Original Article
Development and anti-tumor effects of lung cancer stem cell chaperon molecule antigen peptide vaccine

Hao Wang¹, Changxin Huang², Feiqing Wu¹, Wei Shi¹, Xueyan Fu¹, Keyuan Liu¹

¹Thoracic Surgery, ²Department of Oncology, The Affiliated Hospital of Hangzhou Normal University, Hangzhou, Zhejiang, China

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Abstract: Various antigen peptides including precursors produced by tumor mutation can bind with chaperon molecules to induce anti-tumor immunity by antigen presenting cell (APC) processing. This study thus investigated the preparation of lung cancer stem cell chaperon molecule antigen peptide complex and efficiency of tumor vaccine. Microsphere culture and separation approach was used to enrich lung cancer stem cells, which were sorted for CD133-positive cells. Stem cells after sorting were induced for chaperone molecule antigen peptide expression. C57BL/6 tumor bearing mice received normal lung cancer chaperone molecule antigen peptide, or chaperone antigen peptide complex. Tumor volume and mouse survival time were observed, along with lymphocyte proliferation and IFN-γ or IL-2 levels. Lung cancer microsphere was developed. Khosam oil milk plus radioactive I-125 induced synthesis of chaperone molecule antigen peptide in lung cancer stem cells, with positive correlation with khosam oil milk concentration. Chaperone molecule antigen peptide inhibited tumor growth, elongated survival time of mice and facilitated spleen lymphocyte proliferation, and enhanced secretion of IFN-γ or IL-2. Lung cancer stem cells express more chaperone molecule antigen peptide than cancer cells. Antigen peptide can potentiate antigenicity of tumor cells, and may work as tumor vaccine to suppress tumor growth, thus having implication in preventing lung cancer or its recurrence.

Keywords: Lung cancer stem cells, anti-tumor vaccine, radioactive I-125 particles, khosam milk oil

Introduction

The incidence of lung cancer has been the highest among all malignant tumors in China, with increasing trend by years. Although approaches including chemo-therapy, radio-therapy, molecular targeted treatment have been developed continuously, the overall treatment efficiency of lung cancer is still unfavorable, as shown by drug resistance, radiotherapy insensitivity and recurrence/metastasis [1]. These phenomena can be attributed to so called tumor stem cells with self-renewal, unlimited proliferation and pluripotent differentiation potency, making them the underlying reason behind tumor occurrence, progression, recurrence, metastasis and chemo-/radio-resistance. The targeted clearance of those tumor stem cells may thus bring more effective plan for lung cancer treatment [2, 3]. Recent study has revealed that mutation-produced tumor cell antigen peptide, including the precursor protein, mainly bind with chaperon molecule to form complex, which is processed by antigen presenting cells to induce anti-tumor immune response [4-6]. The extraction of antigen-peptide containing chaperon molecules from tumor cells may induce potent immunity [7-9]. However, few studies have been performed regarding proteomic of chaperone antigen peptide complex tumor vaccines, especially about proteomic property and anti-tumor efficiency. In vitro sequential processing of tumor cells all utilizes high-dosage radiation in short time to potentiate antigen peptide synthesis inside tumor cells, whilst continuous low-dosage radiation significantly elevates radio-therapy sensitivity of tumor cells, enhancing apoptotic rate [10]. Satisfactory enhancement of tumor antigenicity is required for in vitro sequential processing of cancer cells. Therefore, we used low concentration of Khosam oil combined with low dosage radioactive iodine particles for in vitro sequential processing of lung cancer stem...
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cells, and developed optimal processing method for induction of large scale synthesis of chaperone antigen peptide. We further separated lung cancer stem cells enriched with multi-chaperone antigen peptide complex. Our experiment analyzed anti-cancer efficiency of partial chaperone molecule antigen peptide, and obtained initial research results.

