CHAPTER

Urethane-induced lung carcinogenesis

3

Francesca Sozio[a,b,†], Tiziana Schioppa[a,b,†], Silvano Sozzani[c,*], and Annalisa Del Prete[a,b]

[a]*Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy*

[b]*Humanitas Clinical and Research Center-IRCCS, Rozzano-Milano, Italy*

[c]*Laboratory Affiliated to Istituto Pasteur Italia-Fondazione Cenci Bolognetti, Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy*

**Corresponding author: e-mail address: silvano.sozzani@uniroma1.it*

Chapter outline

1 Introduction....................46
2 Urethane preparation and mice treatment....................48
2.1 Preparation of urethane solution (Note 1)....................49
2.2 Injection of mice....................49
3 Tumor lung evaluation....................49
3.1 Lung harvesting and processing....................49
3.2 Preparation of formalin-fixed, paraffin-embedded tissue sections....................50
3.3 Hematoxylin and eosin (HE) staining on lung sections....................52
3.4 Microscopic evaluation of lung nodules....................53
3.5 Lung processing for flow cytometry acquisition and analysis....................53
4 Conclusions....................56
Acknowledgments....................56
References....................56

Abstract

Chemical induced carcinogenesis together with genetically engineered mouse models represent important approaches for the study of the complex mechanisms involving genotype and environmental factors in cancer development, including lung cancer. The induction of lung

[†]These authors have contributed equally to this work.

Methods in Cell Biology, Volume 163, ISSN 0091-679X, https://doi.org/10.1016/bs.mcb.2020.09.005
Copyright © 2021 Elsevier Inc. All rights reserved.

tumor in mice with urethane (ethyl carbamate) is considered a valuable model of *Kras*-driven lung cancer. However, inbred mouse strains show variable susceptibility to lung tumor formation, with C57BL/6 background, widely used to study many transgenic and null mutations, highly resistant to lung carcinogenesis. Here is described a protocol of urethane-induced lung cancer effective in lung tumor induction in C57BL/6J strain. Multiple urethane injections are needed to overcome genetic resistance and induce in a reproducible manner lung carcinogenesis in C57BL/6J background mice.

1 Introduction

Lung cancer is the leading cause of cancer death worldwide (Siegel, Miller, & Jemal, 2020). Two main types characterize lung cancers, the non-small cell lung cancer (NSCLC) which represents about 85% of all lung cancers and the small cell lung cancer (SCLC) (Siegel et al., 2020). Lung adenocarcinoma and squamous cell carcinoma are the most common NSCLC histological subsets (Travis, Brambilla, & Riely, 2013). Mice can develop pulmonary adenocarcinoma with histological and genetic characteristics that recapitulate human adenocarcinomas in response to chemical carcinogens or genetic induction with oncogenes such as *Kras* (Dwyer-Nield et al., 2010; Malkinson, 2001; McFadden et al., 2016). Chemically induced carcinogenesis in mice has proven to be very helpful in the study of the mechanisms underlying tumor formation. These experimental models recapitulate both genetic and environmental features responsible for cancer susceptibility in human population (Kemp, 2015). Urethane (Ethyl Carbamate, EC) was originally reported to induce lung adenomas formation in mice, but it was also shown to initiate tumors in different tissues, including skin, liver, mammary gland and lymphoid organs (Forkert, 2010). Lung is an EC particularly susceptible organ showing tumor formation in 2–6 months, while tumor latency in other tissues was longer (around 1 year) (Forkert, 2010). EC was recognized as carcinogenic in different species and it was classified as a "probable human carcinogen" (Group 2A; IARC Monograph 2010) by the International Agency for Research on Cancer (IARC).

EC is an ethyl ester of carbamic acid and generates spontaneously during fermentation process by the reaction of urea with alcohol (Forkert, 2010). It is widely present in fermented food products and alcoholic beverages and it is also found as a natural compound of tobacco (Forkert, 2010; Gowd, Su, Karlovsky, & Chen, 2018). Metabolism of EC in rodents showed that approximately 90% of EC is hydrolyzed to ethanol, ammonia and carbon dioxide by liver microsomal esterases (carboxylesterases), with only 5% of EC excreted as unchanged (Fig. 1). Very small amounts of EC are transformed by cytochrome P450 enzyme CYP2E1 to vinyl carbamate, resulting in epoxide formation (vinyl carbamate epoxide), which covalently binds to DNA and can result in base misincorporation during DNA replication, leading to point mutations (Forkert, 2010; Gowd et al., 2018).

