

Research Article

Enhancement of Recombinant Human Endostatin on the Radiosensitivity of Human Pulmonary Adenocarcinoma A549 Cells and Its Mechanism

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We observed the effects of endostar on the radiosensitivity of pulmonary adenocarcinoma A549 cells and found that endostar inhibited A549 cell growth under normoxia and hypoxia in time and dose-dependent manners; the D_0 and D_q values in control and endostar groups were (1.36 and 1.30) versus (1.019 and 1.015) under normoxia and (1.693 and 1.39) versus (2.453 and 1.026) under hypoxia, respectively; SER was 1.04 under normoxia and 1.22 under hypoxia in endostar group; under normoxia, the apoptosis rates in control, radiotherapy, endostar and combination groups were $15.9 \pm 0.57\%$, $42.7 \pm 0.37\%$, $19.9 \pm 0.48\%$, and $41.5 \pm 0.38\%$, respectively, with no significant difference between combination and radiotherapy groups; there was significant difference in G_2/M phase cells between combination and radiotherapy groups ($P = 0.028$); under hypoxia, the apoptosis rates in the four groups were $16.7 \pm 0.67\%$, $30.1 \pm 0.95\%$, $26.7 \pm 0.62\%$, and $36.3 \pm 0.71\%$, respectively, with significant difference between combination and radiotherapy groups; G_2/M phase cells were higher in combination group than radiotherapy group ($P = 0.000$); G_2/M phase cells were higher in hypoxic combination group than in normoxic combination group ($P = 0.003$). Based on these results, we conclude that under hypoxia, endostar can enhance the radiosensitivity of A549 cells through G_2/M arrest.

1. Introduction

Lung cancer is one of the most common carcinoma in China. Radiotherapy is a main therapeutic method for lung cancer, especially for locally advanced non-small-cell lung cancer (stage III_{A-B}) [1]. Precise radiotherapy is considered to be the direction of development of radiotherapy in the 21st century. However, precise radiotherapy fails to improve the long-term survival rate of patients with malignant tumors. This may be that local radiotherapy cannot control tumor metastasis and recurrence, and there are a large number of radioresistant cells in tumor tissue. Therefore, finding an effective radiosensitizer to improve therapeutic effects has become a focus in tumor radiotherapy. At present, main radiosensitizers include proelectronic radiation sensitizer, reducing agents, chemotherapeutics, natural drugs, and molecular-targeted drugs. Much attention is paid to molecular targeted drug, endostatin (ES).

Endostatin, a natural protein in animals, was first obtained from the supernatant of mouse hemangioendothelioma cell culture. Endostatin derives from hydrolysis of carboxyl terminal of extracellular matrix collagen protein XVIII. Endostatin contains 184 amino acids with molecular weight of 20 KD. Natural Endostatin is very unstable with shorter half life and lower biological activity. Recombinant human endostatin (RHES, endostar) was obtained by addition of 9 amino acids to Endostatin. RHES is stable with longer half life and higher biological activity. *E. coli* as protein expression system solves the problem of inclusion body renaturation in endostar.

Many preclinical studies show that endostar can improve the radiotherapeutic effects on many malignant tumors [2, 3], but its exact mechanism remains unclear. Jain [4] have found that angiogenesis inhibitors can make tumor blood vascular system normalize, relieving tumor hypoxia. Winkler

et al. [5] have confirmed the presence of blood vascular system normalization. However, Casanovas [6] recently reports that the radiosensitizing effect of angiogenesis inhibitors may be not associated with blood vascular system normalization. In order to further explore the radiosensitizing effects of endostar and its mechanism, we observed the effects of endostar on human pulmonary adenocarcinoma cell line A549 under normoxia and hypoxia in vitro, respectively.

2. Materials and Methods

2.1. Cell Lines and Reagents. Human pulmonary adenocarcinoma cell line A549 was purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). Endostar was provided by Simcere Pharmaceutical Co., Ltd.

2.2. Cell Culture. A549 cells were cultured in DMEM supplemented with 10% of fetal bovine serum and 100 u/mL of penicillin and streptomycin, respectively, at 37°C in an atmosphere of 5% CO₂ under bacteria-free condition with a passage per 2-3 days.

