Indian J Med Res 149, May 2019, pp 627-632
DOI: 10.4103/ijmr.IJMR_1455_17

Quick Response Code:

Apoptosis gene reprograming of human peripheral blood mononuclear cells induced by radioiodine-131 (^{131}I) irradiation

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Received September 6, 2017

***Background & objectives*: The nature of adaptable change of B-cell lymphoma-2 (*BCL-2*) and/or Bcl2-associated X protein (*BAX*) gene expression in the human peripheral blood mononuclear cells (PBMCs) irradiated by radioiodine in thyroid diseases therapy is not fully understood. In this study, the alternation of apoptotic gene expression was evaluated while the PBMCs collected from healthy volunteers were irradiated by the radioiodine-131 (^{131}I).**

***Methods*: Fasting blood samples were obtained from healthy volunteers. PBMCs from group 0 to 6 were incubated and exposed to different doses of ^{131}I in cell suspension for 6, 12, 24 and 48 h. The apoptosis rates and expression of *BCL-2* and *BAX* genes of PBMCs were examined.**

***Results*: The apoptosis rate in the human PBMCs was gradually enhanced after six hour irradiation. The values of *BCL-2* and *BAX* gene expression in groups 1-6 were higher than in group 0 within 6 h of irradiation, and then, these were decreased gradually from 6 to 12 h. *BCL*-2 gene expression increased in groups 1-3 after 12 h irradiation, but there was no difference in groups 4-6. The ratio of *BCL-2/BAX* gene expression among groups 4-6 gradually decreased during the period from 6 to 12 h, and it was significantly lower than in the group 0 at 12, 24 and 48 h.**

***Interpretation & conclusions*: The expression of *BCL-2* and *BAX* genes was initially upregulated following irradiation. Later, the balance of *BCL-2/BAX* genes expression was adjusted, and then, PBMCs underwent apoptosis at higher doses of radiation.**

Key words Apoptosis - gene expression - ^{131}I - mononuclear cell - radiation

Radioiodine has been widely used in hyperthyroidism and post-operative ablation of remnant thyroid tissue or metastasis in thyroid carcinoma patients for several decades[1,2]. The irradiation emitted from radioiodine would harm the peripheral blood mononuclear cells (PBMCs) *in vivo*, which play an important role in the immune response and in host homoeostasis, inflammation and elimination of tumour cells[3]. The results of *in vitro* and *in vivo* study demonstrated that the γ-radiation would induce the programmed cell death (apoptosis) of PBMCs[4]. The apoptosis of PBMCs was induced by γ-irradiation in

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a dose-dependent manner[5]. However, Vokurková *et al*[6] reported that apoptosis was not detected within the first six hours in PBMCs isolated from blood of healthy donors when irradiated by a dose of 7 Gy, but in 50 per cent of the cells after 16 h and almost all cells after 48 h.

The mechanism of PBMC apoptosis induced by radioiodine in thyroid disease therapy is not completely understood[7]. The mechanism of the radiation-induced apoptosis would be associated with the adjustment of apoptosis gene[8]. The key event in the initiation of apoptosis is activation of caspase gene[9,10], which is initiated by two distinct mechanisms mediated by cell surface receptor[11] and mitochondrial factors[12,13]. The event of apoptosis is mainly controlled by several genes[14], including B-cell lymphoma-2 (*BCL-2*) family members, p53, first apoptosis signal (*FAS*) and Fas ligand (*FASL*). The expression of the *BCL-2* family members is a crucial factor in the sensitivity of cells to radiation-induced apoptosis[5].

