Original Article
The role of autophagy and Beclin-1 in radiotherapy-induced apoptosis in thyroid carcinoma cells

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Abstract: Background/Objectives: Thyroid cancer (TC), especially primary squamous cell carcinoma (SCC), is an aggressive malignant tumor which usually resists radiotherapy (RT). We wanted to understand the mechanism of incomplete response of RT in TC cells. Methods: SCC cell line SW579 cells were exposed to X-rays. Apoptosis and autophagy were measured by FACS; 3MA, inhibitor of autophagy, and Beclin-1 siRNA were used to investigate the effect of autophagy on radiation-induced TC cell apoptosis. Results: RT could induce TC cell apoptosis through death receptor as well as mitochondrial pathway. Radiotherapy also induces autophagy and increases expression of autophagy-associated proteins Beclin-1 and LC3. Moreover, inhibition of autophagy by 3MA and Beclin-1 siRNA enhanced radiation-induced apoptosis, through up-regulation of the expression of p53. Conclusion: RT-induced autophagy resists apoptosis by the p53 pathway and an incomplete response to RT may be associated with autophagy in TC patients.

Keywords: Autophagy, Beclin-1, apoptosis, radiotherapy, thyroid carcinoma cells

Introduction

Thyroid carcinoma (TC) is a fairly rare aggressive neoplasm in human, which accounts for about 1% of all new malignant disease. In the past few decades, various approaches such as, surgery, chemotherapy, and radiotherapy (RT), are widely used; however the survival rates have not improved much, which suggests that some approaches should be explored and improved [1, 2].

Primary squamous cell carcinoma (SCC) of the thyroid gland is a more rare and aggressive entity representing < 1% of all primary carcinomas of the thyroid gland. Only a few cases are reported world wide. The overall survival is less than 6 months after diagnosis. Expect surgery, RT is a widely used approach to improve the survival time and life quality [3]. However, RT has limitations including incomplete response and toxicity. Some clinic studies showed that 67% of differentiated TC patients have an incomplete structural response to RT which is associated with significantly worse clinical outcome [4]. Therefore, to improve the response to RT, we are trying to investigate the mechanism by which SCC cells are resistant to RT.

Apoptosis, as one type of antitumor mechanism, is observed in malignant tumor cells in response to RT irradiation. These cells are typically programmed for rapidly induced, rapidly executed, apoptotic death occurring within hours after exposure to radiation [5]. The mechanisms of apoptosis are highly complex and sophisticated, involving caspase cascade events. Two main pathways including the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway mediate cell apoptosis individually or mutually [6]. In response to irradiation, P53 activation induces cell apoptosis by activation of pro-apoptotic genes like Bax and Apaf1, or repression of anti-apoptotic pro-
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Moreover, autophagy is a self-degradative process that is a vital event for the stress response. It plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles, and eliminating intracellular pathogens. Thus, autophagy is thought to be a survival mechanism, although its deregulation also is linked to cell death [9, 10]. RT is believed to induce autophagy through various cellular insults, such as, oxidative stress, endoplasmic reticulum stress, calcium homeostasis imbalance, and altered mitochondrial potential [11, 12]. However, the role of autophagy in RT-induced cancer cell death is controversial.

For instance, autophagy might contribute to regulate viability and survival effects in human glioma cell lines after ionizing radiation (IR), which suggests that IR-induced autophagy might be involved in high-grade glioma prognosis [13, 14]. There is controversy whether berberine, an alkaloid, could improve IR-induced A549 cell apoptosis as well as cause a substantial shrinkage of the tumor volume in a mouse model through enhancement of autophagy [15]. Therefore, we would like to clarify the role of autophagy in RT-induced TC cell death.

Thus, in this study, we would like to investigate how irradiation induces a SCC cell line SW579 to undergo cell death and try to find whether and how autophagy participated in X-ray-induced cell death. Finally, we would like to clarify the molecular mechanism.

Materials and methods

Reagents

5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), monodansylcadaverine (MDC), 3-methyladenine (3-MA), rapamycin (Rap) were purchased from Sigma Chemical (St. Louis, MO, USA). Control siRNA, Beclin-1 siRNA (Santa Cruz, CA, USA). Polyclonal antibodies against Beclin-1, LC3, P53, Caspase-3/8/9, Bax, Bcl-2, β-actin and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human thyroid squamous cell carcinoma SW-579 cells (HTB-107) were obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in Leibovitz’s L-15 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 2 mM glutamine (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37°C without CO₂ in a humidified atmosphere.