2.3. Cytotoxic Effects of Endostar on A549 Cells under Both Normoxia and Hypoxia. (1) A549 cells at log phase were plated into 96-well plate at 5.0×10^3 cells in each well. When the cells were completely adherent after 24 hours, the culture solution was removed, followed by addition of endostar including 500 mg/L, 200 mg/L, 100 mg/L, 50 mg/L, 10 mg/L, 5 mg/L, 1 mg/L, and 0 mg/L (0 mg/L only contained 0.1 % of DMSO), respectively, with 3 wells for each group. At the same time, blank control and zero adjustment wells were set. The cells were incubated at 37°C in a saturated humidity of 5% CO₂ for 24–72 hours, then 10 ul of MTT (5 mg/mL) was added into each well to incubate for 4 h followed by removal of medium. Formazan solution (100 uL) was added into each well to incubate for 4 h, and then light microscope indicated that all Formazan was dissolved. The absorbance (A value) was determined at 570 nm with ELISA. (2) For cell culture in vitro under hypoxia, A549 cells were incubated in DMEM containing 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂, and then CoCl₂ was added to simulate the hypoxic microenvironment in the tumor. The final concentration of CoCl₂ in DMEM was adjusted at 150 umol/L (according to [7]). (3) CoCl₂ was added in hypoxia group, blank control, and the zero adjustment wells, respectively, and the final concentration of CoCl₂ was also adjusted at 150 umol/L. The rest procedures were the same as step (1). The above steps (1) and (3) were repeated three times, respectively, and the results were indicated with average values. The inhibition rates of cell survival in various drug concentrations were calculated according to the following formula: inhibition rate = (A value of control well – A value of tested well)/A value of control well × 100%. Cell growth curve was drawn with various drug concentrations as abscissa and cell growth inhibition rate as ordinate. IC20 was calculated according to the cell growth curve.

2.4. Colony-Forming Assay. After digested by pancreatin and prepared into monocellular suspension, human pulmonary adenocarcinoma A549 cells at log phase were placed into 6-well plate with 10²–10⁵ cells in each well according to various irradiation doses, followed by gently shaking the plate to make the cells well distributed. The cells were incubated at 37°C in a saturated humidity of 5% CO₂ for 24 hours. After the cells were adherent, culture solution was removed and the cells were allocated into four groups including two radiotherapy-alone groups, respectively, under normoxia and hypoxia, respectively, and two combination groups (endostar combined with radiotherapy) under normoxia and hypoxia, respectively. Each group was divided into 5 subgroups according to various irradiation doses (0, 2, 4, 6, and 8 Gy). In combination groups, the final concentration of endostar was 300 mg/L under normoxia and 400 mg/L under hypoxia. After 24 h incubation, the cells were irradiated with high-energy X-ray of various doses: 0, 2, 4, 6, and 8 Gy, respectively, at room temperature followed by removal of the supernatant and addition of endostar-free fresh culture solution. The cells continued to be incubated at 37°C in a saturated humidity of 5% CO₂ for 10–14 d, and the culture solution was changed according to PH value during the 10–14 d. The cells were incubated until 50 or more clones formed. Giemsa staining: (1) reagent 1 of 3–5 droplets was added to quickly cover the cells for one minute. (2) Reagent 2 of 6–10 droplets was directly added, followed by gently shaking the plate to make the staining solution thoroughly mixed for 5–8 min, and (3) washed with water for 30 s, and the clones with ≥0.2 mm diameter was counted. The experiment was repeated twice. The plating efficiency (PE) and the survival fraction (SF) were calculated according to the following formula: PE = (clone count/cell count) × 100%, SF = (PE in observed group/ PE in 0 Gy group) × 100%. The survival fractions at various dose points were calculated. Plotting was made through the curve fitting performed with SigmaPlot software according to single-hit multitarget (SHMT) model $\{S = 1 - (1 - e^{-D/D_0})^N\}$. The mean lethal dose (D_0), quasi-threshold (D_q), extrapolation number (N), survival fraction at irradiation dose of 2 Gy (SF_2), and sensitization enhancement ratio (SER) in each group were calculated, respectively.

2.5. Apoptosis Detected with Flow Cytometer. A549 cells at log phase were inoculated in 6-well plate with 1.8×10^5 cells in each well. Four groups (control group, radiotherapy alone group, endostar group, and combination group) were set under normoxia and hypoxia, respectively. The cells were incubated for 24 h and were adherent; the culture solution was replaced with fresh culture solution. For the hypoxia groups, COCl₂ was added into each well to adjust its final concentration at 150 umol/L followed by 24-hour incubation. Then the cells in radiotherapy alone group and combination group were irradiated (isocenter irradiation with 6MVX-ray, field of 15 cm × 15 cm and dose of 2 Gy) with a linear accelerator (Siemens) for 24 h. After trypsinized and harvested, the cells were washed with PBS twice and resuspended in binding buffer (precooled at 4°C), followed

by addition of AnnexinV (5 μ L) and PI (2.5 μ L) for 15 min at room temperature in the dark. Binding buffer was added, and within the next one hour, the cell apoptosis was evaluated with flow cytometer.

