The Study of Apoptosis-2 Ligand as Radiosensitizer in Lung Adenocarcinima A549 Cell Lines

LI Jing-dong[1], LIU Xi-guang[1△], SONG Hao[1], WANG Yu-kun[1], ZHANG Li-li[1], ZHU Hua[1], LIANG Ye[2]

(1 Department of Oncology, Affiliated Hospital of Medical College, Qingdao University, Qingdao 266061, China;

2 Central Laboratory, Affiliated Hospital of Medical College, Qingdao University, Qingdao 266061, China)

ABSTRACT Objective: To investigate the effect of Apoptosis-2 ligand (Apo-2L) which was also known as Tumor Necrosis Factor-related apoptosis-inducing ligand (TRAIL) on lung adenocarcinima A549 cell lines. Methods: All adenocarcinima A549 cells were randomly divided into four groups: group reaccepted Apo-2L, group reaccepted both Apo-2L and radiation, group reaccepted radiation alone and control group. The two of them were prescribed with different levels of Apo-2L (200ng/mL, 286ng/mL). After 24 hours, three groups were irradiated in different dose level (1Gy, 1.4Gy, 1.8Gy, 2.0Gy and 3.0Gy). The apoptosis rates of all groups were analyzed by Flow cytometry 24 hours later. The inhibition rates of the A549 cell lines were measured by the yellow tetrazolium (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenylterazolium bromide) MTT. Results: The analysis of Flow cytometry showed that the rates of cells apoptosis were escalated from 6.68% to 50% after IC50 for 24 hours. The results of MTT showed that the correlation between the inhibition rate and the concentration of Apo-2L in adenocarcinoma A549 cells is positive. The apoptosis rates of the four groups were 72.8%, 0.12%,50%, 51.5%. Conclusion: The Apo-2L can inhibit the proliferation and promote the apoptosis of adenocarcinoma A549 cells *in vitro* and improve the apoptosis of adenocarcinoma A549 cells significantly if combined with radiation.

Key words: Apoptosis-2 ligand; MTT assay; Flow cytometer; Apoptosis

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Introduction

The Apoptosis-2 ligand (Apo-2L) is also known as Tumor Necrosis Factor-related apoptosis-inducing ligand (TNF-related apoptosis-inducing ligand, TRAIL). It is a new member of tumor necrosis factor superfamily (TNFSF) which was recently discovered. Apo-2L / TRAIL corresponding with the cell surface death receptor can start the apoptotic signal which can strongly, rapidly, broad-spectrumly to induce the transformed cells and the human tumor cells[1]. Unlike TNF or Fas ligand (FASL), Apo-2L / TRAIL without apparent cytotoxicity on normal cells, it is the only known specific apoptosis-inducing molecule that performance of selectively induce apoptosis of tumor cells without systemic cytotoxicity. Thus, becoming one of the hot researches in anticancer drugs areas, TRAIL can selectively induce the apoptosis of tumor cells without significant cytotoxicity, Therefore, in theory, TRAIL combined with radiation therapy can improve the effect of apoptosis and promote the apoptosis of tumor cells which were resistance to radiation.

It was found that radiation can enhance the sensitivity of tumor cells to TRAIL and both with cross-sensitizing effect [2]. TRAIL combined with radiation therapy induced apoptosis of breast cancer was significantly superior to radiotherapy alone or TRAIL alone. *In vivo* experiments also showed that TRAIL combined with radiotherapy can let breast cancer xenografts quickly disappear. The coordination mechanisms may be related to the following pathway, in the first place, radiation can affect cells through the expression of the pro-apoptotic protein Bax to regulate mitochondrial-dependent apoptosis[2]; What is more, it can enhance the response of caspase cascade waterfall which led to apoptosis, this synergistic effect is p53-dependent [3]; Additionally, it also can increase the expression of death receptor DR4,DR5,transfer the death mediated signal and increase the sensitivity of tumor cells to TRAIL[4]. This study was designed to study investigate the effect of Apoptosis-2 ligand (Apo-2L) which was also known as Tumor Necrosis Factor-related apoptosis-inducing ligand (TRAIL) on lung adenocarcinima A549 cell lines.