X-ray irradiation

SW579 were seeded in dishes and incubated overnight prior to irradiation. The cells were irradiated with different doses from 1 Gy to 12 Gy using an X-ray linear accelerator (160 kV, 25 mA; RadSource, Suwanee, GA, USA) at a fixed dose rate of 0.1 Gy/s. The focus skin distance was 40 cm, and the irradiation field was 280 mm × 180 mm with a dose uniformity of > 95%. Control group was sham-irradiated cells. After irradiation, cells were further cultured for indicated periods until analysis.

Growth inhibition assay

SW579 cells were seeded into 96-well plates and incubated overnight. After X-ray irradiation (1-12 Gy) treatment, cells were further cultured for 6, 12, 24 and 48 h and then collected for MTT analysis as previously [16]. Cell was washed three times by PBS and incubated in 100 μL of 0.5 mg/mL MTT solution at 37°C for 3 h. The residual cell layer was dissolved in 150 μL of DMSO, the optical density was measured at 490 nm wavelength using a microplate reader (Thermo Scientific, Shanghai, China). Growth inhibition was calculated using the following equation:

Growth inhibition ratio (%) = (A₄₉₂ control - A₄₉₂ sample)/(A₄₉₂ control - A₄₉₂ blank) × 100

siRNA transfection

2 × 10⁵ SW579 cells were transfected with a mixture of 15 μg of double-stranded siRNA targeted to negative control or Beclin-1 and 60 μl TranSmarter (Abmart) as manufacturer description. The transfected cells were maintained for 24 hours before treatment.

Flow cytometric analysis of apoptosis

After treatment, SW579 cells were labeled with 1 ml PI solution (50 mg/l) and Annexin V (FITC) at dark place for 30 min. After washing, the cells were scraped and suspended in PBS, and

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10,000 cells were analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

**MDC or DAPI staining**

SW579 cells or siRNA-treated were incubated in 6-well culture plates and treated with or without 6 Gy X-ray. The cells were collected at indicated time points and stained in 0.05 mM MDC solution at 37°C for 40 min or 3µM DAPI buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% Nonidet P-40) at RT for 1 h. After washing by PBS, the fluorescent changes were observed by Olympus IX70 reverse fluorescence microscopy (Olympus, Tokyo, Japan).

**Flow cytometric analysis of autophagy**

SW579 cells (2 × 10⁵/well) were incubated in 6-well dishes for overnight and pre-treated with or without 3-MA for 1 h. Then the cells were irradiated with or without 6 Gy X-ray and collected for staining using MDC as above. Then the cells were analyzed with flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with the emission wavelength at 525 nm. The fluorescent intensity of intracellular MDC reflected the level of autophagy. A threshold M1 was set-up to quantify the up-regulated autophagic cell number.

**Western blot analysis**

After treatment, SW579 cells were harvested and washed twice with PBS; then lysed in lysis buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin) at 4°C for 60 min. The supernatants were collected for western blot analysis, and protein concentration was determined by the Folin assay. Equal amounts of protein sample were run in 12% SDS-PAGE and trans-bloting to nitrocellulose membranes. Antibodies against Beclin-1, LC3, caspase 3, caspase 8, caspase 9, bcl-2, Bax, PS3 and caspase 9 were used, and the specific protein level was visualized by using ECL as the HRP substrate.

**Statistical analysis**

All data represent at least three independent experiments and are expressed as mean ± SD. Statistical comparisons were made using Student's t-test. P values of less than 0.05 were considered as significant difference.

**Results**

**RT induced apoptosis in TC cells**

To understand the role of RT in TC cells, we used human thyroid squamous cell carcinoma SW579 cells as a cell model and treated with various doses of X-ray. Post-irradiation, we analyze the growth inhibition ratio at various time points by MTT assay. MTT analysis indicated that RT inhibited SW579 cell growth in a dose-dependent manner (Figure 1A). 6 Gy X-ray irradiation induced 47.9% cell death 24 hours post-irradiation and 78.2% cell death 48 hours post-irradiation. In the case of high doses, 12 Gy X-ray irradiation, 92.3% cells were dead at 48 hours post-irradiation. Therefore, a middle dose, 6 Gy X-ray irradiation, was used in the following assay to mimic RT.

To further clarify the mechanism of RT in SW579 cell growth inhibition, the cell nuclear morphology was observed by DAPI staining and cell apoptosis ratio was analyzed by FACS with Annexin V-PI staining. Post-irradiation for 24 h and 48 h, the cells appeared as apoptotic bodies compared with the control group (Figure 1B). Annexin V-PI staining indicated that Annexin V positive cell number ratio, i.e. apoptosis cell ratio, respectively was increased from 0.47% to 47.13% and 93.96% after X-ray irradiation for 24 h and 48 h (Figure 1B), which was consistent with the MTT analysis data. Since Annexin V positive and PI negative cells are early apoptotic cells, the ratio of early apoptotic cells accounts for the major part. This suggested that X-rays could induce SW579 cell death through apoptosis.