2.6. Cell Cycle Determined with Flow Cytometer. A549 cells at log phase were inoculated in 6-well plate at 1.8×10^5 cells in each well. Four groups (control group, radiotherapy alone group, endostar group, and combination group) were set under normoxia and hypoxia, respectively. After treated for 24 h, cells were digested, centrifuged, washed with PBS two times, and fixed with ice-cold ethanol for 12 h. Cell cycle was determined with flow cytometer according to kit's instructions. DNA analysis and light scattering analysis were performed with CellQuest and ModFit software in cells.

2.7. Statistical Analysis. SPSS 13.0 package was used for statistic analysis. Chi-square test was adopted in comparison of rates. Cell survival curve was fitted with Sigma Plot 10.0 software based on single-hit multitarget equation. ANOVA was used in comparison between variables. *F*-test was used in comparisons between multiple groups. Comparisons between two groups were performed with *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Endostar on the Growth Inhibition Rate of A549 Cells under Both Normoxia and Hypoxia Culture. After A549 cells were treated with endostar of various concentrations for different durations under normoxia and hypoxia, endostar showed the inhibition effect on A549 cells compared with control groups (Tables 1 and 2). A549 cells were treated with endostar of 7 different concentrations under normoxia and hypoxia, respectively, for 24–72 h, and then the cell survival curve was drawn based on the results determined by MTT assay. The IC₂₀ was 330.34 ± 4.18 mg/L under normoxia and 437.23 ± 3.32 mg/L under hypoxia when A549 cells were treated with endostar for 24 h. The concentration of endostar was not higher than that of IC₂₀ in the future experiments, so 300 mg/L of endostar under normoxia and 400 mg/L of endostar under hypoxia were adopted in the future experiments.

3.2. Enhancement of Endostar on the Radiosensitivity of A549 Cells under Both Normoxia and Hypoxia. Under normoxia, after A549 cells were treated with 300 mg/L of endostar for 24 h, the radiosensitivity of A549 cells failed to be significantly improved, the cell survival curves between combination and radiotherapy groups were not markedly changed (Figure 1), and the values of D_0 and D_q were 1.30 Gy and 1.015 Gy in combination group and 1.36 Gy and 1.019 Gy in radiotherapy-alone group (Table 3). Under hypoxia, after A549 cells were treated with 400 mg/L of endostar for 24 h, the radiosensitivity of A549 cells was stronger and the cell survival curve was lower in combination group than in radiotherapy-alone group (Figure 2), and the

TABLE 1: Effects of endostar on growth inhibition rate of A549 cells under normoxia (%).

Group (mg/L)	24 h	48 h	72 h
0	0	0	0
1	3.25 ± 0.03	4.15 ± 0.04	9.87 ± 0.54
5	4.18 ± 0.12	9.87 ± 0.56	15.46 ± 0.87
10	8.15 ± 0.23	12.36 ± 0.72	18.78 ± 0.95
50	10.18 ± 0.32	15.86 ± 0.98	23.67 ± 1.01
100	15.08 ± 0.52	20.15 ± 0.87	29.34 ± 1.05
200	18.32 ± 0.69	23.67 ± 0.98	33.23 ± 0.98
500	23.74 ± 0.98	28.15 ± 0.97	39.45 ± 1.08

TABLE 2: Effects of endostar on growth inhibition rate of A549 cells under hypoxia (%).

Group (mg/L)	24 h	48 h	72 h
0	0	0	0
1	2.87 ± 0.02	3.75 ± 0.05	8.77 ± 0.76
5	3.78 ± 0.13	8.97 ± 0.46	13.56 ± 0.77
10	7.45 ± 0.13	10.37 ± 0.52	15.68 ± 0.89
50	9.28 ± 0.22	13.86 ± 0.88	20.77 ± 1.08
100	13.08 ± 0.62	18.15 ± 0.73	25.34 ± 1.12
200	16.52 ± 0.73	20.37 ± 0.78	30.23 ± 1.08
500	20.74 ± 0.87	25.08 ± 0.87	35.12 ± 1.12

values of D_0 and D_q were 1.39 Gy and 1.026 Gy in combination group and 1.693 Gy and 2.453 Gy in radiotherapy-alone group (Table 3).

3.3. A549 Cell Apoptosis Induced by Endostar under Both Normoxia and Hypoxia. Under normoxia, the apoptosis rates in control group, radiotherapy-alone group, endostar group, and combination group were $15.9 \pm 0.57\%$, $42.7 \pm 0.37\%$, $19.9 \pm 0.48\%$, and $41.5 \pm 0.38\%$, respectively, without statistical significance ($F = 1.22$, $P = 0.479$) between combination group and radiotherapy-alone group (Table 4 and Figure 3). Under hypoxia, the apoptosis rates in the four groups were $16.7 \pm 0.67\%$, $30.1 \pm 0.95\%$, $26.7 \pm 0.62\%$, and $36.3 \pm 0.71\%$, respectively, with statistical significance between combination group and radiotherapy-alone group ($F = 3.34$, $P = 0.036$) (Table 4 and Figure 4).