Materials and methods

Major reagent and materials

$\mathrm{CO}_2$ incubator, -80°C fridge and ultrapure workstation were purchased from Thermo (US). Micro-pipette, cold ultra-centrifuge were produced by Eppendorf (Germany). Cell filter (#200) was purchased from Xinrui Biotech (China). Protein electrophoresis apparatus, semi-dry transfer apparatus and microplate reader were provided by Bio-Rad (US). 4°C fridge and -20°C fridge were produced by Haier (China). Water-bath heater was provided by Jinghong (China). Electronic analytical balance was produced by Toledo (Italy). Micro Chemi 4.2 chem-illumiance gel imaging system was produced by DNF (Israel). Water purification system mill-Q was produced by Millipore (US). Electron microscopy was produced by Olympus (Japan). Flow cytometry was produced by BD (US). Khosam oil was provided by Jiuxu Pharm (China). Lewis mouse lung cancer cell line was provided by Cell Source Center, Chinese Academy of Sciences. C57BL/6 mice were provided by Laboratory Animal Center, Zhejiang Chinese Medicine College. Iodine-125 particle was produced by Junan (China). Anti-CD133-FITC antibody was produced by Miltenyi Biotech (Germany).

Lung cancer tumor stem cell culture and enrichment

Routine method for micro-sphere separation culture was used to separate tumor stem cells. In brief, mouse lung cancer cell line Lewis was incubated in RPMI1640 medium (containing 100 U/ml penicillin and streptomycin) with 10% FBS, in 37°C chamber with 5% $\mathrm{CO}_2$. Cell morphology was observed. Cells at log-phase with 70%~80% confluence were passed at 1:2~1:3 ratio. Freshly prepared serum-free medium (SFM) was prepared by adding pure DMEM/F12 medium with EGF (20 ng/ml), FGF (20 ng/ml) and B27 (20 μg/ml). Attached Lewis cells were digested by trypsin to prepare single cell sus-
pension. After PBS rinsing, Lewis cells were re-suspended into SFM at 10$^3$/ml, and were cultured in vertically placed flask. Cells were gently shake several times per day for observing formation of cell microsphere. Half of medium was changed every 2~3 days. Cells were passed every 6~8 days. Microsphere body was collected by centrifugation after passage and was digested in Accutase. Microsphere was rinsed to prepare single cell suspension, and was re-suspended in SFM after rinsing for further culture, separation, amplification and enrichment.

Identification of sorting of tumor stem cells

Lewis cell microsphere was digested into single cell suspension by Accutase, and was re-suspended into Buffer (containing 0.5% BSA and 2 mM EDTA in PBS) after rinsing. 10$^7$ cells were counted into 0.1 ml buffer, and were centrifuged at 300 g for 10 min. The supernatant was removed. Cells were re-suspended into 0.1 ml Buffer, which contained 10 μl CD133-FITC antibody. After gentle mixture, cells were incubated at 4°C for 10 min, and were rinsed in 1~2 ml Buffer. Cells were then centrifuged again at 300 g for 10 min, and were re-suspended into 1 ml Buffer. Flow cytometry (FACS influx) was used to identify CD133-positive cells as lung cancer tumor stem cells for sorting.

Khosam milk oil processing for tumor stem cells and induction of chaperone antigen peptide expression

Khosam milk oil (1 g/ml) was added into SFM to prepare culture medium containing 0, 1 or 2 g/L Khosam milk oil. Cancer stem cells after sorting were re-suspended into Buffer containing 0.5% BSA and 2 mM EDTA in PBS after centrifugation. 10$^7$ cells were digested in 1 ml Buffer, which contained 10 μl CD133-FITC antibody. After gentle mixture, cells were incubated at 4°C for 10 min, and were rinsed in 1~2 ml Buffer. Cells were then centrifuged again at 300 g for 10 min, and were re-suspended into 1 ml Buffer. Flow cytometry (FACS influx) was used to identify CD133-positive cells as lung cancer tumor stem cells for sorting.

