.05 and **P<0.01, vs. control group. (E) X-box binding protein 1 mRNA splicing in FTC-133 cells, determined by RT-qPCR. Study groups: CTR, control; 1, 2 and 4 Gy, various ionizing radiation doses; TM, 1.25 μg/ml tunicamycin; TG, 100 nM thapsigargin.

Realtime-PCR Reaction System (Bio-Rad Laboratories, Inc.). The relative mRNA concentrations were calculated from the values of the comparative threshold cycle using GAPDH as the control. The primers used were as follows: human GRP78 (GenBank no., NM_005347) forward, 5'-CACGCCGTCCTA TGTCGC-3', and reverse, 5'-AAATGTCTTTGTTTGCCC ACC-3'; human ER degradation-enhancing α-mannosidase-like (EDEM; GenBank no., NM_014674) forward, 5'-GCTGAT GAACACCTGGATTGAC-3', and reverse, 5'-TGGTTGCCT GGTAGAGGAGATA-3'; human C/EBP homologous protein (CHOP; GenBank no., NM_004083) forward, 5'-CTGACC AGGGAAGTAGAGG-3', and reverse, 5'-TGCGTATGTGGG ATTGAG-3'; and human GAPDH (GenBank no., NM_002046) forward, 5'-TGAAGGTCGGAGTCAACGGA-3', and reverse, 5'-CCTGGAAGATGGTGATGGGAT-3'. The reaction was performed using the following cycling conditions: 95˚C for 30 sec, followed by 45 cycles of amplification (denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 33 sec).

XBP1 splicing assay was performed as described in a previous study (22). In brief, the XBP1 forward primer, 5'-CTG GAAAGCAAGTGGTAGA-3', and reverse primer, 5'-CTG GGTCCTTCTGGGTAGAC-3', encompassing the spliced sequences in XBP1 mRNA, were used for PCR amplification. The PCR product sizes were 398 and 424 bp for human XBP1s and XBP1U, respectively. Subsequently, the PCR products were subjected to electrophoresis on a 2.0% agarose gel and were visualized by ethidium bromide staining.

Annexin V-PI apoptosis assay. Apoptosis assays were performed on cells in the following treatment groups: Control, 0 Gy; 1Gy, 2Gy, 4Gy; 0.625 μg/ml TG; 0.625 μg/ml TM + 2 Gy; 0.625 μg/ml TG + 2 Gy. For quantitative analysis of apoptosis, FTC-133 cells were trypsinized and washed with cold PBS. Following washing and passage through a nylon mesh, $1x10^6$ cells were resuspended in 200 μl annexin V binding buffer (Apoptosis Detection kit; Biosea Biotechnology Co., Ltd., Beijing, China) containing 10 μl fluorescein isothiocyanate-conjugated annexin V. After 15 min of incubation in the dark at room temperature, the cells were diluted with 300 μl annexin V binding buffer containing 5 μl PI and then immediately analyzed using a BD FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The annexin V and PI emissions were detected using 515 nm and 560 nm (Hoechst red; Invitrogen Life Tehcnologies, Carlsbad, CA, USA) filters following excitation with a 488 nm UV light. The number of apoptotic cells were counted using the BD CellQuest™ 3.1 software (BD Biosciences).

Table II. Radiobiological parameters of FTC-133, TT, SW579 and ARO cell lines in the various treatment groups.

Cell line	Treatment	N	D_0	D_q	$SERD_q$
FTC-133	Control	1.647±0.142	1.698±0.060	0.847±0.055	
	TM	1.864±0.105	1.789±0.085	1.114±0.035	0.760
	TG	1.911±0.128	2.049±0.098	1.327±0.023	0.638
TT	Control	1.454±0.146	2.331±0.084	0.873±0.053	0.730
	TM	1.648±0.203	2.392±0.117	1.195±0.037	
SW579	Control	1.625±0.176	2.336±0.189	1.134±0.028	0.732
	TM	1.845±0.285	2.532±0.146	1.551±0.046	
ARO	Control	1.591±0.132	2.532±0.067	1.176±0.028	0.581
	TM	2.233±0.176	2.512±0.126	2.024±0.037	

Data are presented as the mean ± standard error of mean (n=3). N, extrapolation number (measuring the width of the survival curve shoulder; D_0, mean lethal dose; D_q, quasi-threshold dose; SER, sensitization enhancement ratio; TM, tunicamycin; TG, thapsigargin.

