Effects of Nicotine on the Progression and Metastasis of Human Ovarian Cancer cells by Exacerbating EMT

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Abstract

Does nicotine, a main constituent of toxicants in Cigarette Smoke, relate to EMT and contribute to the progression of ovarian cancer? Treat SK-OV3 xenograft mice with increasing nicotine and measure TGF-β in blood by ELISA assay and tumor cell metastasis by confocal microscopy, looking for cancer cells in the liver, bone, brain, stomach, ovary and then measure xenograft tumor size by weight. A positive control is benzene treatment, and the negative control is PBS. The results will reveal the effects of nicotine on ovarian cancer cell proliferation, which will improve our understanding of the harm environmental toxicants can do to human health. At the same time, the physiological processes that lead to carcinogenesis will also provide prevention awareness and treatment ideas for ovarian cancer caused by smoking..

Keywords: Ovarian Cancer, Nicotine, Epithelial-Mesenchymal Transition, TGF-β

1. Introduction

Ovarian cancer is one of the three most common malignant tumors in female reproductive organs, accounting for about 4% of all malignant tumors in the female body. Early diagnosis of ovarian cancer is difficult because of the lack of early symptoms and the difficulty of screening for non-specific symptoms. 70% of patients are diagnosed at an advanced stage (i.e., clinical stage III-IV) [1], and advanced cases do not respond well. Therefore, ovarian cancer is the leading cause of death among all kinds of gynecological tumors, and it is the most significant disease that seriously threatens women's health. Although surgery and chemotherapy have recently improved the survival rate of patients with advanced ovarian cancer, treatment failure and disease progression are still inevitable [2-3]. Therefore, identifying the underlying etiology is a top priority in preventing ovarian cancer. The growth and regulation of malignant tumors result from a series of factors, including heredity, environment, and lifestyle. Since heredity cannot be easily changed, the change in environment and lifestyle is fundamental [4]..

Therefore, smoking, a common factor affecting health, began to receive great attention. There is much evidence that smoking increases cancer proliferation and the rate of cancer metastasis [5-7]. With the increase of female smokers worldwide [8], the relationship between smoking and gynecological diseases has become a hot research topic. A growing body of research has found that smoking increases the risk of ovarian cancer, particularly mucinous ovarian cancer [9-11]. Nicotine is the primary addictive component of cigarette smoke, and although it is not considered carcinogenic, it can enhance or inhibit cancer cell proliferation depending on the type of cancer. Several studies have shown a link between nicotine and ovarian cancer cells [12].

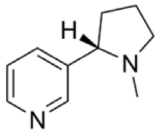

Figure 1. The structure of Nicotine

This study will focus on whether nicotine enhances ovarian cancer cell proliferation through EMT（Epithelial-Mesenchymal Transition）. EMT is a process in tumor cells. EMT also surrounding the internal environment (including matrix and extracellular environment components such as cytokines and growth factors) participate in or influence each other in an autocrine or paracrine manner [13]. EMT plays an important role in cancer metastasis and aggravated statuses of cancer patients. Through this process, cells acquire the capacity of motility, which leads to decreased adhesive ability for embryonic development in various tissues or organs. Most cells undergoing EMT showed down-regulation of epithelial genes, promoting the metastasis of cancer cells [14].

Studies have shown that the development of EMT involves multiple signaling pathways. Among them, TGF-β is the primary inducer of EMT. A TGF-β signaling pathway is one of the major pathways involved in human development. It regulates embryogenesis, maintains human tissue homeostasis, and regulates proliferation, differentiation, apoptosis, and migration in different cell types. Therefore, the content of TGF-β determines whether EMT occurs or not, which also means whether cancer cells proliferate and metastasize..

It is predicted that increasing nicotine concentration for various durations aggravates EMT measured by TGF-β production, which accelerates tumor cell metastasis and promotes the proliferation of Ovarian Cancer cells. The positive control is benzene treatment, and the negative control is PBS.

2. Methods

A. Materials

1) *Toxicants*. Nicotine, dissolved in ethanol at a final concentration of 1mM.
2) *Animals*. SK-OV3 xenograft mice, obtained from the laboratory (measure their weight in advance).
3) *Cells*. Highly invasive human ovarian serous cystadenocarcinoma Ho-8910 cells, cultured in RPMI1640 medium containing 10% serum and 1% dual antibodies in a 37°C incubator containing 5% carbon dioxide.