1 Materials and Methods

1.1 Materials and Cell lines

The A549 cells of lung cancer which is the gift by the Central Laboratory of the Affiliated Hospital of Qingdao Medical University; Apo-2L is from the previous Shanghai Qiaer Biotechnology Co, Ltd, which is white loose body, dissolve as clear liquid and shall not contain visible insoluble. MTT is a product of Sino-American Biotechnology Co., Ltd. We use the Becton Dickinson Company presidium iodide (PI) kit staining, Flow Cytometry is for the company of Becton Dickinson FACS Calibur. A549 cells were subcultured in Gibco'RPMI-1640 that is containing 10% feta calf serum and then adherent growth under the conditions of 37 centi-degree,5% carbon dioxide. After 24 hours, we can put the cells undergoing the conditions that are Varian 23EX-1 linear ac-

Author Introduction: LI Jing-dong (1982-), male, postgraduate student, physician. Research field:comprehensive treatment of tumor. E-mail: ljdserve1@163.com, Telephone:13370855035.

△Corresponding author: LIU Xi-guang, Telephone:0532-82919651, E-mail: liuxiguang1962@yahoo.com.cn

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celerator X-ray.

1.2 Method

A549 cells were subcultured in Gibco'RPMI-1640 which is containing 10% feta calf serum. The pre-experiment was done which took the logarithmic growth phase cells at 1×10^5 per milliliter seeding in the final concentration which were 0,25,50,100,200,400,800 ng per milliliter of 96-well plates for 24 hours and the MTT method was used to decide that the optimum concentration was IC50. Logarithmic growth phase cells were irradiated with 6MV X-ray. The skin source distance (SSD) is 100 centimeters long. Irradiated area is about 400 square centimeters (20× 20 cm). The radiation dose levels were 1Gy, 1.4Gy, 1.8Gy, 2.0Gy and 3.0Gy. The inhibition rate and the sensitizing effect of radiotherapy (SER) was calculated by MTT assay 24 hours after irradiation. The cell cycle was also analyzed by flow cytometry. The results were expressed in the mean addition and subtraction the deviation, test of significance using factor analysis of variance and paired t-test. Statistical analysis were performed by Statistical Product and Service Solutions (SPSS) software (version 17.0) and P<0.05 was considered as statistical significant difference.

2 The Results

2.1 The effect of Apo-2L on lung adenocarcinoma A549 cells (IC50).

Survival of A549 cells underwent different concentrations of Apo-2 after 24 hours were tested by the MTT assay (Table 1). Table 1 showed that the cell apoptosis rate escalated when the concentration of Apo-2L increased. The concentration of Apo-2L were between (25ng/ml) and (800ng/ml), and the Inhibition of cells were significant differences. P<0.05. So 286 ng per milliliter was the optimum concentration of the experiment which is also IC50.(Fig.3).

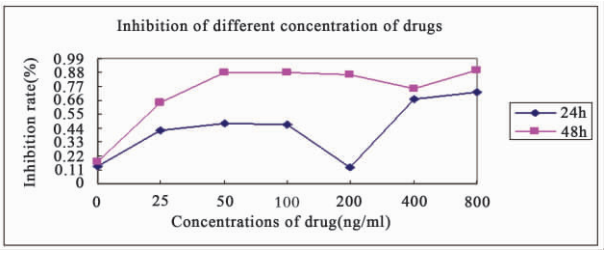

Fig.3 The inhibition rate of different concentrations of Apo-2 to A549 cells after 24 hours and 48 hours

Table 1 Inhibition rate effect of different concentration of Apo-2L on cell line A549