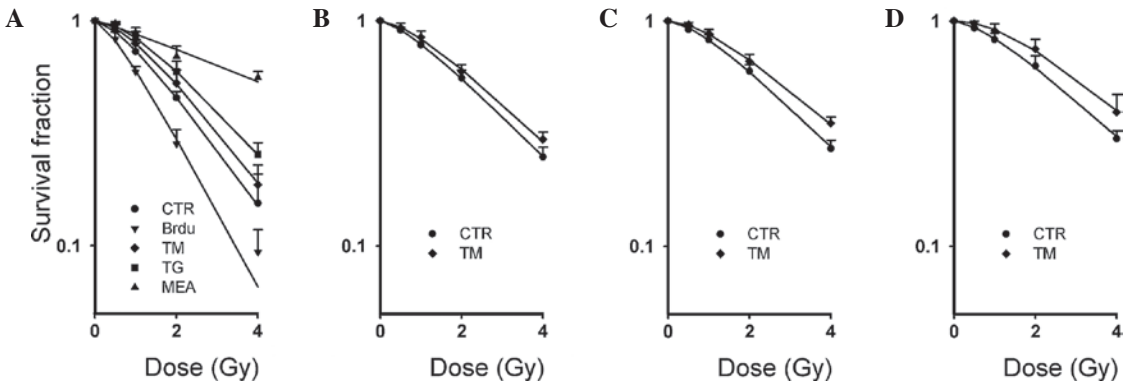

Figure 2. Induction of ER stress protected cultured thyroid cancer cells against the cytotoxicity of IR. (A) FTC-133 cells irradiated following 8 h pretreatment with 1.25 μg/ml TM or 100 nM TG, using Brdu and MEA as the positive controls. (B) TT, (C) SW579 and (D) ARO cells were treated with 1.25 μg/ml TM for 8 h prior to irradiation. The survival curves represent the data fitted in a single-hit multi-targets model. The control cells were irradiated in a drug-free growth medium. Data are presented as the mean ± standard error of meanTM, tunicamycin; TG, thapsigargin; Brdu, bromodeoxyuridine; MEA, mercaptoethylamine.

Statistical analysis. The data are expressed as the mean ± standard error of mean (n=3 to 6). Difference among the mean values were analyzed with one-way analysis of variance. Statistical analyses were performed using the SPSS software (version 17; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

ER stress is induced by tunicamycin and thapsigargin, but not by ionizing radiation in cultured FTC-133 cells. In order to investigate the effects of ER stress on the radiation-induced cytotoxicity of FTC-133 cells, the cells were initially treated with different doses of TM to determine the optimal concentration at which ER stress is induced without leading to cell death. As shown in Fig. 1A, 2.5 μg/ml TM resulted in significant cell death, up to the maximum growth inhibition, compared with untreated cells; however, treatment with 1.25 μg/ml TM resulted in a low cell death rate. TM concentrations as low as 1.25 μg/ml were used in order to diminish the cytotoxicity of TM.

Two ER stress markers, GRP78 and EDEM, were investigated to verify whether the ER stress response was induced by TM (23,24). As shown in Fig. 1C and D, FTC-133 cells treated with 1.25 μg/ml TM for 8 h were found to have increased mRNA expression levels of GRP78 and EDEM compared with the control group. In addition, XBP1 pre-mRNA splicing, in which the splicing process serves as a positive indicator of ER stress, was performed in order to demonstrate that ER stress was induced by TM treatment (25,26). The XBP1 splicing results revealed that treatment with 1.25 μg/ml TM resulted in the formation of spliced XBP1 mRNA. The spliced XBP1 mRNA was also observed in FTC-133 cells treated with a combination of TM and 2 Gy IR (Fig. 1E). However, the results indicated that the 1, 2 and 4 Gy IR treatments had a minimum effect on the development of ER stress. As shown in Fig. 1, the mRNA expression of GRP78 or EDEM and XBP1 pre-mRNA splicing were not induced in FTC-133 cells treated only with IR.

