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Effect of endostar combined with cisplatin on expression of VEGF and Sema3A of Lewis lung cancer rats

Ping Feng[1], Zhi-Lin Zhang[2], Zhi-Hua Zhang[2*], Xiu-Long Zhang[2], Feng Xiang[3], Jian-Hua Tang[3], Bao-Li Xiang[2]

[1]*Master of Respiratory Medicine, Hebei Northern College, Zhangjiakou 075000, Hebei, China*
[2]*Department of Respiratory Medicine, First Affiliated Hospital of Hebei Northern College, Zhangjiakou 075000, Hebei, China*
[3]*Department of Clinical Pharmacology, First Affiliated Hospital of Hebei Northern College, Zhangjiakou 075000, Hebei, China*

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ABSTRACT

Objective: To evaluate the therapeutic effect of endostar (ED) combined with cisplatin(DDP) on model of C57BL/6 rats, and to further investigate the inhibiting mechanism of endostar from tumor angiogenesis. **Methods:** Lewis lung cancer cells were inoculated in C57BL/6 mouse, then the mouse were randomized into control group (group A), ED (group B), DDP (group C) and ED/DDP (group D). They were treated according to the plan. And the expressions of VEGF and Sema3A were evaluated by immunhistochemisty. **Results:** The weight of tumor increased in group A and B. It was decreased in group C and D. The tumor volume was increased in all the 4 groups. The VEGF expression of group D was obviously lower than the other group 3, but the Sema3A expressed of group D was significantly strenghtener than the other group 3. The VEGF expression of group B and group D were obviously low especially in the 4th-8th days. Pearson correlated analysis showed that the expression VEGF and Sema3A were negatively correlated (r=-0.72, P<0.05). **Conclusions:** ED combined with DDP could control the tumor growth effectively, and avoid weight loss. ED could reduce VEGF expression, and enhance Sema3A expression. Tumor vessel presents transient normalization. It is easy for DDP perfusion, and to kill tumor cells.

1. Introduction

Pulmonary adenocarcinoma was one of the most common lung cancer[1], the main treatment of pulmonary adenocarcinoma was platinum combined with other chemotherapeutics. But chemotherapeutic drugs could result in tolerance and reduce treatment effectiveness, micro-environmental hypoxia in tumor cells was the key factor to tolerance. On the contrary endostar[2] targeting endothelial cells could make tumor vessel form temporal "normalization"[3], accordingly improve the efficiency of drug infusion and enhance the effect of chemotherapy. So cisplatin combined with vinorelbine can be used in treatment of stage Ⅲ, Ⅳ non-small cell lung cancer[4]. Recently the mainly research discussed about change of various factors expression before and after treatment, explained the possible enhancing mechanism for endostar chemotherapy sensitivity. Through dynamic observation of weight and tumor growth in different groups of Lewis lung cancer C57BL/6 mice model, and changes in the expression of VEGF and Sema3A, the experiment explained sensitization mechanism of endostar to cisplatin. The results could provide experimental foundation for clinical application of endostar and the mechanism of lung cancer vessel normalization.

*Corresponding author: Zhi-Hua Zhang, Department of Respiratory Medicine, First Affiliated Hospital of Hebei Northern College, Zhangjiakou 075000, Hebei, China.
Tel: 86-313-8043503
E-mail: zzh80018@yahoo.com.cn

2. Materials and methods

2.1. Materials

A total of 56 male C57BL/6 mice were selected, clean level, 6-8 weeks, 18-22 g (from Academy of Military Medical Sciences); Lewis lung cancer cells from Chinese Academy of Sciences; endostar from Shandong Xianshengmaidejin

Co.; DDP from Shandong Qilu Co.; DMEM medium from Gibco Co.; FBS from Zhejiang Sijiqing; rabbit anti–rat VEGF antibody from Wuhan Boshide Co.; rabbit anti–rat Sema3A antibody from Beijing Boaosen Co.; super clean bench SW–CJ–2FD from Suzhou Antai Co.; inverted phase contrast microscope from Japan Olympus Co.; desk centrifuge from Sigma Co.; carbon dioxide incubator HEPA100 from United States; morphological image analysis system from Motic.