Radioactive iodine particle radiation on stem cells for induction of chaperone antigen peptide expression

Iodine 125 particle (activity: 0.3-1.0 mCi (1 mCi = 182 Gy); Half life: 60.2 days) was used with emission of X-ray (27.4-31.4 keV) and $\gamma$-radiation (35.5 keV). The maximal radiation radius was 1.7 cm in tissues. I-125 is one low-dosage radioactive isotope with 0.05-0.10 Gy/h and
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Isolation and identification of chaperone antigen peptide complex

Pierce crosslink IP kit was used to separate chaperone antigen peptide complex from lung cancer tumor stem cells (at $3 \times 10^7$ cell count) and normal lung cancer cells (at $3 \times 10^7$ cell count). This test kit utilized cross-linkage and micro-centrifugation for column filtering, thus expanding the usage of classical immunoprecipitation. Major advantages include purification of targeted proteins without antibody contamination, which can elute and separate samples from bead-like agarose resin with high efficiency.

Tumor bearing assay in C57BL/6 mice

A total of 30 female C57BL/6 mice (6 weeks old) were fed by normal diet. Normal lung cancer cells were re-suspended into saline at $1 \times 10^7$ per 200 μl per animal. Tumor cells were inoculated subcutaneously under the pit of forelimb. Tumor formation was observed every 7 days. Tumor size was measured after 21 days for recording.

Chaperone antigen peptide immunity for tumor bearing mice

Mice with subcutaneously tumors were randomly assigned into three groups: saline, normal lung cancer cell chaperone antigen peptide group, and tumor stem cell chaperone antigen peptide group.

0.0013 Gy/min initial dosage. This study thus selected I-125 particles with 1.0 mCi. Particles were placed under culture flask with 0.5 cm interval. Around 30 particles were placed for one culture flask. Using 0.10 Gy/h dosage rate, a 24-h radiation produced about 2 Gy total dosage. Normal cancer cells were used as control and were treated in identical ways.

Figure 1. Lung cancer cell microsphere at different stages after culture.

Figure 2. HSP70 expression in lung cancer cells and lung cancer stem cells. A. Western blotting detection; B. Gray intensity (*, P<0.05, ***, P<0.001).
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Figure 3. HSP90 expression in lung cancer cells and lung cancer stem cells. A. Western blotting detection; B. Gray intensity (***, P<0.001).

Figure 4. Comparison of chaperone antigen peptide in inhibiting tumor growth. (ns, P>0.05, *, P<0.05, ***, P<0.001).

(N = 10 each). 21 days after inoculation, 200 μl saline (control group), 20 μg normal lung cancer cell chaperon antigen peptide complex, or 20 μg lung cancer stem cell chaperone antigen peptide complex was injected under the pit of contralateral side for tumor cell inoculation.

Tumor formation size and survival time

7 days after subcutaneous inoculation of chaperon antigen peptide, tumor size in all groups was recorded, along with mouse survival rates. Blood was collected from mouse eyes, and was collected for serum on ice, followed by -20°C fridge. Mouse spleen tissues were collected and homogenized in PBS. Cell filtration was collected with PBS to collect suspension, which was centrifuged at 2000 g for 3 min. The supernatant was discarded and cells were rinsed twice in PBS. 8 ml leukocyte lysis buffer was added for 5 min incubation until complete rupture. Cells were centrifuged at 2000 g for 3 min to discard the supernatant, and were rinsed twice in PBS followed by resuspension. Red blood cell lysis buffer (8 ml) was added for 5 min incubation to completely rupture blood cells, which were then centrifuged at 2000 g for 3 min to discard the supernatant. Twice more PBS rinsing were performed for 5 min incubation to remove red blood cells. RPMI1640 culture medium containing 10% FBS was used to re-suspend spleen cells.

Lymphocyte proliferation assay

Cell counting kit-8 (CCK-8) was adopted. In the presence of electron chelating reagent, WST-8 can be reduced by mitochondrial dehydrogenase to produce formazan with orange color, whose depth is positively correlated with cell proliferation level. For the same cell type, darkness of color is linearly correlated with viable cell number.

Statistical analysis

SPSS18.0 software was used for data analysis. Measurement data were presented as mean ± standard deviation (SD) and were compared by student t-test. A statistical significance was defined when P<0.05.
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**Results**

**Preparation of lung cancer cell microsphere**

Microsphere culture and separation method was used to successfully separate lung cancer cell microsphere, which was shown in Figure 1 at different stages. Cultured lung cancer microsphere was sorted in flow cytometry to obtain CD133+ cells at 81.9%, which were separated as lung cancer stem cells for further experiments.