To further verify the cellular ER stress microenvironment model, a further pharmacological ER stress inducer, TG, was used in the same cultured cell system. TG is known to inhibit the endoplasmic reticular Ca^{2+}-ATPase (27). As shown in Fig. 1A and B, 1.25 μg/ml TM or 100 nM TG were found to initiate ER stress response. These results indicated that ER stress may be induced by either TM or TG; however, IR alone was not found to induce ER stress in FTC-133 cells.

Induction of ER stress protects cultured thyroid cancer cells against the clonogenic survival of IR. To determine the

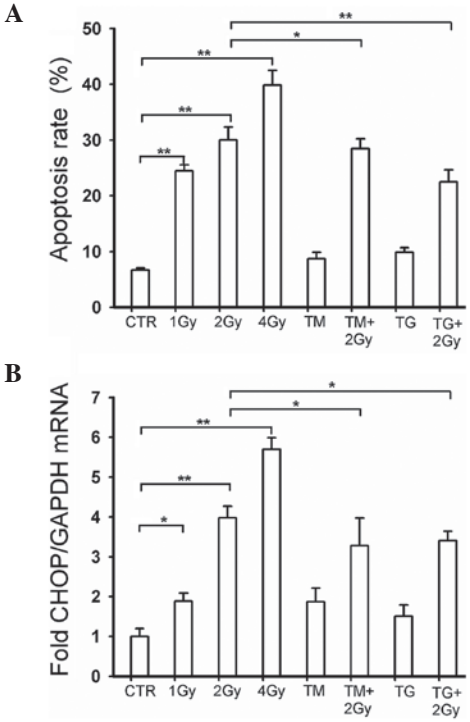

Figure 3. TM-induced or TG-induced ER stress protected FTC-133 cells against IR-induced apoptosis. (A) Apoptosis rate of FTC-133 cells in the various study groups, determined using fluorescence-activated cell sorting. (B) Relative CHOP mRNA ratio of FTC-133 cells in the various study groups, analyzed by reverse transcription-quantitative polymerase chain reaction, using GAPDH mRNA as a reference. The data are expressed as the mean ± standard error of mean for the experiments in triplicate. *P<0.05 and **P<0.01. Study groups: CTR, control; 1, 2 and 4 Gy, various ionizing radiation doses; TM, 1.25 μg/ml tunicamycin; TG, 100 nM thapsigargin.

potential effect of ER stress on the sensitivity of cultured thyroid cancer cells to radiation, FTC-133 cells were pretreated with an ER stress inducer (TM or TG) for 8 h prior to exposure to IR. As shown in Fig. 2A, IR resulted in a dose-dependent inhibition of clonogenic survival in FTC-133 cells. The cell SF values following IR were 72.9±4.5, 45.5±2.4 and 15.5±2.8% at IR doses of 1, 2 and 4 Gy, respectively, in the control group. Treatment with TM (1.25 μg/ml) resulted in resistance of FTC-133 cells to IR and increased the SF values to 79.0±3.4, 52.2±1.7 and 18.6±2.7% at IR doses of 1, 2 and 4 Gy, respectively (Fig. 2A and Table I). These results demonstrated that TM may protect FTC-133 cells against the cytotoxicity of IR. To determine whether the survival curves reflect the effect of TM on IR, radiation-protective and radiation-sensitive reagents were used. The cells were exposed to 30 mM MEA in medium for 30 min or 10 μM Brdu for 48 h prior to irradiation. The survival curves obtained are shown in Fig. 2A. From the curves, the SER of Dq (SERDq) values were calculated. The SERDq values were 0.408 and 2.665 for MEA and Brdu, respectively.

