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Preparation of an Orthotopic, Syngeneic Model of Lung Adenocarcinoma and the Testing of the Antitumor Efficacy of Poly(2-oxazoline) Formulation of Chemo-and Immunotherapeutic Agents

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[Abstract] Tumor xenograft models developed by transplanting human tissues or cells into immune-deficient mice are widely used to study human cancer response to drug candidates. However, immune-deficient mice are unfit for investigating the effect of immunotherapeutic agents on the host immune response to cancer (Morgan, 2012). Here, we describe the preparation of an orthotopic, syngeneic model of lung adenocarcinoma (LUAD), a subtype of non-small cell lung cancer (NSCLC), to study the antitumor effect of chemo and immunotherapeutic agents in an immune-competent animal. The tumor model is developed by implanting 344SQ LUAD cells derived from the metastases of *Kras*^{G12D}; *p53*$^{R172H\Delta G}$ genetically engineered mouse model into the left lung of a syngeneic host (Sv/129). The 344SQ LUAD model offers several advantages over other models: 1) The immune-competent host allows for the assessment of the biologic effects of immune-modulating agents; 2) The pathophysiological features of the human disease are preserved due to the orthotopic approach; 3) Predisposition of the tumor to metastasize facilitates the study of therapeutic effects on primary tumor as well as the metastases (Chen *et al.*, 2014). Furthermore, we also describe a treatment strategy based on Poly(2-oxazoline) micelles that has been shown to be effective in this difficult-to-treat tumor model (Vinod *et al.*, 2020b).

Keywords: Xenograft, Immune-competent, Immune-deficient, Orthotopic, Syngeneic

[Background] NSCLC has a poor prognosis because most patients have advanced stage of cancer at the time of diagnosis, and patients with early-stage tumors are very likely to encounter post-surgical metastasis and recurrence (Zappa and Mousa, 2016; Renaud *et al.*, 2016). Transplanted tumors grown subcutaneously in immune-deficient nude mice do not faithfully recapitulate the metastatic disease, and therefore, better models are needed (Manzotti *et al.*, 1993). The 344SQ LUAD cell line forms

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spontaneous metastasis due to the suppression of the microRNA-200 (miR-200) expression, resulting in an epithelial-mesenchymal transition (EMT) phenotype having increased motility (Chen *et al.*, 2014). Moreover, with a considerably low number of tumor-infiltrating cytotoxic T lymphocytes, the Kras/p53 mutant LUAD model exhibits an 'immunologically cold' phenotype. The low number of anticancer lymphocytes renders it less receptive to treatments like anti-PD1 that depends on pre-existing T cells, making it an ideal model to test alternative strategies for the treatment of “immunologically cold tumors” (Pfirschke *et al.*, 2016; Espinosa *et al.*, 2017).

Platinum-based chemotherapy is a standard of care in NSCLC. However, acquired resistance to platinum drugs presents a serious challenge in NSCLC management (Galluzzi *et al.*, 2012). Here, we describe a procedure to adopt the Poly(2-oxazoline) (POx)-based nanomicelle formulation strategy for the coadministration of agents that reverse drug resistance (a.k.a. chemosensitizers) with platinum drugs and assess their efficacy in the LUAD model of NSCLC. Further, we describe an immunotherapeutic approach for treating LUAD by using POx micelle formulation of a small molecule biologic response modifier, Resiquimod (administered alone or in combination with checkpoint blockade therapy). POx micelle formulation of poorly soluble drugs has been previously demonstrated to be safe and effective in various tumor models (He *et al.*, 2016). POx micelles are easy to prepare (Vinod *et al.*, 2020a) and can be used to solubilize a broad range of poorly soluble compounds for drug delivery applications.

Materials and Reagents

1. Alcohol swab (B.D. Biosciences)
2. Nair hair removal cream
3. Cotton swab
4. Sterile gauze pads
5. Tuberculin syringe (B.D. Biosciences)
6. 344SQ-green fluorescent protein/Firefly luciferase (GFP/fLuc) cells (source: J. Kurie, MD Anderson Cancer Center)
7. Hanks' Balanced Salt solution (Gibco)
8. Matrigel (Corning)
9. D-luciferin (PerkinElmer)
10. 1× DPBS (Gibco)
11. Isofluorane (VetOne)
12. Ketamine (Vedco)
13. Xylazine (Acorn animal health)
14. Acepromazine (Vedco)
15. Anesthetic cocktail (see Recipes)

Note: Refer to Vinod et al. (2020a) (protocol based exclusively on Poly(2-oxazoline) preparation for reagents for Sections C and D.

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Equipment

1. Surgical instruments
   a. Autoclip Wound Closing System – Staples + Applier (Braintree Scientific, Inc)
   b. Iris Surgical Scissors, 4½ inch, curved (Fisher Scientific)
   c. Delicate Specialty Dissection Forceps, Serrated, 5" (Fisher Scientific)
2. IVIS-Lumina II optical imaging system (PerkinElmer Inc., Hopkinton, MA)
3. Small Animal induction chamber

Software

1. Living Image® software

Procedure

A. Preparation of animal tumor model of NSCLC
   1. Prepare a suspension of 344SQ-green fluorescent protein (GFP)/fLuc cells in 50% Hanks' Balanced Salt Solution and 50% Matrigel (v/v).
   2. Anesthetize the mice by injecting the anesthetizing cocktail intraperitoneally and lay it in the right lateral decubitus position.
   3. Apply hair removal cream using cotton swab in the chest area and leave for 1 min (be sure not to exceed 1 min to avoid giving the mice a chemical burn). Wipe away with dry sterile gauze pad. Use damp sterile gauze pad to remove any residual cream.
   4. Clean the skin surface with an alcohol swab and make an incision between ribs 10 and 11 by placing the blade parallel to the rib cage (**see Figure 1A**).
   5. Transfer the cell suspension to a 1-ml tuberculin syringe and inject 50 µl of 2.5×10^3-5×10^3 cells into the left lung parenchyma in the lateral dorsal axillary line (**see Figure 1B**).
   6. Close the incision using wound closure clips and place the animal in the left lateral decubitus position until recovery.
      Note: Day 1 is defined as the day of tumor inoculation.