BCL-2 family members belong to two main categories[15]. One is restraining apoptosis genes, such as *BCL-2*, *BCL*-extra-large (*BCL-XL1*) and Bag family molecular chaperone regulator 1 (*BAG1*). The upregulated expression of *BCL-2* enhances the ability of lymphocytes in resisting γ-radiation[16]. *BCL-2* gene family plays an important role in radiation-induced apoptosis and its interaction determines the regulatory biological effect[5,17]. Another category is promoting apoptosis genes, for example, *BCL-2*-associated X protein (*BAX*). Bax proteins exist in the cytoplasm or attach loosely at membrane as monosomes[17,18]. When stimulated by the death signal, these protein monosomes transpose to mitochondrial membrane, transform into intrinsic membrane protein and cross-link as homodimer and finally lead cell to death. Nakagawa *et al*[19] demonstrated that expression of *BAX* was upregulated in NIH3T3 cells within eight hours after ultraviolet light irradiation, and more than 50 per cent of the NIH3T3 cells underwent apoptosis 48 h after irradiation. Bcl-2 and Bax proteins are always co-ordinately expressed in cells[17], and the elevated Bcl-2/Bax ratio promotes cell survival and vice versa. Bcl-2/Bax change plays a vital role in determining cell fate[20,21]. Increasing level of Bcl-2/Bax heterodimer may prevent Bax inserting into mitochondrial membrane and inhibit the release of apoptosis factors, *e.g*. cytochrome complex (Cyt c) from mitochondria into cytosol, thereby inhibiting apoptosis[22].

It is contentious whether the radioiodine irradiation would damage human body's immune system during the radionuclide therapy in thyroid diseases[1,7]. The sequence of events and nature of adaptable change in B *BCL-2* and *BAX* genes following irradiation of human PBMCs by radioiodine in thyroid diseases therapy are not fully understood. We here present the change in apoptotic genes expression in human PBMCs following irradiation of radioiodine (^{131}I).

Material & Methods

The study was conducted in the departments of Nuclear Medicine and Breast and Thyroid Surgery, Third Affiliated Hospital of Sun Yat-Sen University, PR China. This study was approved by the clinical medical research Ethical Clearance Committee in the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, PR China. Informed written consents were obtained from healthy volunteers for the collection of blood specimens. The PBMC samples obtained from blood sample (5 ml) of 12 volunteers were divided into seven groups, and each group had four-hole plate sample for four test points. The study was repeated three times for apoptosis, *BCL-2* and *BAX* gene analysis, respectively.

Human PBMCs were obtained from fresh whole blood after differential migration with Ficoll Paque[1]. Washed twice with cold phosphate buffered saline[3], the cells were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) supplemented with 10 per cent foetal bovine serum (Zhejiang Tianhang Biotechnology, PR China) at a density of 1.5×10^6 cells/ml unless otherwise noted. The viability of these cells was greater than 98 per cent as estimated by trypan blue exclusion test[1,3]. The fluorescent dye 4′, Annexin V/PI double-staining kit were purchased from Keygen, PR China. Total RNA Extraction Kit I was from Omega, USA. *BCL-2* primer was procured from Takara, Japan. *BAX* primer was from Invitrogen, USA.

Exposure of human PBMC samples to ^{131}I: PBMC samples with cell density of 1.5×10^6/ml were placed in each well of a 12-well culture plate. The radioactive concentration in the blood was approximately 74 kBq/ml in the hyperthyroidism patients administrated with 370-555 MBq radioiodine in the next morning and was approximately 1332 kBq/ml in differentiated thyroid carcinoma patients with 7400 MB ^{131}I in our preparatory experiments. Hence, the PBMC samples were divided into six groups (0-6). The

group 0 was set as control group without radioiodine, and groups 1-3 were set for lower irradiation dose and treated with 18.5, 37 and 74 kBq ^{131}I/ml cell suspension, respectively. Groups 4 to 6 were set for higher irradiation and treated with 333, 666 and 1332 kBq ^{131}I/ml cell suspension, respectively. Immediately, these were incubated at 37°C in five per cent CO_2 humidified atmosphere for 6, 12, 24 and 48 h and collected for analysis. Each experiment was repeated thrice.

Cell apoptosis detection: Annexin V-FLUOS assay was used for apoptosis detection according to the manufacturer's instruction. Briefly, 5 µl FITC-Annexin V and 5 µl propidium iodide (PI) were added to 5×10^5 cells suspended in 500 µl binding buffer. The mixture was incubated for 15 min in the dark at room temperature and was analyzed using FACS Aria flow cytometry (BD Company, USA) within one hour.

Semi-quantitative RT-PCR detection of BCL-2 and BAX mRNA: Briefly, total RNA was isolated using Total RNA Extraction Kit I (Omega, USA) according to the manufacturer's protocol. Reverse transcription-generating cDNA was performed using first strand cDNA Synthesis Kit (Fermentas, USA). PCR of *BCL-2* and *BAX* cDNA using Go-Taq Green Master Mix (Promega, USA) was performed, with β-actin as internal standard.