Apo-2 concentration(ng/ml)	Inhibition rate(%)
The control group	0.1185± 0.024
25	0.2305± 0.267
50	0.4565± 0.028
100	0.4635± 0.004
200	0.1003± 0.581
400	0.6255± 0.061
800	0.7135± 0.0162

2.2 Comparing the value of each detection in Flow Cytometry

Analysis of variance showed that when cells undergone the same concentration of Apo-2L but different apoptosis of radiation dose, the tumor inhibition rate of radiotherapy combined drug group was significantly higher than that of the control group. Thus, the effect that different radiation dose on adenocarcinoma A549 cells was significantly different,such as P<0.003, (in Table 2). Different concentration of Apo-2L combined with same radiation dose also had very significant statistical difference (P<0.001),(in Table 3).

Table 2 The Inhibition rate of effect with radiation of different doses and same drug concentration

Radiation dose	Inhibition rate of Lung adenocarcinoma A549 cells		
	Concentration 286ng/ml	200 ng/ml	0 ng/ml
0 Gy	22.413± 23.969	10± 23.969	6.68± 23.969
1 Gy	51.223± 7.190	54.41± 7.190	42.99± 7.190
1.4Gy	61.493± 3.602	60.56± 3.602	58.45± 3.602
1.8Gy	65.926± 4.291	64.21± 4.291	62.76± 4.291
2.0Gy	72.790± 4.041	72.26± 4.041	69.04± 4.041
3.0Gy	77.650± 8.979	76.81± 4.041	69.12± 4.041

Table 3 The Inhibition rate of different drug concentration and same radiation dose

Apoptosis-2ligand concentration(ng/ml)	Inhibition rate of Lung adenocarcinoma A549 cells	
	Radiation dose(2Gy)	Radiation dose(0Gy)
0	51.5067± 23.97690	6.68± 23.97690
200	56.4683± 23.88157	10.56± 23.88157
286	67.77 ± 13.562	50± 13.562

2.3 Analysis Radiosensitizer

Apo-2L can significantly enhance the radiation effect that the radiosensitizer could be up to (11.5374)(P=0.002). Thus, the radiosensitizer was positive to radiation dose and drug concentration, it has a very significant statistical difference(P=0.001).(shown in Table 4, 5).

Table 4 The radiosensitizer of different radiation dose and same drug concentration

Radiation dose	The sensitizing effect of radiotherapy	
	Apoptosis-2 ligand concentration(286 ng/ml)	Apoptosis-2 ligand concentration(0ng/ml)
0 Gy	7.4850	1.0000
1 Gy	8.4236	6.4356
1.4Gy	9.8008	8.7500
1.8Gy	10.600	9.3952
2.0Gy	11.5374	10.3353
3.0Gy	13.0269	10.3473

Table 5 The radiosensitizer of different drug concentration and same radiation dose

Apoptosis-2 ligand concentration(ng/ml)	The sensitizing effect of radiotherapy (SER)	
	Radiation dose (2Gy)	Radiation dose (0Gy)
0	10.3353	1.0000
25	10.3997	1.9451
50	10.5926	3.8523
100	10.7001	3.9113
200	10.8173	1.0042
400	11.6872	5.2784
800	12.0266	6.0210

2.4 Flow Cytometry analysised the cell cycle

The results of analysis of variance that most of cells were in G1 phase and S phase after the effects of radiotherapy combined TRAIL, and other groups. Apoptosis rate was statistically significant, (P=0.03),but the proportion of different cell cycle were not statistically different, (P＞0.05). (Fig.2 and Table 6). The apoptosis rates of the four groups were 72.790%, 0.1185%, 50%, 51.5067%, (Fig.1). Analysis of variance showed that when cells undergone the different concentration of Apo-2L but same apoptosis of radiation dose, the tumor inhibition rate of radiotherapy combined drug group was significantly higher than that of the control group.