To verify that TM preincubation reduced the cytotoxic effect of IR on tumor cells, the TT, SW579 and ARO thyroid cancer cell lines were pretreated with TM for 8 h using a clonogenic assay. The results revealed a similar radiation resistance tendency to that observed in FTC-133 cells (Fig. 2B-D). Subsequently, a further ER stress inducer (TG) was used to further confirm that the protection of FTC-133 cells following TM pretreatment was due to ER stress induction. TG pretreatment of FTC-133 cells for 8 h was found to markedly increase the levels of SF to 83.9±4.2, 59.5±3.6 and 25.5±1.4%, compared with the control SF levels of 72.9±4.5, 45.5±2.4 and 15.5±2.8%, at IR doses of 1, 2 and 4 Gy, respectively (Fig. 2A and Table I). Therefore, a similar protective effect was observed in the TG pretreatment group, as in the TM pretreatment group.

The values of D_0, N and D_q were analyzed through the application of the single-hit multitarget model. As shown in Table II, cells pretreated with TM or TG produced higher values of D_q compared with the control group, indicating that enhanced repair of sublethal damage may contribute to a higher cell surviving fraction upon pre-incubation with an ER stress inducer.

TM- and TG-induced ER stress protects FTC-133 cells against IR-induced apoptosis. Apoptosis is the process of cell death in tumors following radiotherapy. A previous study has revealed that the majority of radiation resistance results from failure of radiation-induced apoptosis pathways (28). In addition, previous results indicated that ER stress inducers, TM or TG, can promote resistance to the cytotoxicity of IR. To determine the effect of ER stress on the sensitivity of IR-induced apoptosis, flow cytometric analysis was performed on FTC-133 cells that were double labeled with annexin V and PI, in order to identify whether the cells underwent apoptosis. As shown in Fig. 3A, the apoptotic cell death rate of FTC-133 cells pretreated with 1.25 μg/ml TM or 100 nM TG was not found to be significantly increased compared with the control group. FTC-133 cells irradiated with 2 Gy IR showed an average of 30.0±2.7% of annexin V^+/PI^+ cells, while a combination of IR and TM or TG pretreatment produced a significant reduction in the number of apoptotic cells (28.5±1.6 and 22.7±2.4%, respectively). These results demonstrated that TM and TG pretreatment inhibited the apoptosis caused by IR, which is consistent with the protective effect of TM and TG on IR-induced cytotoxicity (Fig. 2).

The ER stress-associated apoptosis pathway was investigated, in order to further verify that ER stress protects FTC-133 cells against IR-induced apoptosis. CHOP, a UPR downstream effector, is known to alter the balance between the prosurvival and proapoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family and activate growth arrest and DNA damage-inducible gene 34 (GADD34), thus promoting apoptosis (4,29,30). Furthermore, the expression of CHOP was detected in order to investigate whether TM and TG pretreatment inhibited apoptosis in the FTC-133 cells. As shown in Fig. 3B, irradiation with 1, 2 and 4 G IR was found to increase the mRNA expression of CHOP in a dose-dependent manner. Coincidentally, pretreatment of FTC-113 cells with TM or TG was shown to markedly decreased the mRNA expression level of CHOP following irradiation. These results indicated that ER stress-induced radiation resistance may be associated with the inhibition of apoptosis.

Discussion

Treatment of tumors that are resistant to radiation therapy is a major clinical challenge, and the underlying mechanism of the radiation resistance is not fully understood. In the current study, the results indicated that the induction of endoplasmic reticulum (ER) stress may be a possible mechanism for the thyroid cancer cell resistance to radiation.

ER plays a crucial role in normal cellular functioning, particularly in the synthesis, folding and modification of secretory and membrane proteins (28); in addition, a wide variety of cellular stress stimuli, which are related to cancer and tumor development may result in the disruption of the ER function. The metabolic environment of tumors is often acidic, hypoxic and nutrient deprived, which may be due to the tumor growth rate exceeding the ability of the vasculature to maintain an adequate supply of amino acids and glucose (9,31). These pathophysiological conditions result in the disruption of the protein folding capacity and the buildup of unfolded and misfolded proteins within the ER, activating the UPR signaling pathway (5,6). Previous studies have demonstrated that the microenvironment of tumor cells resembles the physiologic ER stress, indicating that UPR plays an important role in cancer progression, malignancy and resistance to cancer therapy (15-17). Therefore, endogenous ER stress from the tumor microenvironment may contribute to the resistance of thyroid cancer cells to radiotherapy. In order to verify this hypothesis, the current study initially established a cellular ER stress microenvironment model in FTC-133 cells using exogenous ER-stress inducers.