3.4. Effects of Endostar on A549 Cell Cycle under Both Normoxia and Hypoxia. Under normoxia, each group had different number of G₂/M phase cells ($F = 1350$, $P = 0.0$), and there was significant difference between combination and radiotherapy groups ($P = 0.028$), suggesting that endostar influences the distribution of A549 cell cycle under normoxia. Under hypoxia, each group had different number of G₂/M phase cells ($F = 493$, $P = 0.000$), and G₂/M phase cells were higher in combination group than radiotherapy group ($P = 0.000$). G₂/M phase cells were higher in hypoxic combination group than in normoxic combination group ($t = 15.48$, $P = 0.003$). The results above suggest that

TABLE 3: Parameters of A549 cells in single-hit multitarget model under different conditions.

Parameters	Normoxia		Hypoxia	
	Radiotherapy	Endostar + Radiotherapy	Radiotherapy	Endostar + Radiotherapy
D_0 (Gy)	1.36	1.30	1.693	1.39
D_q (Gy)	1.019	1.015	2.453	1.026
N	2.67	2.61	4.295	2.798
SF_2	0.521	0.519	0.793	0.532
SER	1	1.04	1	1.22

Notes: D_0 : the mean lethal dose; D_q : quasi-threshold; N : extrapolation number; SF_2 : survival fraction at irradiation dose of 2Gy; SER: sensitization enhancement ration.

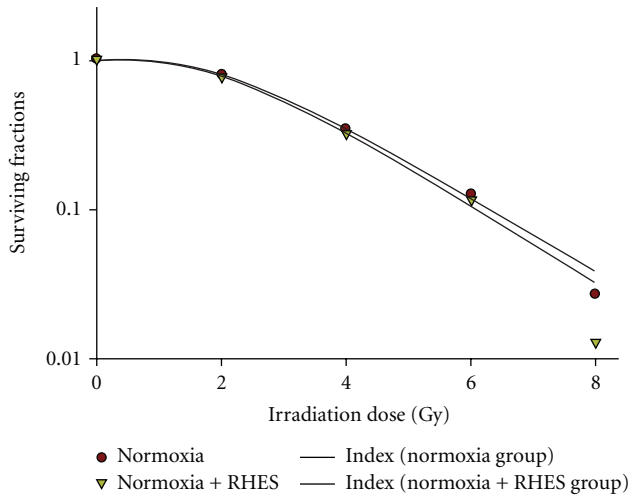


FIGURE 1: Cell survival curves of A549 cells under normoxia.

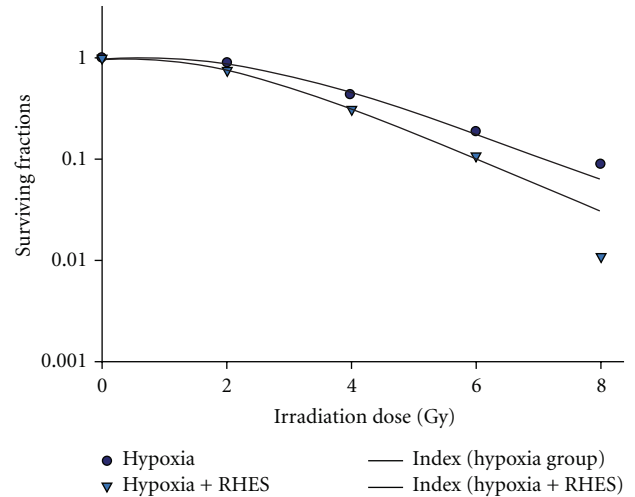


FIGURE 2: Cell survival curves of A549 cells under hypoxia.

endostar allows A549 cells to produce G_2/M arrest, especially under hypoxic conditions (Table 5 and Figure 5).

4. Discussion

Our results indicated that endostar had no radiosensitizing effect and failed to increase the apoptosis rate under normoxia, but under hypoxia, endostar could improve A549 cell radiosensitivity and increase A549 cell apoptosis. Endostar allowed A549 cells to produce G_2/M phase arrest, especially under hypoxic conditions. The mechanism of radiosensitizing effect of endostar may be associated with G_2/M arrest.

The morbidity and mortality of lung cancer takes the first place among malignant tumors in the world [8], and its incidence is increasing, especially non-small-cell lung cancer (NSCLC) accounting for 80–89% of lung cancer [9]. Radiotherapy is a main therapeutic method for lung cancer, especially for locally advanced NSCLC [1]. However, partial lung cancer cell's resistance to radiotherapy affects therapeutic effects, and 5-year survival rate of radiotherapy alone is only 5%–10%, local recurrence occurs in 80% of patients, and metastasis occurs in 60% of patients [10]. Therefore, it is necessary to find an effective radiosensitizer to enhance tumor radiosensitivity.

