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Original Research

Influence of the vaccinating density of A549 cells on tumorigenesis and distant organ metastasis in a lung cancer mice model

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Abstract: Lung metastasis of malignant tumors, such as lung carcinoma, is a major cause of cancer-related deaths worldwide. The commonly used lung tumor models were established by subcutaneous or intravenous injection of the non-small cell lung cancer cell line A549 in mice. However, the influence of cell densities on tumorigenesis and distant organ metastasis remains poorly investigated. In this study, A549 cells were subcutaneously injected into mice at 1×10^7 cells/mL, 5×10^6 cells/mL, and 1×10^6 cells/mL or intravenously at 1×10^6 cells/mL, 5×10^6 cells/mL, and 1×10^6 cells/mL. Then, histology analysis, immunohistochemistry staining, and *in-situ* TUNEL assay were performed to evaluate tumor growth and metastasis. Results showed that subcutaneously injecting the A549 cells could develop tumors and that fewer apoptotic cells were found in the 5×10^6 cells/mL group than in the other two groups. In groups intravenously injected with A549 cells, there were tumor nodules in all groups, and the 1×10^5 cells/mL group showed longer survival time than the other two groups without any distant organ metastasis. There were tumor nodules formed in the liver in the 1×10^6 cells/mL group at 14 d. Together, our results demonstrated that 5×10^6 cells/mL and 1×10^5 cells/mL are the optimal cell concentrations for the subcutaneous and experimental metastatic models, respectively.

Key words: Lung cancer model; Vaccinating density; Tumor growth; Organ metastasis.

Introduction

Lung cancer is a malignant tumor with one of the highest mortality rates and minimal responses to therapeutic treatments worldwide, and more than 85% of the lung cancer-related deaths are caused by non-small cell lung cancer (NSCLC)(1). Its morbidity is rising every year in China(2). The predicted 5-year survival rate is only 15.9%, and most lung cancers are diagnosed at an advanced stage(3, 4). The lung is central to the circulatory system and is the most common metastasis site of malignant tumors, which is the main cause of death due to many malignant tumors. NSCLC is diagnosed in early stages in less than one-third of the cases, and treatment is rarely effective (5, 6). Therefore, lung cancer and pulmonary metastasis have become a focus of research worldwide(7). To simulate the pathogenesis and development process of lung cancer, it is necessary to establish multiple experimental animal models with good stability, reproducibility, obvious clinical characteristics, and objective evaluation index, which are the basis for studying lung cancer and metastasis(8). Currently, subcutaneous xenograft models inoculated with tumor cells and metastatic lung cancer models established by tail vein injection of tumor cells are being widely used in early pharmacodynamic evaluation(9, 10). In the modeling process, the concentration of the tumor cells directly affects the growth kinetics of the tumor, the living state, survival time of the mice, and distant organ metastasis. Tumor cells injected into the mice seem to flush into the circulatory system and probably reach to distant organs. Thus, the most critical step in this modeling process is to determine whether subcutaneously injected tumor cells can grow in situ and whether intravenously injected tumor cells directly recruit to the lung or if some of the cells simultaneously diffuse around the body through the vascular system.

In this study, different concentrations of human A549 cells were applied to establish models via subcutaneous inoculation at the armpit of a forelimb and injection via the tail vein in nude mice. The relationship between tumor cell concentration and tumor development process was assessed to offer the appropriate window period for research(11). The distant organs such as the heart, liver, spleen and the kidney were checked for metastatic nodule formation. These experiments support the development of innovative drugs aimed at targeting lung cancer and diffused metastasis.

Materials and Methods

Cell lines

The human NSCLC cell line A549 was incubated in RPMI-1640 (Thermo Scientific) containing 10% fetal bovine serum (FBS) (Thermo Scientific) and 1% penicillin/streptomycin (Hyclone, USA). Cells were main-

tained in an incubator with a humidified 5%CO_2 atmosphere at 37°C. The medium was changed every other day, and cells were passaged at sub-confluence after trypsinization with 0.25% trypsin (Thermo Scientific).

Animals and treatments

BALB/c nude mice aged 5–6 weeks weighing 16–22 g were purchased from the Laboratory Animal Center of Sichuan University (Chengdu, Sichuan, China). All animal experiment procedures were approved by the Animal Ethics Committee of Sichuan University.