In addition, following radiation treatment for 24 h and 48 h, the two pathways of apoptosis, intrinsic and extrinsic pathways, were analyzed by western blotting. Compared with non-irradiation cells, X-rays could induce up-regulation of caspase 3 and caspase 8 as time passed (Figure 1C). Further, the pro-apoptosis protein Bax was up-regulated and anti-apoptosis protein Bcl-2 was repressed by radiation treatment. These results present that radiation treatment might induce thyroid carcinoma cell apoptosis both by the death receptor pathway and the mitochondria-dependent pathway.
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To understand the role of autophagy in RT-induced TC cells apoptosis, autophagy level was quantified by FACS following MDC staining. X-ray irradiation induced an increase of MDC positive SW579 cell population, which was respectively increased to 6.33%, 22.8% and 11.67% at 12, 24 and 48 h (Figure 2A). Further, the intense punctuate MDC fluorescence which represented the autophagic vacuoles were clearly increased in X-ray irritated SW579 cells (Figure 2B). Moreover, the expression of autophagy associated protein Beclin-1 and conversion of LC3 I to LC3 II were raised by radiation treatment (Figure 2C). These dates indicate that autophagy could be induced by RT in TC cells.

Inhibition of autophagy by 3MA and Beclin-1 siRNA promotes RT-induced apoptosis

To further document the relationship between RT-induced autophagy and apoptosis, we introduced 3MA, an inhibitor of autophagy and siRNA which targets the autophagy associated protein Beclin-1 to study its effect on RT-induced apoptosis. 3MA significantly inhibited the autophagy level; but it also increased X-ray-induced cell death ratio from 44.5% to 61.0% (Figure 3A). This assay suggests that RT-induced autophagy could play a productive role in TC cells.

Then Beclin-1 siRNA was used to block autophagy, and apoptosis was analyzed by FACS with Annexin V-PI staining. Compared to X-ray treatment group, Beclin-1 siRNA treatment further increased the Annexin V-positive cell population from 28.2% to 47.6% (Figure 3B). From the nuclear morphology observation, Beclin-1 siRNA treatment clearly enhanced X-ray-induced apoptotic bodies in SW579 cells (Figure 3B). This suggested that autophagy-induced by RT could decrease apoptosis in TC cells.

In addition, we analyzed the apoptosis-associated protein expression under X-ray irradiation in Beclin-1 sliced SW579 cells. Caspase 3, 8 and 9 was upregulated when SW579 cells were treated with X-ray, however this increase was blocked in Beclin-1 knock down cells. X-ray-induced an increase of pro-apoptosis Bax and a decrease of anti-apoptosis Bcl-2 which was not observed in siBeclin-1 SW579 cells (Figure 3C). In sum, our data indicate that autophagy could repress RT-induced apoptosis in TC cells.
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Since RT could induce double strand DNA breaks and activate P53, we analyzed the role of P53 in RT-induced autophagy resists apoptosis in TC cells. Here, we found that X-ray irradiation could induce up-regulation of P53 expression (Figure 4A). Moreover, when autophagy was inhibited by 3-MA and siBeclin-1, P53 was further up-regulated under irradiation in SW579 cells (Figure 3A). Then pifithrin-α (PFT-α) which clearly inhibited irradiation, and induced an increase of P53 expression (Figure 4A) was introduced to document the role of P53 on apoptosis. When PFT-α was added, the X-ray-induced apoptosis rate was significantly reduced, and this reduction was partly reversed in siBelin-1 group (Figure 4B). Thus, RT-induced autophagy may inhibit P53 and help resist apoptosis in TC cells.

Discussion

The role of autophagy is complex and controversial in different cancer cells. In thyroid cancer, autophagy-related proteins such as LC3, BNIP-3 and Beclin-1 were expressed, and their expression differs according to TC subtype [17, 18]. Especially, Beclin-1 is highly expressed in papillary thyroid carcinoma and associates with lymph node metastasis [19]. A drug called pseudolaric acid B (PAB) shows ability to inhibit the proliferation of SW579 cells and PAB-induced autophagy plays a protective role [19]. Here, by using the autophagy inhibitor and Beclin-1 siRNA, a higher amount of apoptosis cancer cells was observed in response to RT. We suggest that autophagy plays a survival role in thyroid cancer, especially under RT.