2.2. Animal model and groups

Mice were adapted to live in the room for one week after bought back. Lewis lung cancer cells of logarithmic growth phase (cell confluence 80%) were collected, and cell density was adjusted to 1×10^7/L with saline solution, then the cells were injected in mice right axillary, per 0.2 mL. After the tumor size up to about 8 mm, 56 tumor–bearing mice were randomly assigned to 4 groups: group A: normal saline (control group); group B: endostar; group C: DDP; group D: ED/ DDP.

2.3. Treatment methods

Group A: 0.2 mL, intraperitoneal injection once a day,14 mice; group B: 2.0 mg/kg, 0.2 mL, intraperitoneal injection once a day, 14 mice; group C: 2.0 mg/kg, 0.2 mL, intraperitoneal injection once a day, 14 mice; group D: the doses as above, 0.2 mL, intraperitoneal injection once a day, 14 mice. Before dosing every time, the dosage was adjusted according to weight. After dosing, at the 2nd, 4th, 6th, 8th, 10th, 12th, 14th day, two mice were sacrificed each group. The tumor blocks were taken out and fixed in 4% paraformaldehyde.

2.4. Calculating weight and volume of tumor

Weight was measured daily after treatment, and the maximum diameter a (mm) and the minimum diameter b (mm) of the tumor blocks were calculated with vernier caliper. The tumor volume was calculated based on V=ab2/2. Time was taken as the horizontal axis and volume as the vertical axis, which depicted tumor growth curves.

2.5. VEGF and Sema3A detection

After all tumor tissue was removed, then fixed, dehydrated, paraffin was embedded, sliced. Expression of VEGF was detected by SP immunohistochemical method, and expression of Sema3A was detected by SABC method. Dyeing method was strictly used according to its statement. Sema3A antibody concentration was 1:200. Both were used microwave antigen hot fix, and antigen retrieval solution was citrate buffer of pH6.0. Criterion were as following: expression of factors were observed by light microscope, to VEGF, tumor cell cytoplasmic appeared brown yellow particles for positive; to Sema3A, cell pulp and mesenchyme presented brown yellow particles for positive. Brown yellow particles were more, and dyeing was more deeply, so its expression also was stronger. Dyeing slices was analyzed by morphology image analysis system, 5 high–power fields randomly were selected in each slice, and positive signal average gray value was determined.

2.6. Statistical analysis

Statistical analysis software SPSS16.0 was used. The measurement data was expressed as mean±standard deviation (±SD). Correlation analysis was analyzed by Pearson correlation. When $P<0.05$, the difference was statistically significant.

3. Results

3.1. Comparison of weight and tumor volume

For weight, group A and B were increased, and group A was more significant; group C and D were decreased, and group D decreased slowly.

After the Lewis lung cancer cells inoculated, tumor blocks at inoculation could be touched on the 8th–10th days. And the blocks were increased gradually. After treatment, tumor growth was the fastest in group A and group B, C, D by turns (Figure 1).

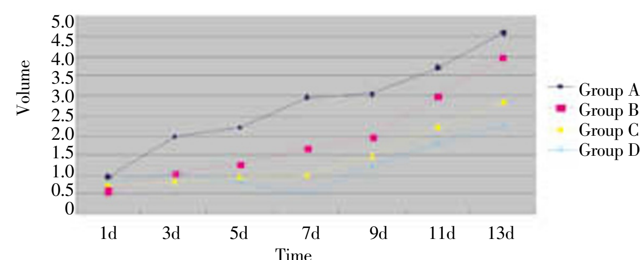

Figure 1. Tumor volume change after treatment.

3.2. Pathological observation of tumor tissue

General observation: the characteristic of tumor blood vessel was rich, quality crisp, bleeding easily, tumor cells infiltrating surrounding tissue and skin, boundaries not clear, splited difficultly, bleeding obvious, cut fish–shaped, necrotic area in the middle. HE dyeing: a large number of cancer cells, the cells arranged in close, cell volume and nuclear big, stromal vascular rich (Figure 2).