A549 cells were harvested and resuspended in RPMI-1640 at concentrations of 1 × 10^7 cells/mL (high-concentration group, SC-HC), 5 × 10^6 cells/mL (medium-concentration group, SC-MC), and 1 × 10^6 cells/mL (low-concentration group, SC-LC) for the subcutaneous tumor model and at concentrations of 1 × 10^6 cells/mL (high-concentration group, IV-HC), 5 × 10^5 cells/mL (medium-concentration group, IV-MC), and 1 × 10^5 cells/mL (low-concentration group, IV-LC) for the experimental metastatic mice model involving intravenous injection. BALB/c nude mice were randomly divided into these six groups according to the different concentration of cells and modes of introducing tumor cells. About 100 μL of cell suspension was injected subcutaneously into the armpit of a forelimb or intravenously in the tail vein of the mice. In the subcutaneous tumor model, the tumor size was measured with a vernier caliper twice a week, and the tumor volume was calculated as follows: tumor volume (mm^3) = length (mm) × width $(mm)^2$ × 0.5(12). In the experimental metastatic mice model, mice were euthanized at 7 d, 14 d, 21 d, and 28 d after injection, tumor nodules were counted (13), and the survival time was measured. Moreover, all mice were weighed twice a week and all surviving mice were euthanized at the end of the fourth week after the cell injection.

Histological analysis

Tumor tissues, lung tissues, and distant organ tissues of the heart, liver, spleen, and kidney were fixed in 4% paraformaldehyde and embedded in paraffin. Then, 5-μm sections were cut using a microtome and stained with hematoxylin–eosin (HE). Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) was performed with rabbit anti-mouse antibody (Abcam, UK) to detect tumor cell proliferation, as previously described (14); this is briefly performed as follows: sections were blocked with 3% hydrogen peroxide for 10 min and antigen retrieval with sodium citrate (10 mM, pH 6.0) was done under high pressure for 3 min. Sections were stained with the rabbit anti-mouse antibody after incubation with bull serum albumin and were washed three times with PBS between each procedure. PCNA-positive cells were counted under a microscope and the percentage was calculated. *In-situ* Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed for detecting the fluorescence of apoptotic cells *in situ* using the TUNEL kit (Roche) following the manufacturer's protocol. The apoptotic cells were counted for magnification.

Statistical analysis

All data were statistically analyzed using SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL, USA) and were represented as mean ± SD. Between-group differences were tested by one-way analysis of variance (ANOVA). $p < 0.05$ (two-tailed) was considered to indicate statistical significance.

Table 1. Time of the tumor formation.

Cell Concentration (mL^{-1})	Time (d)
1×10^6(Low)	16 ±0.63
5×10^6(Medium)	9.4 ±1.02**
1×10^7(High)	6.6±0.8**

** P<0.01.

Results

Establishment of the subcutaneous transplantation tumor

After subcutaneous injection of different concentrations of A549 cells in the armpit of a forelimb, we checked the nude mice daily until neoplasms were accessible. Only one nude mouse among the SC-HC, SC-MC and SC-LC groups did not form a tumor. The tumor formation time point was considered as the time point when the neoplasm was palpable. The results show that the tumor formation time was positively correlated with the inoculated cell concentration. Tumor in the SC-HC group occurred at the earliest on 6.6 ± 0.8 d, followed by the SC-MC and SC-LC groups which showed tumor formation at 9.4 ± 1.02 d and 16 ± 0.63 d, respectively ($p < 0.01$)(Table 1).

To investigate the tumor growth characteristics, tumor size was measured with a vernier caliper at 7 d after inoculation. The tumor growth curves showed that tumor growth was slower in the SC-MC group than in the SC-HC and SC-LC groups. Thus, in the SC-MC group, the experimental process was not interrupted due to mice death induced by a fast-growing tumor and was not delayed because of a slow-growing tumor as in SC-