B. Cell culture

(1) Cell culture: Cell samples were cultured in RPMI1640 medium containing 10% serum and 1% dual antibody in an incubator containing 5% carbon dioxide at 37°C. The medium was changed every other day and the cells were subcultured every 3 to 4 days. When the degree of cell fusion reached 80%-90%, discard the culture medium, wash with PBS twice, add 0.25% trypsin LML for digestion for 2min, add fresh RPMI1640 culture medium to neutralize the trypsin, blow into a single cell suspension with a pipetting gun, transfer to a new cell culture dish, shake and mix in a rice shape to make the cells evenly distributed. Culture in an incubator containing 5% carbon dioxide at 37°C.
(2) Cell freezing: Select the logarithmic growth phase cells with 80% cell fusion and good growth status 48 hours after passage, and change the culture medium once a day before freezing. The culture dish was digested by adding 0.25% trypsin LML, adding a new fresh RPMI1640 culture medium, collecting the blown single cell suspension, centrifuging at 1000rpm for 5min, and discarding the supernatant. Add the frozen solution (10%DMSO+70%RIPM 1640 medium +20% FBS) to the centrifuge tube and blow gently. Mix the cells, transfer 1ml of each tube into a 2ml frozen storage tube, tighten the cap, and mark the cell name, time, and other markers. It was stored at -4°C for 2h, -20°C for 4h, and -80°C overnight. Finally, it was frozen at -196°C in a liquid nitrogen tank.
(3) Cell resuscitation: HO-8910 cells were removed from the liquid nitrogen tank and quickly placed in a 37°C water bath. The cryopreserved tube was gently shaken to melt the cryopreserved liquid as soon as possible. After rewarming, sterilize and seal the cryogenic tube with 75% alcohol, absorb the cryogenic liquid into the centrifuge tube, add 5ml culture medium and gently blow it to mix it, centrifuge it at 1000rpm for 5min, discard the supernatant, add 5ml culture medium RPMI1640 with 10% fetal bovine serum, and mix the cells repeatedly. The cultures were grown in an incubator containing 5% carbon dioxide at 37°C.

C. Nicotine exposure to mice

Let the SK-OV3 xenograft mice be exposed to air filled only with nicotine as an impurity. Different groups are divided according to the different concentrations of nicotine (0.5mg/L, 2mg/L, 10mg/L), also for different durations(5min/1hr/6hrs). The mice were monitored at all times, and blood samples were taken regularly to measure the carcinogenesis of cells in the body.

D. ELISA analysis for TGF-beta

Measure the concentration of the TGF-beta in blood after exposure to nicotine using ELISA assay. Analyses were carried out according to the manufacturer's protocol, in triplicate, and read at 450 nm using a Molecular Devices microplate reader for each ELISA kit. Special software would determine the standard curves and individual proper concentrations. All the treatment groups are compared with the control group which does not receive any treatment. Two-tailed P values were calculated through paired T-test.

E. Confocal microscopy analysis for cancer cells

Blood samples were taken from each experimental organ of the mice, and the ovarian cancer cells were observed in the liver, bone, brain, stomach, and ovary by confocal microscopy according to the instructions of the instrument.

F. Measuring xenograft tumor size by weight

The volume and weight(estimated) of the tumor in mice were dynamically detected by infrared scanning or micro-CT imaging at regular intervals every day. At the end of the experiment, the xenograft mice were vivisected and then weigh the treated tumor to obtain accurate data on weight.

3. Statistical analysis

A student t-test was used to examine the data. The data were entered into Minitab and a significant difference P was obtained, and the resulting P-value was compared with the significance level α (0.05). A P value equal to or less than 0.05 indicates that an association between nicotine and ovarian cancer can be verified, not vice versa. Then the experiment will be repeated three times and the three groups of data were tested according to the same method as above to reduce the error of the data and ensure

that the results are true.

A. A. Combination of Possible Results (CR)

Table 1. Possible combination of results

Possible observations	CR1	CR2	CR3	CR4	CR5	CR6	CR7	CR8
Increased TGF-β by ELISA?	+	+	+	-	+	-	-	-
Increased metastasis by confocal microscopy?	+	+	-	+	-	+	-	-
Increased tumor size in xenograft mice	+	-	+	+	-	-	+	-
Supporting Hypothesis ?	YES	Partially	Partially	Partially	Partially	Partially	Partially	NO

Note. "+" represents "Yes, when comparing nicotine treated to untreated, similar to the positive control which is benzene". "-" represents "No, when comparing nicotine treated to untreated, similar to the negative control which is PBS".

B. Results

Possible Result 1: An increase in TGF-β content, cancer metastasis, and tumor size were detected simultaneously, fully verifying the hypothesis.

Possible Result 2: In this case, increased TGF-β content and metastasis of cancer cells were both detected, but no change in tumor size was detected. Thus, the result partially supports our hypothesis.

Possible Result 3: Both increased TGF-β content and increased tumor size were detected. But the increase in cancer metastasis was not detected. Thus, the result partially supports our hypothesis.

Possible Result 4: Metastatic growth of cancer cells and increased tumor size were detected. But there was no significant change in TGF-β levels. Thus, the result partially supports our hypothesis.