Further, our data showed that autophagy could inhibit P53 expression and block RT-induced apoptosis in thyroid cancer. P53 is a well-known anti-tumor transcription factor which regulates various gene expression. RT-induced and P53-dependent cell apoptosis is well understood in various type of cancer cells and normal tissue. Briefly, radiation induces double-strand DNA breaks, which leads to activation of DNA damage checkpoints so that P53 initiates the target gene expression inducing apoptosis-associated genes and cell arrest genes [20]. However, the relationship between autophagy and P53 is

Figure 2. Autophagy was induced by X-ray in SW579 cells. A. Autophagy positive rate was increased by X-ray irradiation in SW579 cells. The autophagy positive rate was measured by FACS with MDC staining. B. Autophagic vacuoles were observed in X-ray-irradiated SW579 cells at 24 h post-irradiation by MDC staining. C. Autophagic protein change was analyzed in SW579 cells under X-ray irradiation. Western blot was performed to detect autophagy-associated protein expression including Beclin-1 and LC3. SW579 cells were treated by X-ray as Figure 1B.
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Figure 3. Autophagy may inhibit X-ray-induced apoptosis in SW579 cells. A. X-ray-induced cell death rate was increased when 3MA was added in SW579 cells. SW579 cells were pre-treated with or without 3 mM 3MA for 1 h and then irradiated by 6 Gy X-radiation. Growth inhibition and autophagy rate were measured by MTT and FACS with MDC staining at 24 h after X-ray exposure. Results are expressed as mean ± SD, n = 3. *P < 0.05 vs Rad group. B. X-ray-induced cell apoptosis ratio was increased when Beclin-1 was silenced in SW579 cells. SW579 cells transfected with Beclin-1 or control siRNA were exposed to 6 Gy X-radiation. Cell apoptosis rate was measured by FACS with Annexin V-PI staining at 24 hr after exposure. C. Autophagy may induce apoptosis-associated protein changes in SW579 cells. SW579 cells transfected with Beclin-1 or control siRNA were exposed to 6 Gy X-radiation. Western blot was performed at 24 h post-irradiation to analyze apoptosis-associated proteins.
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Figure 4. X-ray-induced autophagy can resist apoptosis in SW579 cells by P53. A. X-ray-induced autophagy could enhance P53 expression. SW579 cells were pre-treated with or without 3 mM 3MA or 10 μM pifithrin-α for 1 h and then irradiated by 6 Gy X-radiation. Western blot was used to analyze P53 expression. B. Inhibition of P53 could repress X-ray-induced apoptosis in SW579 cells. SW579 cells transfected with Beclin-1 or control siRNA were used to pretreat with or without 10 μM pifithrin-α for 1 h and then irradiated by 6 Gy X-ray. Apoptosis rate was analyzed by FACS with Annexin V-PI staining at 24 hr after exposure. Results are expressed as mean ± SD, n = 3. #, P < 0.05 vs negative control group; *, P < 0.05 vs Rad group, **, P < 0.01 vs Rad group.

complex: either induction or repression have been reported [21]. Autophagy mediates tumor promotion by suppressing the p53 response, maintaining mitochondrial function, sustaining metabolic homeostasis and so on [22]. For example, autophagy opposes the p53-mediated breast cancer barrier to promote tumorigenesis [23]. In thyroid cancer, our work showed that RT-induced autophagy can repress P53 expression since autophagy inhibitor by siRNA can enhance RT-induced P53 expression.

Moreover, our work demonstrated X-ray irradiation could induce autophagy which resists apoptosis via P53 pathway in SW579 cells. This indicated that an incomplete response to RT could occur through autophagy and the P53 pathway in TC patients, especially SCC patients. P53 expression level was reported to contribute to an incomplete response to RT in various cervical cancer patients, and only some of the patients show up-regulation of P53 in response to RT [24, 25]. Moreover, P53 polymorphism was reported to influence individual responsiveness to chemo-radiotherapy in advanced head and neck cancer [26]. Further, radiation-induced autophagy contributes to cytoprotection and radio-sensitization in various cancer cells including prostate cancer cells [27], human glioblastoma multiforme cells [28], and others [29]. Therefore, we believe the autophagy-p53 pathway might contribute to an incomplete response to RT in TC patients, especially in SCC patients.

In conclusion, the present study supports that RT can induce autophagy which represses the P53-mediated cell apoptosis in TC, especially SCC. A combination of anti-autophagy drugs and RT could be a good strategy for treatment of TC by enhancement of the response to RT.

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Disclosure of conflict of interest

None.

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