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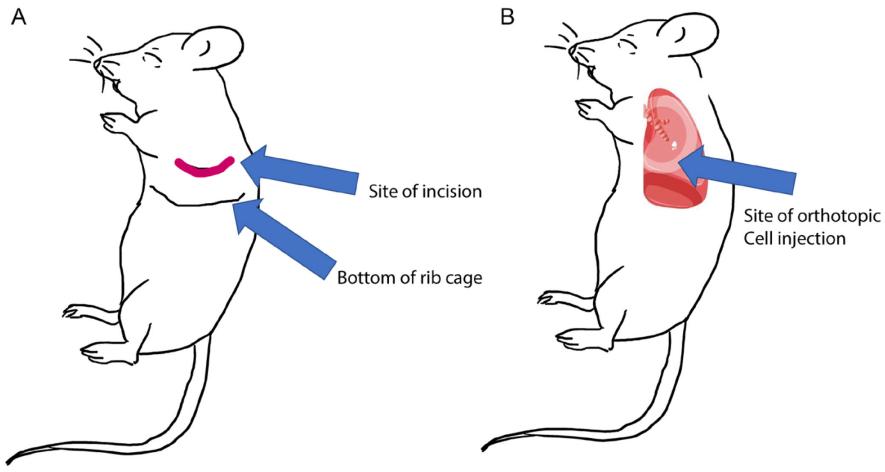

Figure 1. Schematic showing the site of incision (A) and the site of orthotopic injection (B)

B. Bioluminescence imaging

1. Inject 150 mg/kg of 15 mg/ml (in 1× DPBS) D-luciferin solution intraperitoneally and start imaging after 15 min.
2. Anesthetize the mice by placing them in an inhalation induction chamber (for ~5 min) comprising 2-3% isoflurane in oxygen.
3. Place the anesthetized animals in the sample stage of the imaging chamber of the IVIS lumina optical imaging system in the supine position.
4. Acquire luminescent images using the following acquisition settings in the Living Image® software:
   a. Pixel Width: 1
   b. Pixel Height: 1
   c. Image units: counts
   d. Luminescent exposure (seconds): 60
   e. Field of view: 24
   f. Emission filter: open
   g. Subject size: 1.5

   Note: Bioluminescence imaging (B.L.I.) is done once prior to the commencement of the treatments to obtain baseline bioluminescence to confirm the presence of the tumor. Following the start of treatments (Day 8), B.L.I. is performed once every week to monitor tumor growth.

C. Chemotherapy in conjunction with chemosensitizers

1. Randomize mice into four groups of 10 mice each
2. Administer a total of 4 doses (on days 8, 12, 15, and 19) of either of the formulations intravenously (unless indicated otherwise):
   a. Normal saline (volume: 100-200 µl).

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b. C_6CP/AZD7762 PM (POx micelles; coloaded with C_6CP and AZD7762 at a weight ratio of 10/20, yielding a final dose of 10 mg/kg of C_6CP and 20 mg/kg of AZD7762).
c. C_6CP/VE-822 PM (POx micelles; coloaded with C_6CP and VE-822 at a weight ratio of 10/10, yielding a final dose of 10 mg/kg of C_6CP and 10 mg/kg of VE-822).
d. Anti-PD-1 antibody (250 µg per mouse; administered intraperitoneally).

D. Immunotherapy alone and in combination with immune checkpoint blockade

1. Randomize mice into four groups of 13 mice each.
2. Administer a total of 4 doses (on days 8, 12, 15, and 19) of either of the formulations intravenously (unless indicated otherwise):
   a. Normal saline (volume: 100-200 µl).
   b. Resiquimod PM (5 mg/kg).
   c. Anti-PD-1 antibody (250 µg per mouse; administered intraperitoneally and continued after day 17 for four more times – days 22, 26, 29, and 33).
      Note: Since the mice in this group reached the study endpoint before day 33, they did not receive the fourth dose.
   d. Resiquimod PM (5 mg/kg) + anti-PD-1 (250 µg per mouse; administered intraperitoneally and continued after day 17 for four more times – days 22, 26, 29, and 33).

Data analysis

Analyze the B.L.I. data by specifying a region of interest outlining the tumor and quantify the total radiance using Living Image Software to measure the luciferase activity, which is representative of the tumor burden. Refer to Vinod *et al.* (2020b) for representative BLI images.

Recipes

1. Anesthetic cocktail
   To prepare the anesthetic cocktail, dilute stock solutions of Ketamine, Xylazine and Acepromazine in 1× DPBS and administer 100 µl of the cocktail per 10 g of mouse body weight. Use the following doses of each anesthetic:
   Ketamine: 80 mg/kg (stock conc. 100 mg/ml)
   Xylazine: 8 mg/kg (stock conc. 100 mg/ml)
   Acepromazine: 1 mg/kg (stock conc. 10 mg/ml)

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Excellence).

Competing interests

A.V.K. is co-inventor on patents pertinent to the subject matter of the present contribution and A.V.K. and M.S.P. have co-founders' interest in DelAqua Pharmaceuticals Inc. having intent of commercial development of POx based drug formulations. The other authors have no competing interests to report.

Ethics

The protocol (IACUC# 18-174) was approved by The University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. The validity period of IACUC# 18-174 is of 3 years.

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