3 Discussion

Tumor necrosis factor-related apoptosis-inducing ligand is a new clone members of the TNF family in 1995, it was also known as apoptosis-2 ligand, which is widely expressed in human tissues, such as spleen, prostate, liver, kidney, ovary and peripheral blood lymphocytes [5]. The current study showed that full-length membrane protein and soluble of the TRAIL can rapidly induce apoptosis in various cancer cell lines, in vitro it can induce a variety of tumor cells apoptosis, while normal cells are not sensitive to TRAIL. The reason for TRAIL selectively induced apoptosis of tumor cells may be that its receptor selectively expressed in various

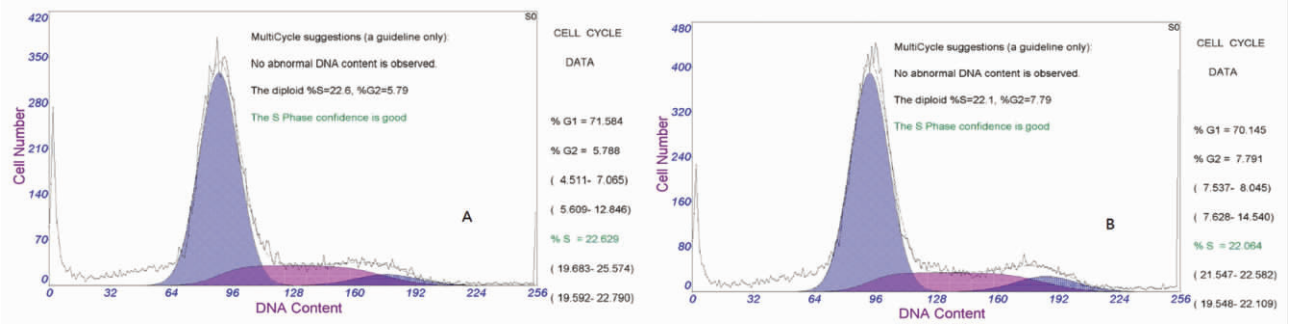

Fig.2 Cell cycle distribution of two concentrations of drugs by Flow cytometry: A The cell cycle distribution of IC10 combined with radiotherapy; B The cell cycle distribution of IC50 combined with radiotherapy

Table 6 Cell cycle and apoptosis of cell line A549 in each group by FACS

Group	Cell cycle			Apoptosis (%)
	G0/G1	S	G2/M	
The control group	69.803	21.301	8.896	6.68
Drug group	77.203	11.563	11.234	56.27
Radiotherapy	73.776	15.656	10.568	69.04
Radiation and drug	78.028	13.604	8.363	87,02

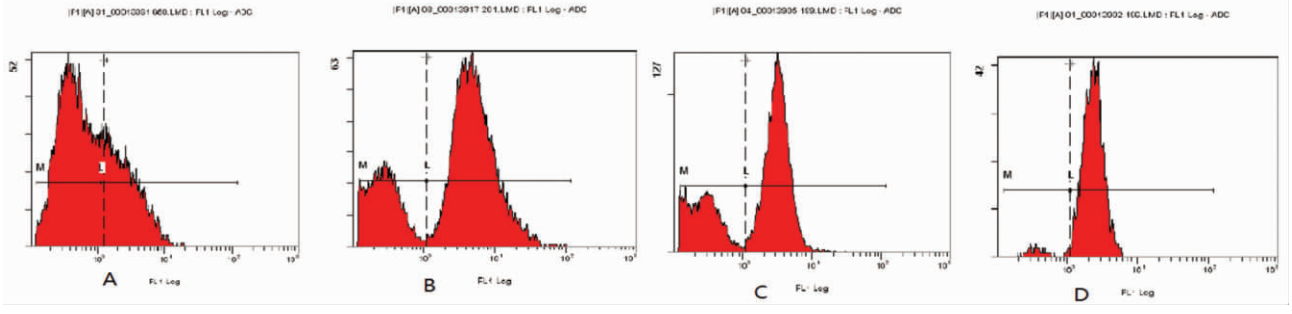

Fig.1 The apoptotic rate of cells by Flow cytometry:A: The apoptosis of Control group; B: The apoptosis of Drug group; C The apoptosis of Radiotherapy; D The apoptosis of Radiation and drug