GRP78 has been widely used as a marker of ER stress since was identified in the 1980s. GRP78 is regarded as a major ER chaperone that facilitates protein folding and ER Ca^{2+} binding and regulates ER stress signaling (5,6). Upon induction of ER stress, GRP78 dissociates from three ER transmembrane stress sensors, which are then activated. The activation of IRE1, which is one of these ER sensors, leads to the splicing of a 26 nucleotide sequence from XBP1, resulting in a translational frame-shift and read-through of a stop codon in order to generate activated/spliced XBP1 (5,6). Alternatively spliced XBP1 encodes a number of transcription factors, including the EDEM protein (5,6). In the present study, the mRNA expression levels of GRP78, EDEM and spliced XBP1 were used as indicators of ER stress. The results revealed that the inhibition of protein glycosylation by TM or the inhibition of endoplasmic reticular Ca^{2+}-ATPase by TG can induce an ER stress response in FTC-133 cells, which is characterized by increasing the mRNA expression levels of GRP78 and EDEM, and induce the splicing of XBP1.

IR has been shown to induce ER stress in rat intestinal epithelial cells, IEC-6, and neuronal PC12 cells (32,33) at a dose of 10 Gy, indicating that ER may be a cytoplasmic target of IR. However, in the current study, FTC-133 cells treated with 1, 2, and 4 Gy of radiation was not found to result in an increase in the mRNA expression of GRP78 and EDEM or XBP1 mRNA splicing. By contrast, pretreatment with 1.25 μg/ml TM and 100 nM TG was found to markedly increase the mRNA expression levels of GRP78 and EDEM, as well as induce XBP1 mRNA splicing. Therefore, at least in the present experimental system, radiation-induced ER stress response can be ruled out at a low dose of IR. Thus, the ER stress response, which was demonstrated through the increased mRNA expression levels of GRP78 and EDEM and the XBP1 mRNA splicing in FTC-133 cells, was evidently induced by TM and TG, but not IR.

ER stress response represents an adaptive mechanism for tumor cells under stressful conditions (34). In the present study, an ER stress microenvironment model was used to investigate the effects of ER stress on radiation-induced cytotoxicity in FTC-133 cells. Usually, radiation sensitivity is expressed in terms of clonogenic cell survival using colony assays (35). The intrinsic radiosensitivity for a single dose was measured by the surviving fraction (SF). The SF of colony-forming cells that are exposed to radiation is plotted against the absorbed dose on a semi-logarithmic scale, generating a clonogenic cell survival curve. The curve consists of a gradually bending component within the low dose range, usually referred to as the shoulder region, and an exponential component in the high dose range (35). A steeper curve indicates more radiosensitive cells. The results of the current study demonstrated that pretreatment with TM or TG protected against IR-induced cell death, which was characterized by a reduction in the steepness of the survival curve and an increase in SF at each dosage. These results indicated the important role of ER stress in the resistance of thyroid cancer cells to IR.

Apoptosis may be induced following the irradiation of tumors. Previous studies have indicated that the apoptotic response following irradiation may be used to evaluate the radioresponse of tumors; in addition, several studies have revealed that the majority of radiation resistance results from the failure of radiation-induced apoptosis pathways (28, 36,37). Furthermore, ER stress-associated pathways in tumor cells have been shown to be an adaptive response to promote survival under stress conditions (7, 34). A number of studies have demonstrated that GRP78 protects cancer cells against apoptosis by inactivating proapoptotic components, such as Bcl-2-interacting killer and caspase-7 (38,39). In addition, Nawrocki *et al* reported that bortezomib induced apoptosis via the inhibition of ER stress in the downstream IRE1/XBP1 pathway (40). Considering the aforementioned evidence, ER stress induction was hypothesized to contribute to the resistance of thyroid cancer cells to radiation-induced apoptosis. The results of the current study indicated that pretreatment with TM or TG significantly decreased the IR-induced apoptosis in FTC-133 cells. Furthermore, the expression of CHOP, which is required to initiate proapoptotic pathways, was found to be inhibited by pretreatment with TM or TG prior to IR exposure.