At present, there has not been an ideal radiosensitizer in clinical practice. Therefore, it is important to find an effective radiosensitizer in radiotherapeutics. Endostar is becoming a hot spot of present research.

In 1994, O'Reilly doctor in Folkman laboratory discovered angiostatin followed by the discovery of endostatin, which can specifically inhibit vascular endothelial cell growth. O'Reilly et al. [11] found that endostatin had inhibition activity on endothelial cell proliferation of bovine capillary and aortopulmonary. However, natural endostatin is very unstable, half life is short and biological activity is very low. These limit its application in clinical practice. Endostar is obtained by addition of 9 amino acids to endostatin, which changes amino acid sequence of natural endostatin. Endostar is more stable with longer half life and higher biological activity.

Are there any synergistic effects of endostar combined with radiotherapy? Many clinical studies show that endostar can improve radiotherapeutic effects on many kinds of malignant tumors, but the mechanism remains unclear. Jain [12] and Winkler et al. [13] have found that angiogenesis inhibitors can make tumor blood vascular system normalize in vivo, relieving tumor hypoxia. Huang and Chen [14] have also confirmed that endostar can make tumor blood vascular system normalize within a "time window," relieving

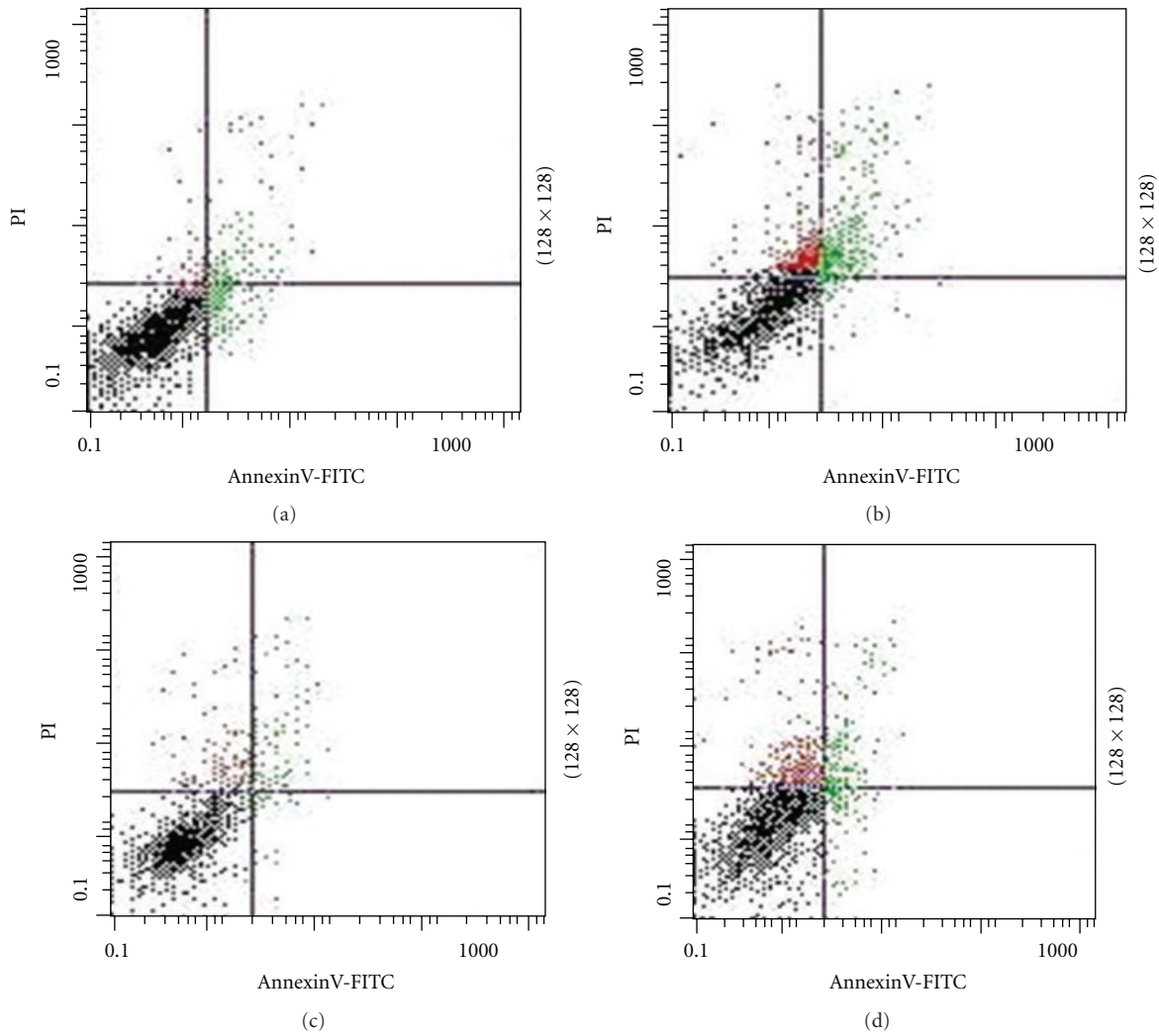


FIGURE 3: A549 cell apoptosis under normoxia. (a) control group; (b) Radiotherapy alone group; (c) Endostar alone group; (d) Endostar + radiotherapy.