Possible Result 5: Only increased TGF-β content was detected, but neither the number of metastases nor tumor size changed. Such results only weakly partially support the hypothesis.

Possible Result 6: Only the amount of cancer cells metastasized was detected to increase. There was no change in TGF-β content or tumor size. Such results only weakly partially support the hypothesis.

Possible Result 7: Only tumor size was detected to increase, while TGF content and cancer cell turnover were both changed. Such results only potentially support the hypothesis.

Possible Result 8: No change was found in any of the three data sources, suggesting that nicotine and ovarian cancer are not associated and cannot support the hypothesis at all.

4. Discussion

For possible result 1, tumor enlargement was detected in xenograft mice, accompanied by tumor metastasis in vivo. At the same time, increased TGF-β levels were detected. It has been hypothesized that nicotine will promote TGF-β production, thereby promoting the development of EMT, which leads to cancer metastasis, and thus ovarian cancer cells. The results obtained from the experiment are consistent with this process because the rise of TGF-β pathway content leading to the occurrence of EMT and ultimately to the generation of ovarian cancer were verified in the experiment. This result suggests that the hypothesis is valid, i.e., that nicotine does have a direct association with ovarian cancer formation.

For possible result 2, the increase in TGF-β content and metastasis of cancer cells were monitored, but there was no significant increase in tumor size. According to the hypothesis, EMT is induced by TGF-β, which is demonstrated by this experimental result, and then the development of EMT will lead to the development of ovarian cancer, that is, tumor enlargement. However, according to the experiment, we did not find significant tumor enlargement, which also indicates that EMT does not directly cause the occurrence of ovarian cancer, and also proves that nicotine is not one of the direct causes of ovarian cancer, thus does not fully support the hypothesis.

For possible result 3, even though both TGF-β content and metastasis were detected to increase, tumor size did not increase significantly. Based on the hypothesis, basically what we're looking at is the association between nicotine and ovarian cancer development, which is ultimately measured by the change in tumor size. In this case, the absence of size change indicates that nicotine does not have a direct effect on ovarian cancer development and does not fully support the hypothesis.

For possible result 4, growth in metastasis and tumor size were both detected, suggesting an effect of nicotine on ovarian cancer development. However, the absence of significant changes in TGF-β content suggests that

the induction of EMT does not occur due to TGF-β as hypothesized. The hypothesis that nicotine causes ovarian cancer is correct, but the mechanism involved is not completely proven, so the results only partially support the hypothesis.

For possible result 5, since there was no change in the number of metastasized cancer cells or tumor size, the increase in TGF-βcontent only demonstrated the hypothesized relationship between nicotine and TGF-β, not its effect on ovarian cancer. Such experimental results explain only a small part of the hypothesized relationship.

For possible result 6, since only the number of metastases was detected to increase. The hypothesized link between nicotine and ovarian cancer has not yet been proven to be direct. The results showed that nicotine could cause EMT and metastasis of cancer cells, but did not directly cause ovarian cancer. Also, the hypothesis that nicotine boosts TGF-β levels has not been proven. As a result, such results do not fully account for the entire hypothesized mechanism of the nicotine effect.

For possible result 7, the results do point to the potential role of nicotine in ovarian cancer. However, the hypothesized mechanism of increasing TGF-β content, thereby inducing EMT and ultimately leading to ovarian cancer was overturned. Such results only point to a potential relationship between nicotine and ovarian cancer, and the mechanism of action is still unclear and only partially justifies the hypothesis.

For possible result 8, TGF-β content, cancer cell turnover, and tumor size were all unchanged. This completely shows that the process mentioned in the hypothesis is incorrect and reveals that there is no hypothesized relationship between nicotine and ovarian cancer. Such experimental results reject the hypothesis.

A. For different concentrations or durations

If low concentrations of nicotine have no significant effect on the development of ovarian cancer (little growth in tumor size), but higher concentrations of nicotine (same duration) cause greater tumor growth, then nicotine concentration has a direct effect on the development of ovarian cancer.

Similarly, if the tumor size increased much less after a short exposure than after long exposure (at the same concentration), the time of exposure also had an effect on the experiment.

Since concentration and exposure time are considered as two separate variables and are hereby interpreted separately from regular operations above.

5. Conclusion

In conclusion, this study aims to reveal the link between nicotine and ovarian cancer and the physiological process of carcinogenesis. Some of the possible results may reveal EMT occurring in the absence of a change in TGF-β content, or an increase in tumor size in the absence of EMT. Future studies will also focus on the association between other signaling pathways that induce EMT and nicotine, as well as other causes of ovarian cancer lesions. By analyzing the specific effects, we can not only get constructive inspiration in the prevention work but also play a key role in the treatment of ovarian cancer. Cases of ovarian cancer caused by smoking or secondhand smoke may further help us understand the mechanism.

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