BCL-2 cDNA was amplified using forward (5′-ACAACATCGCCCTGT GGATGAC-3′) and reverse (5′-ATAGCTGATTCGACGTTTTGCC-3′) primers, which produced a 409 bp product. Amplification of *BCL-2* cDNA was performed at 94°C for five minutes for preheating, followed by 40 cycles of 94°C for 30 sec, 63°C for 30 sec, 72°C for 10 sec and a final extension of 72°C for five minutes.

BAX cDNA was amplified using forward (5′-GGACGAACTGGACAGTAACA-3′) and reverse (5′-ACCACCCTGGTCTTGGAT-3′) primers, which produced a 271 bp product. Amplification of *BAX* cDNA was performed at 94°C for five minutes for preheating, followed by 31 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 10 sec and a final extension of 72°C for five minutes.

β-actin was amplified using forward (5′-GCTCGTCGTCGACAACGGCTC-3′) and reverse (5′-CAAACATGATCTGGGTCATCTTCTC-3′) primers, which produced a 359 bp product. All primers used in this study were synthesized by Invitrogen.

All PCR products were electrophoresed on two per cent agarose gels containing ethidium bromide. The resulting bands were photographed under ultraviolet light and analyzed using a Gel Imaging System (Gel Doc2000, Bio-Rad, Hercules, CA, USA). The relative intensity of bands of interest was represented as the ratio to β-actin mRNA bands. The ratio of fluorescence intensity of target-specific product to the internal control product was represented as the relative levels of target mRNA expression.

Statistical analysis: Data were presented as percentages, mean and standard deviation. Significance was assessed by analysis of variance for repeated measures and least significant difference test to compare the groups. Statistical analysis was performed using SPSS for Windows Release 13.0 (SPSS Inc., USA).

Results

Apoptosis rates of PBMCs after radioiodine irradiation: The data of apoptosis rate from groups 0 to 6 are presented in the Table while the PBMCs were irradiated within 6 to 48 h. The apoptosis rate from group 1 to 6 was not significantly different from that in the group 0 at six hours but increased gradually during the time period of irradiation from 6 to 48 h. The apoptosis rates in groups 4-6 were higher at 48 h than at 24 h irradiation (P<0.001). The apoptosis rate

Table. Percentage change of apoptosis rates after radioiodine irradiation

Time (h)	Group 0	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
6	6.26±1.30	6.87±0.51	5.80±1.57	6.37±0.85	5.47±0.84	6.23±1.17	6.63±0.41
12	9.55±0.78	11.79±1.59	10.9±0.77	11.49±1.24	11.24±1.15	12.35±0.79	14.73±1.32
24	13.69±4.24	12.09±3.27	12.52±3.23	13.42±2.91	16.84±2.25	20.79±3.26	26.47±3.45
48	22.23±4.67	22.96±5.79	22.45±4.61	23.63±1.86	34.80±1.01***†††	41.32±2.64***†††	51.79±8.51***†††

Values are mean±SD (n=3)
$^{***}P$<0.001 compared to respective values in the same group at 24 h
$^{\dagger\dagger\dagger}P$<0.01 compared to values in groups 0-3 at 48 h

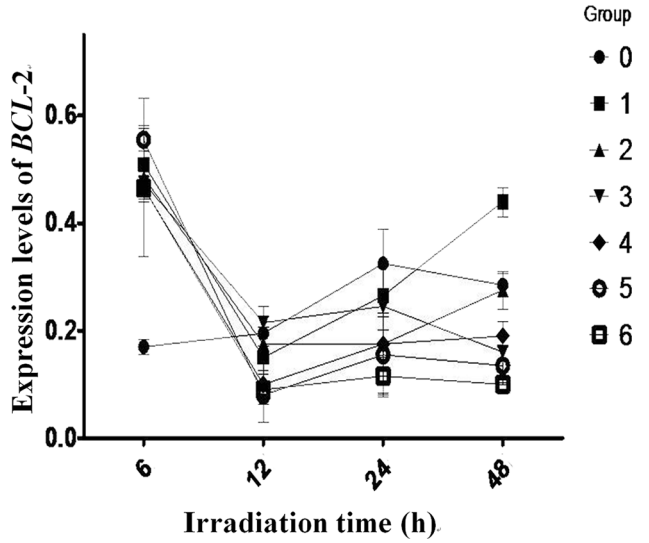

Fig. 1. Expression of B-cell lymphoma-2 (*BCL-2*) in the treatment groups decreased gradually from 6 to 12 h and increased gradually in groups 1-3 from 12 to 48 h but remained unchanged in groups 4-6. The expression of *BCL-2* in treatment groups increased significantly than the control group at six hours (P<0.01). Values are mean±SD of three observations.