tumor hypoxia. Above results suggest that the synergistic effect of endostar combined with radiotherapy is related to the improvement of tumor hypoxia. However, in the above studies, the results were derived from the direct effects of endostar on endothelial cells. It has been unclear whether endostar also possess radiosensitizing effects on tumor cells in vivo or in vitro. Previous studies suggest that endostar specifically acts on vascular endothelial cells and have no effect on tumor cells. However, recent studies indicate that the inhibitory effect of endostatin is not only on endothelial cell, but also on tongue squamous cell carcinoma [7] and on head and neck squamous cell carcinoma (HNSCC) [15]. There is also evidence, which confirms that human endostatin can inhibit the proliferation of bladder carcinoma cell line EJ and promote its apoptosis [16]. Dkhissi et al. [17] also found that endostatin could inhibit colon carcinoma cell line C51 and HT29 in vitro.

In order to explore the mechanism, we observed the effects of Endostar on A549 cell proliferation, radiosensitivity, apoptosis, and cell cycle distribution.

TABLE 4: A549 cell apoptosis under different conditions.

Groups	Apoptosis rate (%)	
	Normoxia	Hypoxia
Control	15.9 ± 0.57	16.7 ± 0.67
Radiotherapy	42.7 ± 0.37*	30.1 ± 0.95 ^Δ
Endostar	19.9 ± 0.48	26.7 ± 0.62
Endostar + radiotherapy	41.5 ± 0.38*	36.3 ± 0.71 ^Δ

Note: * indicates $F = 1.22$, $P = 0.479$ in comparison between radiotherapy group and endostar + radiotherapy group under normoxia. ^Δ indicates $F = 3.34$, $P = 0.036$ in comparison between radiotherapy group and endostar + radiotherapy group under hypoxia.

In this study, endostar showed an inhibitory effect on A549 cell proliferation in time and concentration-dependant manners under both normoxia and hypoxia.

In colony-forming assay, at the absence of endostar, the survival fraction was higher under hypoxia than under normoxia ($P = 0.000$), but it was lower in hypoxic endostar