cancer cell lines. TRAIL has four receptors, namely the death receptors DR4, DR5, false receptors DcR1, DcR2 [6,7]. TRAIL combined with DR4 and DR5 can rapidly induce apoptosis in multiple sources of transformed cell lines. Meanwhile, DR4 and DR5 transcription in normal tissue and tumor cell lines were seized, but the viruses can encode a false receptor combined with molecules attracting immune cells. So DcR1 and DcR2 were following this measure to protect normal cells from attacked by TRAIL. They have two pseudo-repeat sequences combined with TRAIL, which were highly homologous cysteine to DR4 and DR5 outside the cell membrane and they widely expressed in normal tissues but not in tumor cell lines. At the same time, false receptors of DcR1, DcR2 and death receptors of DR4, DR5 competitively combined with TRAIL. Normal tissue is not sensitive to TRAIL-induced apoptosis [8-10]. It is expected to be selective anti-tumor treatments. In this regard, the death-receptor ligand TNF-a-related apoptosis-inducing ligand (TRAIL/Apo2L) and TRAIL-receptor agonistic antibodies were shown to display a high selectivity for tumor's cells and act synergistically with conventional chemotherapy drugs and radiation therapy. Up to now it has been shown that radiation strongly sensitizes malignant cells to TRAIL and TRAIL-agonistic antibodies. Synergistic induction of apoptosis was demonstrated in majority of malignant cell types and xenograft models, especially in those cells types displaying only weak response to either treatment alone, strong sensitizing effects were described.

It was found that TRAIL exerted a highly selective cytotoxicity towards malignant cells was the trigger to investigate the potential of TRAIL as an anti-neoplastic agent [12-14]. Currently it has been shown that recombinant TRAIL and monoclonal TRAIL-receptor agonistic antibodies possess a favorable safety-profile in a number of clinical phase I/II trials [15-17]. An overview of published and ongoing clinical studies can be found in a review by Ashkenazi and in the chapter by Elisabeth de Vries in this issue [18]. Unfortunately, many cancer cells are resistant to TRAIL induced apoptosis even at high doses, limiting the clinical use of TRAIL as a mono-therapeutic agent [19]. The following section will focus on

the experimental results of combined treatment with ionising radiation and TRAIL-agonists in vitro.

The experimental results showed that apoptosis-2 ligand and radiation were both inhibitory effect on adenocarcinoma A549 cells and the effect was both time and concentration dependent. But after addition of TRAIL most of the tumor cells were decomposition 48 hours later, which was not meet the condition of experimental study. For this reason this study selected the cells which were given TRAIL after 24 hours as the experimental material. The IC50 was 286ng per milliliter, while, Apo-2L combined with radiotherapy significantly enhanced the effect of radiation ($P<0.05$). The radiosensitizing effect was direct correlation with radiation dose level and concentration of Apo-2L. Low concentration of Apo-2L may increase sensitizing effect on A549 cells. The evidences collected by flow cytometry which detecting the apoptosis and cell cycle showed that the Apo-2L significantly increase the rate of apoptosis compared with the control group (50± 13.562 Vs 0.1185± 0.024)%. The Apo-2L can not only improve the radiosensitivity but also promote apoptosis of A549 cells. Most of cells were in their G1 phase and S phase after the effects of radiotherapy combined Apo-2L. The cells in G2-M phase were sensitive to radiation, considering this, the radiosensitivity of A549 cells can not be explained by Apo-2L's regulation of the cell cycle. The potential mechanisms we deduced from experimental data was that the Apo-2L may play an important role in reducing the ability of repair after radiation injury and promoting apoptosis and inhibition of cell clonal proliferation. The contribution of major apoptosis signaling molecules in TRAIL and irradiation-induced apoptosis was analysed in detail.