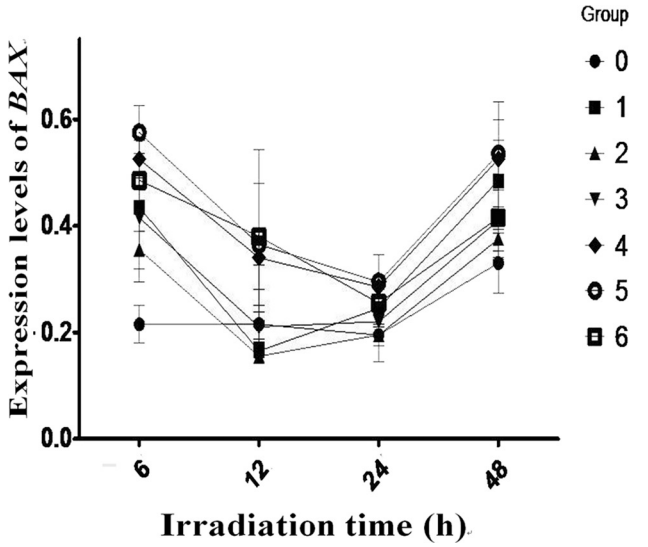

Fig. 2. Expression of Bcl-2 associated *BAX* protien in all treatment groups decreased gradually from 6 to 24 h and decreased more obviously in groups 4-6 than others. However it increased more obviously in groups 4-6 at 48 h. Values are mean±SD of three observations.

among groups 4-6 was higher than for groups 0-3 at 48 h (P<0.001).

BCL-2 gene expression after radioiodine irradiation: The expression value of *BCL-2* gene in human PBMCs from groups 1 to 6 increased compared to the control group at six hours (P<0.01). However, the expression value of *BCL-2* in human PBMCs from groups 1 to 6 decreased gradually in the period from 6 to 12 h and increased in groups 1-3 from 12 to 48 h but had no significant change in groups 4-6 (Fig. 1). The gene expression value of *BCL-2* in all groups had no significant difference at 12 and 24 h, but these were lower in groups 4-6 than in groups 0-2 at 48 h (P<0.001).

BAX gene expression after radioiodine irradiation: The value of *BAX* gene expression in the human PBMCs from groups 1 to 6 was significantly higher than in the control group 0 at 6 h (P<0.01). The value of *BAX* gene expression in groups 1-6 was decreased from 6 to 12 h. However, there was no significant difference in *BAX* gene expression among groups 1-6 compared with group 0 at 12, 24 and 48 h, though the value of *BAX* gene expression in all groups had an ascendant trend after 24 h (Fig. 2).

Ratio of BCL-2/BAX gene expression after radioiodine irradiation: The ratio of *BCL-2/BAX* gene expression in the human PBMCs in groups 4 to 6 was decreased from 6 to 12 h while that in groups 0-3 was relatively unchanged. However, it was significantly lower in groups 4-6 than in the group 0 at 12, 24 and 48 h (P<0.05). The ratio of *BCL-2/BAX* in groups 1 to 6 was similar to group 0 at 6 h.

Discussion

Programmed cell death, also known as apoptosis, plays a vital role in regulating the cell number of human body and function of organs[3,16,23]. The major mechanism for cell injury and damage caused by ionizing radiation is regarded as being the radiation-induced apoptosis[8,16]. Apoptosis of PBMCs could be induced by γ-irradiation in a dose-dependent manner[4]. In the present study, the apoptosis rates among irradiated cells increased gradually during the time period of irradiation from 6 to 48 h. Though, the apoptosis rate of PBMCs in groups 1-3 had no increase compared with that in control group, but the apoptosis rate of PBMCs in relatively higher dose group (groups 4-6) was higher than in the lower dose groups and control group after the PBMCs were irradiated within 48 h. Those results indicated that the apoptosis event in PBMCs was dose dependent. Micronucleus formation in the lymphocytes was increased with the increasing radiation dose[24]. The apoptosis rate of PBMCs induced by irradiation was not only associated with radiation intensity but also with the exposure time[25].