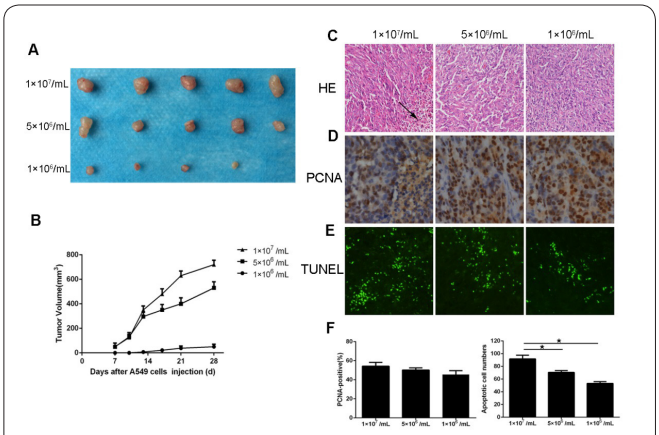

Figure 1. Establishment of the subcutaneous transplantation tumor. A. Tumor tissues from the three groups were separated from the mice at 28 day after A549 cells injection; B. Tumor growth curve reveals that tumor in the low concentration group (1×10^6 cells/ml) grows much slowly than the medium group (5×10^6 cells/ml) and high concentration group (1×10^7 cells/ml), p<0.05; C. H&E staining of tumor sections; D. Immunohistochemistry of PCNA (brown-positive) were performed in the tumor sections; E. In situ TUNEL was done to detect the apoptosis cells in the tumor, (original magnification, C, and D, ×100; E, ×400). F. Percentages of the PCNA-positive cells and apoptotic cells in the total number of neoplastic cells.

HC and SC-LC groups, respectively. Therefore, the medium concentration (5×10^6 cells/mL) may be a suitable cell concentration for the subcutaneous transplantation model (Figure 1A, B).

Tumor cell proliferation and apoptosis

To further confirm whether the medium concentration mimics the tumor growth mostly, all mice were euthanized 28 d after subcutaneous inoculation of tumor cells. The tumors were separated and sectioned for histological evaluation with HE staining (Figure 1A). As shown in Figure 1C, the closely packed tumor cells have an irregular shape with big, distinct, and intensely dyed nucleoli and distinct boundaries between tumor tissue and alveolus tissue. An evidently necrotic area was observed in the tumor tissue of the high-concentration group.

PCNA expression was detected in tumor tissue by immunohistochemical staining. Actively proliferating tumor cells (brown) were seen in all groups, and necrotic tissue can be seen in the SC-HC group. No significant differences were found in three groups (Figure 1D).

TUNEL assay was performed to detect apoptosis-associated DNA fragmentation in the tumor tissue. As shown in Figure 1E, a scattered fluorescence signal was seen in the sections of all groups, and the number of the cells with fluorescence in the high-concentration group was significantly more than in the other two groups.

Establishment of the experimental pulmonary metastasis model

After injecting different concentrations of A549 cells via the tail vein, mice in the IV-LC group (1×10^5 cells/mL) began to die at 23 d, and all the mice of this group died within 33 d. The mice in the IV-MC group (5×10^5 cells/mL) began to die at 11 d, and all mice died within 28 d. The mice of the IV-HC group (1×10^6 cells/mL) began to die at 10 d, and the last mouse died at 18 d. The survival curve is shown in Figure 2C. Then, the dead mice were dissected, and the evident tumor nodules on the surface of the lung were observed. As shown in Figures 2A and 2B, the number of lung tumor nodules at different time points was calculated. No tumor nodule was observed in any group in the first 7 d after the tumor cells injection. From 14 d to 21 d, the tumor nodules in the IV-LC group were fewer than in the other two groups ($p < 0.01$). These results explain lower mortality rate and prolonged survival of the mice in the low-concentration group.

Then, all lungs were fixed in 4% paraformaldehyde and were paraffin-embedded. Sections were stained with HE (Figure 2D). Tumor nodules were dense and scattered in the pulmonary alveolus. Immunohistochemical staining was used to detect PCNA expression in tumor nodules. As shown in Figure 2E, there are comparatively proliferating tumor cells (brown) in all groups. No significant differences were found between the three groups. The *in-situ* TUNEL assay was performed to detect the apoptotic cells from the tumor nodules in the lung. The results show that no obvious fluorescent signal was found in any group, indicating that the tumor nodules formed in the lung proliferate actively with significantly apoptotic cells (Figure 2F).