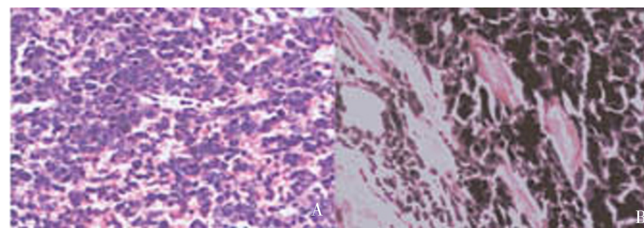

Figure 2. HE Straining.
A: Vascular rich; B: Muscle infiltration.

3.3. Expression level changes of VEGF

Results of immunohistochemical revealed that expression of VEGF was mainly in the cytoplasm, and the positive expression was visible in all groups. The expression of A was the highest (153.67±5.40), and D the lowest (78.79±7.83). In group C, its expression was stronger than group B. Expression of VEGF was supreme on the 2nd day, minimum at the 4th–8th days in group B and D after chemotherapy. Expression of VEGF gradually increased on the 9th–14th days. But it was decreased than it on the 2nd day (Figure 3 and Figure 4).

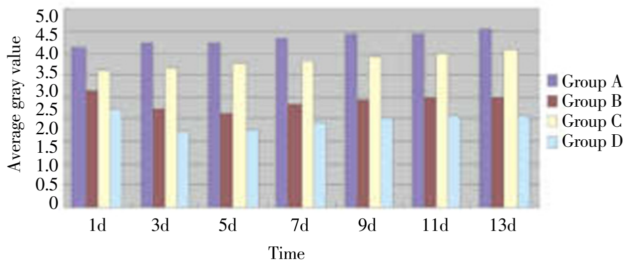

Figure 3. Change of VEGF average gray level value.

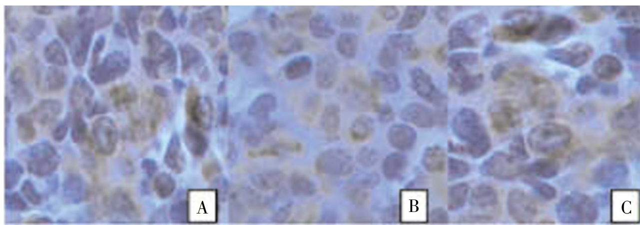

Figure 4. VEGF immunohistochemical staining results in group D (400×).
A: 2nd day after treatment; B: 4th day after treatment; C: 10th day after treatment.

3.4 Expression level changes of Sema3A

Results of immunohistochemical revealed that expression of Sema3A was mainly in the cell cytoplasm and interstitial. It had no expression in group A and C. In group D, expression of Sema3A was the highest (75.48±35.77), in group B was (66.65±30.64). Sema3A was not expressed on the 2nd day after chemotherapy. Sema3A had high expression on the 4th–8th days. On the 9th–14th days, expression of Sema3A decreased gradually (Figure 5 and Figure 6).

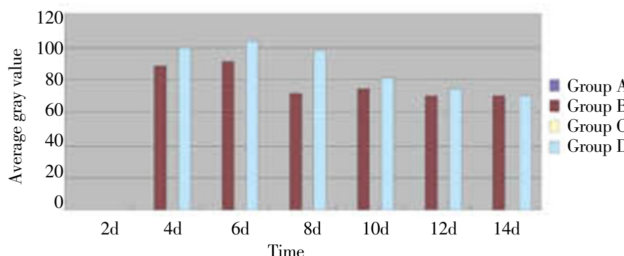

Figure 5. Change of Sema3A average gray level value.

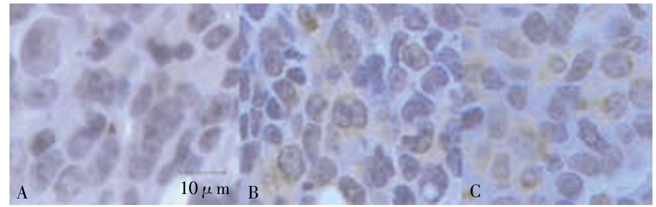

Figure 6. Sema3A immunohistochemical staining results in group D (400×).
A: 2nd day after treatment; B: 4th day after treatment; C: 10th day after treatment.