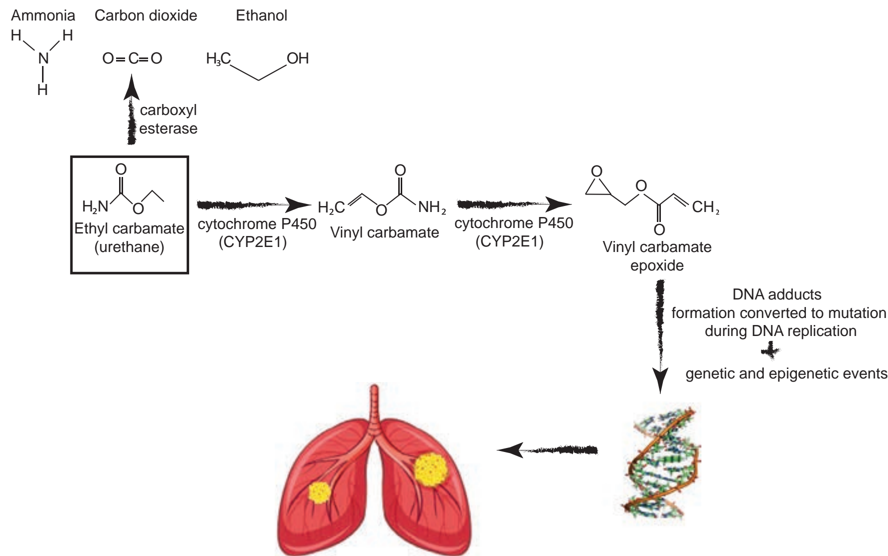

FIG. 1

Urethane metabolism and carcinogenic action. Vinyl carbamate epoxide formed by CYP2E1 activity, covalently binds to DNA leading to the formation of DNA adducts. Genetic mutations and epigenetic events contribute to lung cancer development and progression.

EC-induced carcinogenesis is involved in the development of both benign and malignant tumors, mainly lung tumors, and is considered a valuable experimental model of human adenocarcinoma (Dwyer-Nield et al., 2010; Malkinson, 2001; Narayan & Kumar, 2012). Exposure of mouse to EC primarily induces tumors in lung with a single driver mutation in *Kras* gene at position Q61 with one substitution L or R according to mouse strain (Q61L more frequent in A/J mouse strain, Q61R in the C57BL/6 strain, B6) (Dwyer-Nield et al., 2010; Forkert, 2010; Nuzum, Malkinson, & Beer, 1990; Westcott et al., 2015). Even if the activation of an oncogenic *Kras* allele in mice was shown to be tumorigenic in multiple organs, such as liver and pancreas, pulmonary lesions are the primary tumors arising in mice after intraperitoneal injection of urethane. The specific lung tropism seems to be due to differences in mutagenesis between tissues, rather than to differential carcinogen exposure (Li, MacAlpine, & Counter, 2020). Indeed, the higher transcription levels of *Kras* in the lung, compared to other mouse organs, are responsible for the increased susceptibility of this gene to mutagenesis (Li et al., 2020).

Inbred mouse strains show different susceptibility to urethane-induced lung tumor formation. A/J mice are a particularly susceptible strain, which develops also spontaneous lung tumors during lifespan. On the contrary, C57BL/6 mice are resistant to both carcinogen-induced and spontaneous lung tumor formation because of a

polymorphism in an intron within the *Kras* protooncogene (Dwyer-Nield et al., 2010; Lin et al., 1998). In addition, the enzymatic bioactivation of EC and vinyl carbamate in vinyl carbamate epoxide is responsible for mediating DNA alkylation and the formation of nucleic acid adducts (Forkert, 2010). A strain-related capacity of vinyl carbamate bioactivation has been described (Titis & Forkert, 2001). Accordingly, significantly higher levels of DNA adduct formation are found in A/J mice compared to C57BL/6J due to the greater capacities of vinyl carbamate epoxide bioactivation in A/J mice. The lower capacity for vinyl carbamate oxidation observed in the lung of C57BL/6 mice is associated with increased resistance to lung tumorigenesis (Forkert, 2010).