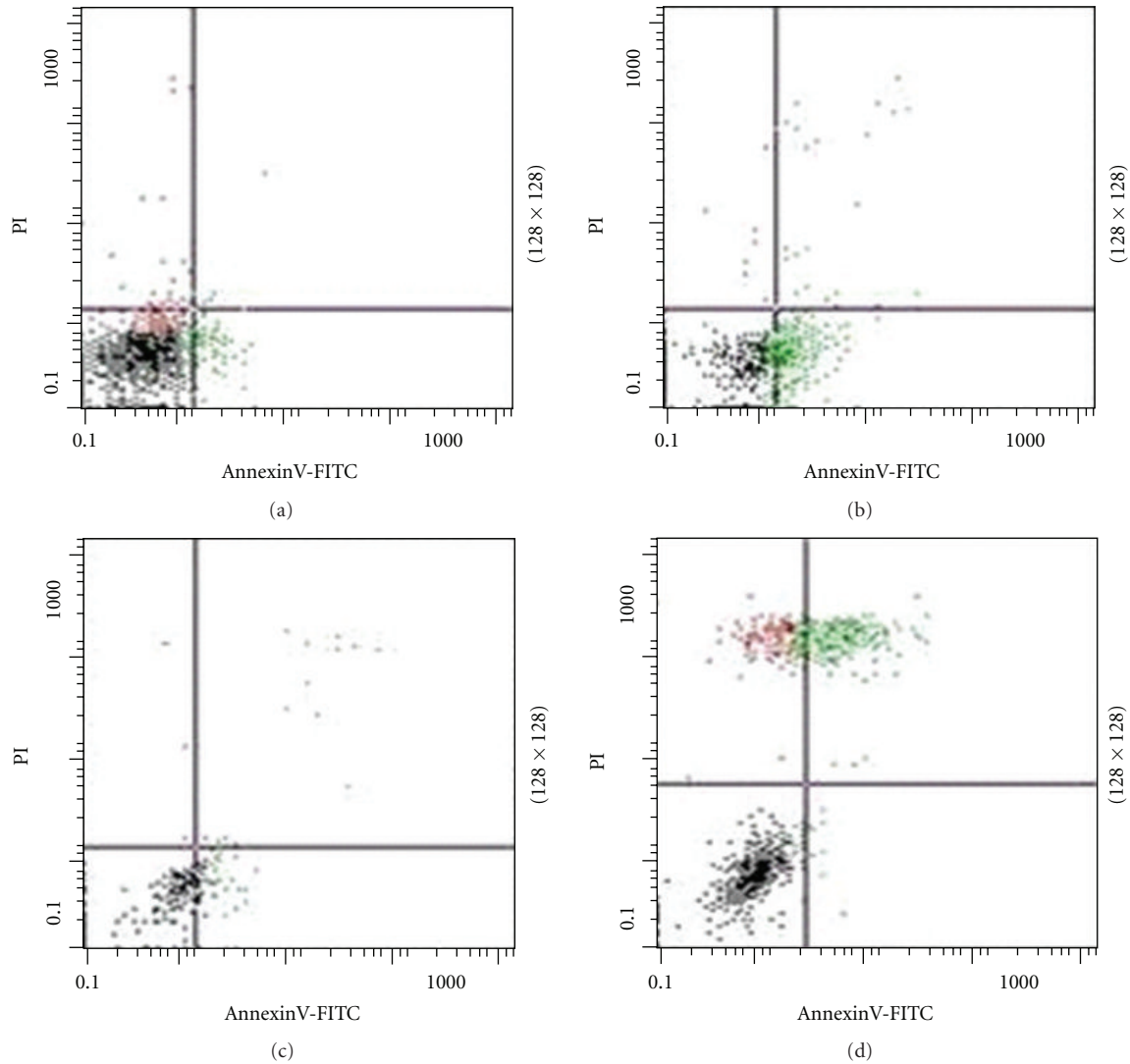


FIGURE 4: A549 cell apoptosis under hypoxia. (a) control group; (b) radiotherapy alone group; (c) endostar alone group; (d) endostar + radiotherapy.

TABLE 5: Cell cycle in each group under normoxia and hypoxia (% , $\bar{x} \pm S$).

Groups	Normoxia			Hypoxia		
	G ₀ /G ₁	S	G ₂ /M	G ₀ /G ₁	S	G ₂ /M
Control	61.54 ± 0.98	37.81 ± 0.25	5.43 ± 0.38	59.73 ± 0.91	36.23 ± 0.23	5.49 ± 0.39
Radiotherapy	65.74 ± 0.49	27.91 ± 0.89	8.71 ± 0.61	55.12 ± 0.84	39.19 ± 0.74	6.19 ± 0.26
Endostar + radiotherapy	66.55 ± 0.77	27.16 ± 0.93	8.87 ± 0.62*	69.12 ± 0.54	11.01 ± 0.83	22.41 ± 0.77 [△] *

Note: * indicates $P = 0.028$ compared with radiotherapy group under normoxia; [△] indicates $P = 0.000$ compared with radiotherapy group under hypoxia; * indicates $P = 0.003$ compared with endostar + radiotherapy group under normoxia.

group than in hypoxic group without endostar ($P = 0.000$), demonstrating that endostar can enhance the radiosensitivity of A549 cells under hypoxia. The values of D_0 and D_q were higher under hypoxia than under normoxia, suggesting that the mean lethal dose was higher, the sublethal damage repair was stronger, and radiosensitivity was poorer in hypoxic cells than in normoxic cells. After endostar was added, the values of D_0 and D_q were decreased from 1.693 to 1.39 and

from 2.45 to 1.02, respectively, suggesting that endostar can decrease the mean lethal dose and sublethal damage repair capacity in hypoxic cells. After endostar was added, SER was 1.22 more than one, demonstrating that endostar has the radiosensitizing effect on A549 cells.

In this study, apoptosis rate was lower in hypoxic radiotherapy-alone group than in normoxic radiotherapy-alone group ($t = 10.822$, $P = 0.002$), suggesting hypoxia