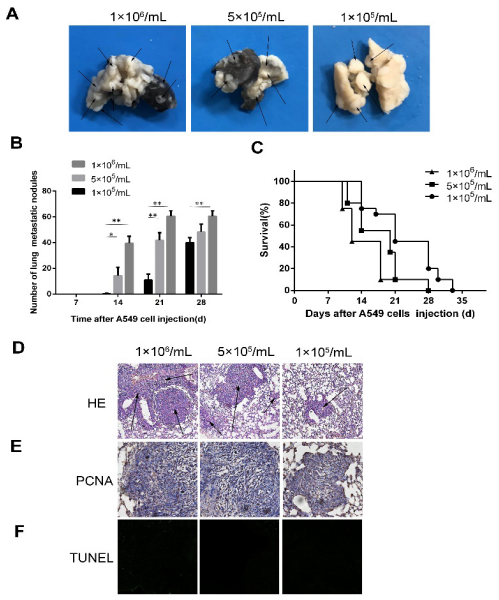

Figure 2. The establishment of experimental pulmonary metastasis model.A. Representative lungs of the experimental pulmonary metastasis model. Lungs were fixed with 4% paraformaldehyde after separated from mice. Arrows point at the tumor nodules in the lungs; B. Average metastatic nodules, *$p<0.05$, ** $p<0.01$; C. Survival time of the mice; D. H&E staining; E. Immunohistochemistry of PCNA were performed in the lung sections, arrows point at the tumor metastatic nodules in the lungs; F. In situ TUNEL was done to detect the apoptosis cells in the lung. (original magnification, D, and E, ×100; F, ×400). No fluorescence signal was seen in all groups.

Tumor cell colonization on distant organ

To confirm whether a metastasis nodule had formed in the distant organs, the heart, liver, spleen, kidney, and lung were separated and analyzed. In the subcutaneous transplantation tumor model, no nodule or nodule-like growth was seen, and the same results were proven by analyzing the pathological sections of these organs (data not shown). In the experimental pulmonary metastasis model, no nodule was found in the distant organs except the lung under stereoscopy. However, the pathological sections showed that small nodules formed in the liver of the IV-HC group (Figure 3).

Discussion

Lung cancer is one of the most frequently diagnosed cancers that in most cases is at an advanced stage at diagnosis, and it is the leading cause of cancer-related death worldwide(15). About 85% patients of all the new lung cancer cases are classified as having NSCLC (16, 17). NSCLC is still considered as a difficult disease to manage because of its aggressiveness and resistance to common therapies. Malignant cells from lung cancer might rapidly acquire activities that confer both infiltration and colonization, thus making the tumor cell migrate and cause metastatic relapse easily, leading to poor prognosis(18). Although the clinical therapeutic effect is discouraging, chemotherapy remains the gold standard treatment in nearly 80% of lung cancer cases (19). Therefore, innovative therapeutics for controlling the growth and metastasis of tumors in lung cancer patients are necessary (9). However, before that, it is

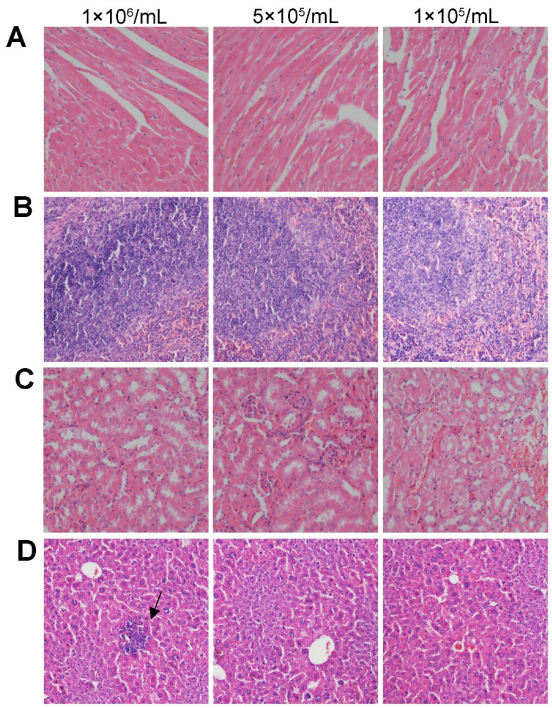

Figure 3. Tumor cell colonization on distant organs. Representative distant organs in the experimental pulmonary metastasis model. Organs were fixed with 4% paraformaldehyde after separated from mice and observed under stereoscopy. A, Heart; B, Spleen; C, Kidney; D, Liver. Original magnification,×100. Arrow points at the small tumor nodule formed on the liver of the high concentration group.

crucial to have an appropriate animal model that accurately mimics the pathologic events of lung cancer (20, 21). We suspect whether the injection concentration of tumor cells play a key role in the invasive metastatic process of malignant tumors (9, 22).