**Induced expression of chaperone antigen peptide complex in lung cancer cells and cancer stem cells**

Khosam oil milk and radioactive iodine-125 particle were sequentially used to process and induced the expression of chaperone antigen peptide complex from lung cancer cells and cancer stem cells. By cross-linking immune co-precipitation, HSP70 and HSP90 antigen peptide complex were separated and purified, with Western blotting for quantification of expression (Figures 2, 3). Results showed that Khosam oil milk coupled with radioactive iodine-125 particle induced synthesis of chaperone antigen peptide from lung cancer cells and cancer stem cells, indicating that lung cancer stem cells expressed more chaperone antigen peptide compared to lung cancer cells.

**Chaperon antigen peptide inhibited tumor growth and elongated tumor bearing mouse survival period**

We further used purified chaperone antigen peptide for
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Chaperone antigen peptide enhanced body immune defense level

We further tested immune index of mice after using chaperone antigen peptide to study its tumor suppressor mechanism. Results showed that chaperone antigen peptide could facilitate proliferation of spleen lymphocytes, with more potent effects in lung cancer stem cell group (Figure 6). Moreover, after treating with chaperone antigen peptide complex, mouse serum level of IFN-gamma and IL-2 were all elevated, with more potent change in lung cancer stem cell group than lung cancer cell group (Figures 7-10). These results suggested that chaperone molecule indirectly facilitated generation of IFN-gamma and IL-2 production in tumor cell-lymphocyte co-culture system via potentiating spleen lymphocyte proliferation, thus exerting tumor growth inhibitory roles.

Discussion

Recent studies revealed that various antigen peptides including their precursor proteins that are produced by tumor vaccine antigen-bearing tumor cell mutations can bind with chaperone molecules to generate chaperone antigen complex, which is processed by antigen presenting cells to induce anti-tumor immune response [11-14]. Clinicians thus tried immune therapy using antigen peptide containing chaperone antigen peptide complex from tumor cells, and found potent immune response under trace amounts of such complex [15]. Chaperone molecule tumor vaccine thus has become one focus and has promising future of clinical usage [15, 16]. Comparing with single chaperone tumor vaccine, those tumor cell lysate containing multiple chaperone antigen peptide complex has more potent
tumor bearing assay on C57BL/6 mice. Results showed that no significant difference of tumor volume before treatment. After using chaperone antigen peptide, however, normal lung cancer cell and stem cell group had significantly decreased tumor volume compared to control group, especially in stem cell group, strongly supporting that chaperone antigen peptide inhibited tumor growth (Figure 4). Survival analysis showed that after using chaperone antigen peptide, survival rate of both normal cancer cell group and cancer stem cell group were elevated compared to control group, proving that chaperone antigen peptide could retard the death of tumor bearing mice (Figure 5).
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showed that immune processing by lung cancer stem cell-originated chaperone antigen peptide enriching vaccine significantly potentiated proliferation rate of spleen cells, most of which are lymphocytes, containing 60% B cells and 40% T lymphocytes. Further collection of supernatant of spleen cell-lung cancer cell co-culture system and mouse serum, followed by ELISA assay showed that lung cancer stem cell-originated chaperone antigen peptide enriching vaccine strongly stimulated the production of cytokines such as IL-2 and IFN-gamma [19-21], thus facilitating proliferation and activation of T lymphocytes inside the body and inducing differentiation of Th0 toward Th1 cell sub-population, causing strong anti-tumor immune response, thus significantly elongated survival period of tumor bearing mice and decreased tumor volume.

Conclusion

This study has promising in clinical application. Via further investigation of mechanism of lung cancer stem cell-originated chaperone antigen peptide enriching vaccine, one can provide more evidences and references for application of anti-tumor vaccine in treating late to terminal stage of tumors.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Changxin Huang, Department of Oncology, The Affiliated Hospital of Hangzhou Normal University, No. 126 of Wenzhou Road, Hangzhou, Zhejiang, China. Tel: +86-0571-88303665; Fax: +86-0571-88303665; E-mail: changxinhuangyh@163.com

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