Depending on the cell type, induction of apoptosis is mediated either by direct activation of effector caspases through caspase -8 or caspase -10, or by amplification of the apoptosis signal via the mitochondria [11,20]. Using a Jurkat-T cell system with Bcl-2 under control of a tet-regulated promoter, after low dose stimulation with recombinant TRAIL, Bcl-2 over-expression rendered cells apoptosis-resistant, while high doses of TRAIL overruled the inhibitory influence of Bcl-2 and resulted in substantial cell kill. Thus the induction of apoptosis by TRAIL depends on the trigger dose, incubation time and complex thresholds imposed on the mitochondria by members of the Bcl-2 family. In contrast to death receptor mediated apoptosis, radiation and chemotherapy induced apoptosis is primarily dependent on the mitochondrial pathway, namely the equilibrium between pro- and anti-apoptotic Bcl-2 proteins.

Radiation therapy is one of the main treatments of malignant tumors, but the effect of radiotherapy may be influenced by the tumor cell's heterogeneity of radiation-sensitive and difference in its repair capacity after radiation injury and the other factors. Based on the experimental results, Apo-2L combined with radiotherapy can not only improve the radiation sensitivity of A549 cells but also inhibit the cells potential proliferation and reduce their repair capacities. The Apo-2L may be a potential sensitizer for radiotherapy.

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重组人凋亡素 2 配体对放射线诱导肺腺癌 A549 细胞凋亡作用的研究

李敬东[1] 刘希光[1△] 宋 浩[1] 王玉坤[1] 张丽丽[1] 朱 华[1] 梁 晔[2]

(1 青岛大学医学院附属医院肿瘤科 山东 青岛 266003;2 青岛大学医学院附属医院中心实验室 山东 青岛 266003)

摘要 目的:研究凋亡素 2 配体(Apo-2 ligand,Apo-2L),或称肿瘤坏死因子相关凋亡诱导配体(TNF-related apoptosis-inducing ligand,TRAIL) 在体外对人肺腺癌 A549 细胞系的放射增敏作用的研究。**方法**:MTT 法检测 Apo-2L 单药或与放射线联合对腺癌 A549 细胞的抑制率,将细胞分为 4 组,对照组、Apo-2L 组、Apo-2L+ 放射照射组、单纯照射组,200ng/ml、286 ng/ml 的 Apo-2L 作用 24 小时后给予放射照射,照射剂量分别为:(1Gy、1.4 Gy、1.8Gy、2 Gy、3 Gy).然后进行流式细胞仪分析照射后 24h 各组细胞凋亡率变化。**结果**:MTT 结果显示,腺癌 A549 细胞的抑制率与 Apo-2L 的浓度成正相关,凋亡素 2 配体作用 24h 后 IC50 为 286 ng / ml.流式细胞仪分析显示 286ng / ml 的 Apo-2L 处理 24h 后,细胞凋亡率从(6.68)%上升至(50)%,照射后 24h Apo-2L+ 照射组凋亡率明显升高,为 72.790%,对照组 0.1185%,Apo-2L 组 50%,单纯照射组 51.5067%。**结论**:Apo-2L 在体外对腺癌 A549 细胞有抑制增殖和促进凋亡作用,并且 Apo-2L 联合放射线可以明显提高腺癌 A549 细胞的凋亡率。

关键词:凋亡素 2 配体;凋亡;MTT 法;流式细胞仪

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作者简介:李敬东(1982-),男,硕士研究生,肿瘤综合治疗。

E-mail:jdserve1@163.com, **电话**:13370855035

△**通讯作者**:刘希光,青岛大学医学院附属医院肿瘤科主任,副主任医师。**电话**:0532-82919651,

E-mail:liuxiguang1962@yahoo.com.cn

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