In conclusion, the present study demonstrated that the ER stress response renders thyroid cancer cells more resistant to IR-induced apoptosis. Due to the various stresses that can induce an ER stress response in a thyroid tumor microenvironment, the results of the current study indicated that targeting the ER stress pathway may be beneficial in improving the clinical efficiency of radiotherapy.

References

1. Ishikura S, Ito Y and Hiraoka M: JCOG Radiation Therapy Study Group: history and achievements. Jpn J Clin Oncol 41: 1241-1243, 2011.
2. Early Breast Cancer Trialists' Collaborative Group (EBCTCG); Darby S, McGale P, Correa C, *et al*: Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. Lancet 378: 1707-1716, 2011.
3. Bourhis J, Overgaard J, Audry H, *et al*; Meta-Analysis of Radiotherapy in Carcinomas of Head and Neck (MARCH) Collaborative Group: Hyperfractionated or accelerated radiotherapy in head and neck cancer: a meta-analysis. Lancet 368: 843-854, 2006.

4. Wang HQ, Du ZX, Zhang HY and Gao DX: Different induction of GRP78 and CHOP as a predictor of sensitivity to proteasome inhibitors in thyroid cancer cells. Endocrinology 148: 3258-3270, 2007.
5. Boyce M and Yuan J: Cellular response to endoplasmic reticulum stress: a matter of life or death. Cell Death Differ 13: 363-373, 2006.
6. Rutkowski DT and Kaufman RJ: A trip to the ER: coping with stress. Trends Cell Biol 14: 20-28, 2004.
7. Ma Y and Hendershot LM: The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 4: 966-977, 2004.
8. Lee AS and Hendershot LM: ER stress and cancer. Cancer Biol Ther 5: 721-722, 2006.
9. Koumenis C: ER stress, hypoxia tolerance and tumor progression. Curr Mol Med 6: 55-69, 2006.
10. Zhang LH, Yang XL, Zhang X, Cheng JX and Zhang W: Association of elevated GRP78 expression with increased astrocytoma malignancy via Akt and ERK pathways. Brain Res 1371: 23-31, 2011.
11. Lee E, Nichols P, Spicer D, *et al*: GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. Cancer Res 66: 7849-7853, 2006.
12. Zhang J, Jiang Y, Jia Z, *et al*: Association of elevated GRP78 expression with increased lymph node metastasis and poor prognosis in patients with gastric cancer. Clin Exp Metastasis 23: 401-410, 2006.
13. Uramoto H, Sugio K, Oyama T, *et al*: Expression of endoplasmic reticulum molecular chaperone Grp78 in human lung cancer and its clinical significance. Lung Cancer 49: 55-62, 2005.
14. Daneshmand S, Quek ML, Lin E, *et al*: Glucose-regulated protein GRP78 is up-regulated in prostate cancer and correlates with recurrence and survival. Hum Pathol 38: 1547-1552, 2007.
15. Zhou H, Zhang Y, Fu Y, Chan L and Lee AS: Novel mechanism of anti-apoptotic function of 78-kDa glucose-regulated protein (GRP78): endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BCL-2) from BCL-2-interacting killer (BIK). J Biol Chem 286: 25687-25696, 2011.
16. Dong D, Stapleton C, Luo B, *et al*: A critical role for GRP78/BiP in the tumor microenvironment for neovascularization during tumor growth and metastasis. Cancer Res 71: 2848-2857, 2011.
17. Wu MJ, Jan CI, Tsay YG, *et al*: Elimination of head and neck cancer initiating cells through targeting glucose regulated protein 78 signaling. Mol Cancer 9: 283, 2010.
18. Bi M, Naczki C, Koritzinsky M, *et al*: ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. EMBO J 24: 3470-3481, 2005.