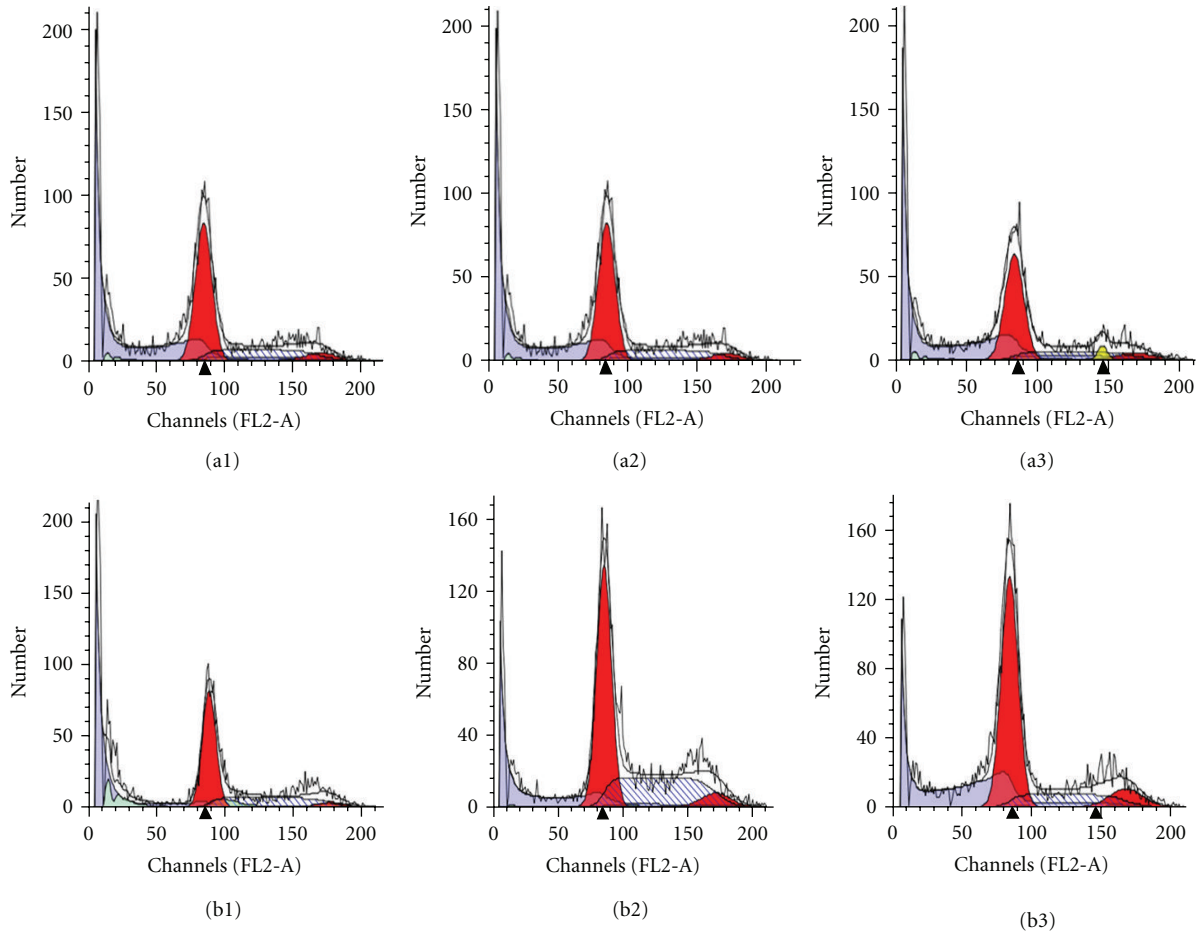


FIGURE 5: The distribution of cell cycle under normoxia and hypoxia. (a1)–(a3): The distribution of cell cycle in control group, radiotherapy-alone group and Endostar + radiotherapy group under normoxia. (b1)–(b3): The distribution of cell cycle in control group, radiotherapy-alone group and Endostar + radiotherapy group under hypoxia.

can decrease apoptosis. There were no statistical differences in apoptosis rates among normoxic control and endostar groups and hypoxic control and endostar groups ($F = 3.768$, $P = 0.148$), indicating that endostar alone has no marked effects on apoptosis. There was no significant difference in apoptosis rate between normoxic combination group and normoxic radiotherapy-alone group ($P = 0.553$), but there was a significant difference between hypoxic combination group and hypoxic radiotherapy-alone group ($P = 0.000$), suggesting that endostar promotes apoptosis only under hypoxic conditions. Our study also found that G_2/M phase cells were lower in hypoxic radiotherapy-alone group than in normoxic radiotherapy-alone group ($F = 24.572$, $P = 0.000$), displaying that hypoxic cells are not sensitive to radiotherapy; G_2/M phase cells were higher in hypoxic combination group than in normoxic combination group ($t = 15.48$, $P = 0.003$), further demonstrating that endostar has radiosensitizing effects on A549 cells only under the conditions of hypoxia combined with radiotherapy. The mechanism may be that hypoxia changes cell cycle.

Our results suggest that endostar-combined radiotherapy possesses the radiosensitizing effect on human pulmonary

adenocarcinoma cell line A549 under hypoxia, while the radiosensitizing effect is not found under normoxia. The mechanism may be that endostar changes cell cycle under hypoxia. Our study provides an experimental basis for the clinical application of endostar combined with radiotherapy. The genesis and progression of tumor are a complex process. There are differences between in vitro and in vivo environments, so the mechanisms of in vitro and in vivo radiosensitization may be not the same. The exact molecular mechanism remains to be further studied.

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