BCL-2 gene is considered as one of the strongest anti-apoptosis genes[26,27], and the *BAX* gene is one of main apoptosis promoting genes[19]. Earlier reports[28] demonstrated that upregulating expression of *BCL-2* might enhance the ability of lymphocytes in resisting to γ-radiation. In the present study, the PBMCs upregulated the *BCL-2* and *BAX* gene expression within six hour of irradiation. It implied that the PBMCs might upregulate the expression of *BCL-2* gene to prevent apoptosis induced by irradiation in early period. However, it was not clear as to why the level of both *BCL-2* and *BAX* gene expression decreased during the time point from 6 to 12 h, while the PBMCs were irradiated by radioiodine in all group. This might be because all gene expressions in cells would be inhibited by the initial irradiation.

The *BCL-2/BAX* ratio plays an important role in mitochondria-mediated apoptosis[18]. The expression of *BCL-2* and *BAX* genes in PBMCs can be changed after radiation[16,29]. In our study the gene expression value of *BCL-2* in PBMCs increased gradually in lower dose groups 1-3 during the time points from 12 to 48 h of irradiation. Gradual rise in *BCL-2* expression in PBMCs receiving lower doses of irradiation (as in groups 1-3) may be due to an adaptive upregulation of *BCL-2* to prevent apoptosis; however, at higher doses with greater extent of damage, the pro-apoptotic regulators prevail and cells undergo apoptosis.

BAX is one of main apoptosis promoting genes. In the present study, the *BAX* gene expression was up-regulated in the PBMCs irradiated within six hours also, but later the *BAX* gene expression was down-regulated close to the level in control group, and there were no significant difference in different radioactive dose samples compared with the control group after 12 h irradiation. These results indicated that the *BAX* gene expression might not be the key event while the apoptosis was induced by the radioiodine irradiation.

Bcl-2 and Bax proteins are co-ordinately expressed in cells; Bcl-2 and Bax ratio plays a vital role in determining cell fate[20]. In the present study, the ratio of *BCL-2/BAX* gene expression in higher dose group was lower than that in lower dose group after 12 h. These results implied that the mechanism of apoptosis induced by radioiodine radiation in PBMCs would be due to the decrease of *BCL-2/BAX* ratio. In the lower dose group, cells did not undergo apoptosis because the *BCL-2* gene expression was upregulated and the *BCL-2/BAX* heterodimer was increased to prevent apoptosis.

The present study had certain limitations. The actual irradiation dose was not calculated because it was difficult and complicated to accurately calculate the irradiated dose of radioiodine in the PBMCs[30]. Further, the radioactive concentration in PBMCs was set according to the rough results of the radioactive concentration in the blood in several hyperthyroidism and differentiated thyroid carcinoma patients. The biological factor of radioiodine drainage was not considered while cumulative dose was evaluated in cytological experimental of PBMCs.

In conclusion, the expression of the *BCL-2* family members is a crucial factor in the sensitivity of cells to radiation-induced apoptosis. The apoptosis rate in human PBMCs gradually increased after six hour of irradiation in the present study. The values of *BCL-2* and *BAX* gene expression were enhanced at initial irradiation while the PBMCs were irradiated by radioiodine (^{131}I), and then decreased gradually from 6 to 12 h. Later, the balance of *BCL-2/BAX* gene expression was adjusted, and PBMCs underwent apoptosis at higher cumulative doses.

***Acknowledgment*:** Authors thank the technicians in the Nuclear Medical Department of the Third Hospital Affiliated Sun Yat-Sen University for technical support.

***Financial support & sponsorship*:** This work was supported in part by the Foundation (No.2016A020215070) of Guangdong Provincial Science and Technology plan project (Department of Science and Technology of Guangdong Province, PR China).

***Conflicts of Interest*:** None.

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