19. Romero-Ramirez L, Cao H, Nelson D, *et al*: XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. Cancer Res 64: 5943-5947, 2004.
20. Kaur M, Singh K, Rup PJ, *et al*: A tuber lectin from *Arisaema helleborifolium* Schott with anti-insect activity against melon fruit fly, *Bactrocera cucurbitae* (Coquillett) and anti-cancer effect on human cancer cell lines. Arch Biochem Biophys 445: 156-165, 2006.
21. Xia S, Zhao Y, Yu S and Zhang M: Activated PI3K/Akt/COX-2 pathway induces resistance to radiation in human cervical cancer HeLa cells. Cancer Biother Radiopharm 25: 317-323, 2010.
22. Martinon F, Chen X, Lee AH and Glimcher LH: TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. Nat Immunol 11: 411-418, 2010.
23. Austin RC: The unfolded protein response in health and disease. Antioxid Redox Signal 11: 2279-2287, 2009.
24. Yang W, Tiffany-Castiglioni E, Koh HC and Son IH: Paraquat activates the IRE1/ASK1/JNK cascade associated with apoptosis in human neuroblastoma SH-SY5Y cells. Toxicol Lett 191: 203-210, 2009.
25. Kaser A, Lee AH, Franke A, *et al*: XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell 134: 743-756, 2008.
26. Lin JH, Li H, Yasumura D, *et al*: IRE1 signaling affects cell fate during the unfolded protein response. Science 318: 944-949, 2007.
27. Urano F, Wang X, Bertolotti A, *et al*: Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 287: 664-666, 2000.
28. Verheij M and Bartelink H: Radiation-induced apoptosis. Cell Tissue Res 301: 133-142, 2000.
29. Puthalakath H, O'Reilly LA, Gunn P, *et al*: ER stress triggers apoptosis by activating BH3-only protein Bim. Cell 129: 1337-1349, 2007.
30. McCullough KD, Martindale JL, Klotz LO, Aw TY and Holbrook NJ: Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21: 1249-1259, 2001.
31. Lee AS: GRP78 induction in cancer: therapeutic and prognostic implications. Cancer Res 67: 3496-3499, 2007.
32. Zhang B, Wang Y, Pang X, *et al*: ER stress induced by ionising radiation in IEC-6 cells. Int J Radiat Biol 86: 429-435, 2010.
33. Lee KR, Kim SW, Kim YK, *et al*: Silkworm Hemolymph Down-Regulates the Expression of Endoplasmic Reticulum Chaperones under Radiation-Irradiation. Int J Mol Sci 12: 4456-4464, 2011.
34. Yadav RK, Chae SW, Kim HR and Chae HJ: Endoplasmic reticulum stress and cancer. J Cancer Prev 19: 75-88, 2014.
35. Hall EJ: Radiobiology for the radiologist. 5th ed. Lippincott Williams and Wilkins, Philadelphia, pp32-50, 2000.
36. Ivanov A, Krysov S, Cragg MS and Illidge T: Radiation therapy with tositumomab (B1) anti-CD20 monoclonal antibody initiates extracellular signal-regulated kinase/mitogen-activated protein kinase-dependent cell death that overcomes resistance to apoptosis. Clin Cancer Res 14: 4925-4934, 2008.
37. Jantschitsch C, Majewski S, Maeda A, Schwarz T and Schwarz A: Infrared radiation confers resistance to UV-induced apoptosis via reduction of DNA damage and upregulation of antiapoptotic proteins. J Invest Dermatol 129: 1271-1279, 2009.
38. Reddy RK, Mao C, Baumeister P, *et al*: Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem 278: 20915-20924, 2003.
39. Fu Y, Li J and Lee AS: GRP78/BiP inhibits endoplasmic reticulum BIK and protects human breast cancer cells against estrogen starvation-induced apoptosis. Cancer Res 67: 3734-3740, 2007.
40. Nawrocki ST, Carew JS, Dunner K Jr, *et al*: Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. Cancer Res 65: 11510-11519, 2005.