Heterologous subcutaneous cancer models in nude mice are the most commonly used lung cancer models. The main advantage of the subcutaneous model is the convenience of monitoring tumor growth(11). Nevertheless, the injection concentration of tumor cells proved to be an important factor for the cancer model, with a high concentration leading to a fast tumor growth and inducing animal death before the end of the experiment and low concentration leading to either a slow tumor growth or nor even forming a tumor. Therefore, it is necessary to explore the suitable inoculation concentration of cells to establish the appropriate experimental time window. Moreover, whether high cell concentration could lead high metastasis needs to be clarified.

In the subcutaneous transplantation model, all concentrations of A549 cells could lead to tumorigenesis. However, the tumor growth of the SC-LC group was too low to simulate tumor growth well. The tumor growth rate in the SC-MC and SC-HC groups was almost exponential, which appropriately mimics the actual growth of a tumor making it suitable for the study of anti-tumor drugs. Nevertheless, the tumor grows faster in the SC-HC group, leading to necrosis of the tumor tissues, which is not conducive to scientifically evaluate the efficacy of anti-tumor drugs. Therefore, the SC-MC group was optimal for the subcutaneous transplantation model. We also checked all nude mice for distant organ metastasis. No metastasis was found in any group. The infiltration ability of tumor cells is determined by the distant organ microenvironment and different classes of metastasis genes, such as metastasis initiation gene, metastasis progression gene, and metastasis virulence gene (23, 24). Primary tumor cells need different intervening latency to accomplish distant organ infiltration and colonization(10). In this study, the observation period was only four weeks because this period is the common treatment course for preclinical anti-cancer drug testing in the subcutaneous transplantation model.

For the experimental pulmonary metastasis model, tumor nodules in the lung were found in all concentration groups. However, more tumor nodules formed in IV-MC and IV-HC groups, and the tumor growth was fast, leading to the formation of pulmonary embolism and respiratory disorders in mice, resulting in a short survival period of 28 d and 18 d in the two groups, respectively. Histopathology examinations showed that the lung tissue was infiltrated by the tumor, and the tumor cells were seen to proliferate actively with no apoptotic cell observed. IV-LC group mice showed fewer lung nodules and prolonged lifetime than other groups; this meets the demand of anti-cancer drug research and development.

Intravasation and survival in the circulation are beneficial to tumor cell diffusion to distant organs, and theoretically, this may lead to metastatic nodules forming everywhere in the body. However, we only found metastatic nodules in the IV-HC group of the experimental pulmonary metastasis model in the pathological sections of the liver. This may be due to the structural features of capillary walls in different organs, which affects tumor cell infiltration(10). The liver is rich in blood, and the capillaries are fenestrated and readily colonized by the circulating tumor cells(25). The tumor cells first get to the lung via blood circulation when they are injected into the tail vein. The basement membrane of the pulmonary alveoli is an obstacle that circulating tumor cells can bypass only by expressing specific mediators of transendothelial migration(10, 26, 27). Therefore, we believe that the IV-LC group has the optimum cell concentration for an experimental model of pulmonary metastasis.

This study successfully establishes the A549 subcutaneous transplantation tumor model and experimental pulmonary metastasis model and suggests suitable cell concentrations. This information provides a model for exploring lung cancer and tumor metastasis mechanism and provides a foundation for the development of anti-tumor drugs. 5×10^6 cells/mL and 1×10^5 cells/mL are the optimal concentrations for the subcutaneous transplantation tumor model and experimental pulmonary metastasis model, respectively. Further studies should take advantage of the new technologies to mark and track tumor cells to expound the accurate molecular mechanisms of tumor metastasis so we can improve the animal model for more innovative anti-cancer drug development.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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