3.5. Correlation analysis

In the experiment, VEGF and Sema3A had correlation. Pearson correlation analysis showed that Sema3A and VEGF expression showed negative correlation (r=–0.72, P<0.05).

4. Discussion

Tumor growth demands angiogenesis to provide nutrition and metabolic waste. And tumor invasion and metastasis also depend on angiogenesis. So anti–angiogenesis treatment become a research hot spot. Endostar is synthesis endostatin as endotheliocyte–targeted. Many studies had showed that endostar could inhibit the generation of tumor angiogenesis *in vivo*[5] and normalize the tumor tissue short time window. The experiment showed that the tumor volume of ED/DDP (group D) was obviously smaller than DDP (group C), but the weight slowly declined in ED/DDP (group D). It showed that endostar could enhance treatment effect, meanwhile also could reduce perfusion for normal tissue, decrease side effects and improve quality of mouse life. Whether or not endostar combined with DDP had effect on the angiogenesis, specific mechanisms had not been reported. The experiment revealed that expression change of the key angiogenesis–related factors in a different time period by transplantation tumor model, so as to provide a scientific evidence for in–depth study on endostar sensitization mechanism.

VEGF is accepted as the strongest factor which promoted angiogenesis at present, it could be expressed in a variety of tumors. It stimulated in many link of tumor angiogenesis[6], such as degradation of blood vessel endothelium basal lamina, migration and multiplication of endothelial cell, it could still directly contribute to the growth of tumor cells, *etc*. Sema3A[7] is a kind of inhibiting angiogenesis factor, belonging to the Semaphorins family. Recently there is not reports about the expression in the lung tissue. The experiment found that its expression was different in different groups and different time, there was connected with its mechanism of action. In tumor tissues, Sema3A expression reduced significantly, and Sema3A–NRP1–Plexins[8] was interrupted. Because NRP1 is VEGF's receptor, the competitive strength of Sema3A and VEGF weakened

and bonding force of NRP1 and VEGF is strengthened, which promoted the formation of vessels[9]. And other researches showed that[10] long–term expression of Sema3A could significantly improve the tumor vessels cell coverage. So this could normalize the tumor tissue for a short time and make more effective to kill tumor cells. At last it reducesthe VEGF secretion, and weakens its competitiveness to Sema3A. Sema3A is a inhibitory angiogenesis factor, it could promote the normalization of tumor vessels. It might bring benefit if it was developed to clinical preparation of therapy of lung cancer.

The experiment found that the VEGF expression was the most weak in group B and group D, and the Sema3A expression was the strongest in the 4th–8th days. Their expression showed negative correlation (r=–0.72, P<0.05). It is related to tumor vessels normalization, and it is almost unanimously on 3rd–7th days[11]. In normalization time window, endostar runs away immature vessels, strengthened the residual vessels, increases tumor tissue perfusion, improves the uniformity of tumor blood supply in time and space, makes more chemotherapeutic drugs into tumor tissue. In all groups, the effect of group D was the best, it was connected with different targets[12] of two drugs except normalization. The target of DDP was tumor cells, it could directly kill tumor cells, reduce tumor cells to secrete VEGF. And the target of endostar was vascular endothelial cell[13,14], it could interrupt the activity of endothelial $\alpha 5\beta 1$ integration protein[15] (cellular adhesive molecular), metal protease 2, 9 and 13[16] and activity of endothelial cell selection element, indirect inhibit VEGF secretion[17]. They could inhibit tumor growth at the same time. This could also explain sensitization mechanism of endostar to DDP.

Endostar combined with DDP have become first–line drug to treat with non–small cell lung cancer, but the sensitization mechanism was not clear. The results showed that endostar reaches sensitization by influencing the angiogenesis correlation factor, but the specific mechanism and the key targets still need to be further discussed. It provids experimental basis for widely clinical application of endostar.

Conflict of interest statement

We declare that we have no conflict of interest.

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