Urethane induces bronco-alveolar adenomas and less frequently adenocarcinomas with histological characteristics of NSCLC, depending on genetic background. The sensitive A/J strain develops multiple adenomas after a single intraperitoneal urethane injection that become visible by 25 weeks; the appearance of adenocarcinomas is observed by 40 weeks. A longer latency period is needed to see very few lung tumors in less sensitive strains, such as C57BL/6 mice (Gurley, Moser, & Kemp, 2015; Miller et al., 2003). Since mice with B6 background are commonly used to generate transgenic and null mutations of potential interest for lung carcinogenesis, a protocol for EC-induced lung carcinogenesis in this resistant strain was developed (Miller et al., 2003). To increase the incidence of lung tumors in C57BL/6 mice, 10 weekly intraperitoneal administration of EC were used. This modification causes nearly 100% of lung tumor incidence with a tumor multiplicity >2 (Del Prete et al., 2019; Miller et al., 2003).

Here we describe the multiple EC dose-based protocol for EC-induced lung tumorigenesis in C57BL/6J mice. This protocol is easy to manage, relatively safe to handle and low cost (Del Prete et al., 2019). A detailed description of procedures to handle urethane, to treat mice, to harvest and process lungs for histological and flow cytometric analysis is also discussed.

2 Urethane preparation and mice treatment

Materials

- C57BL/6J mice (21/28-day-old)
- Phosphate buffered saline (PBS 1×, w/o calcium and magnesium)
- Urethane (Ethyl Carbamate, EC, Sigma-Aldrich U2500)
- Chemical fume hood
- Freezer set to −20°C
- Insulin syringe with 28-gauge needle
- Microcentrifuge tubes (1.5 mL)
- Electronic scale

Methods

2.1 Preparation of urethane solution (Note 1)

1. In a chemical fume hood, dissolve urethane in PBS 1 × at 100 mg/mL (Note 2).
2. Make aliquots of EC solution in 1.5 mL microcentrifuge tubes and stock them at −20 °C for no longer than 6 months.

2.2 Injection of mice

1. Mark animals with earmarking and ensure sufficient group size (Note 3).
2. Weigh the animals.
3. Calculate the amount of urethane needed (1 mg urethane per gram of body weight) and thaw as many aliquots of stock solutions as necessary.
4. Inject each mouse intraperitoneally with 1 mg of urethane per gram of body weight: for example, inject 10 g mouse with 10 mg of urethane (100 μL) (Note 4).
5. Sacrifice mice after 30 weeks from the first urethane injection.

Note 1: It is important that you consult the appropriate Material Safety Data Sheets and your Institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.
Note 2: Urethane has been classified as a group A carcinogen by the International Agency for Research on Cancer (IARC) and can be carcinogenic to humans (Hazard statement H302, H350). Plan with your institution and your animal facility the best practice to safety handle urethane and to properly dispose waste.
Note 3: All mice are maintained on hardwood bedding on a 12 h light/dark cycle and given antioxidant-free lab diet chow (Teklad Global 19% Protein extruded rodent diet, ENVIGO) and water ad libitum (Chen et al., 2017; Miller et al., 2003). Ten weekly EC doses cause in C57BL/6J mice nearly 100% lung tumor incidence but low tumor multiplicity. To achieve statistical significance, large (such as 7–10 mice) groups of animals are required (Del Prete et al., 2019; Miller et al., 2003).
Note 4: Because urethane is also an anesthetic, mice will lie torpid after injection. Monitor the mice according to Institutional Animal Care and Use Committee (IACUC) animal facilities rules and regulations.

3 Tumor lung evaluation

3.1 Lung harvesting and processing

Materials

- Scissors
- Tweezers
- Saline solution
- 5 mL syringe
- Ice

- Laminar flow hood
- Personal protective equipment

Methods

1. Sacrifice mice.
2. Spray down mouse with 70% ethanol.
3. Expose the thoracic cavity and abdomen by cutting and gently pulling back the outer skin of the peritoneum. Open the abdomen with forceps, move gently down the liver to cut the inferior cava (mouse will start bleeding). Proceed to open the diaphragm by cutting the rib cage to expose both heart and lungs.
4. Perfuse gently the lungs using a 5 mL syringe filled with cold saline solution. Hold the heart with tweezers, insert the syringe into the right ventricle and gently start the perfusion. Accurate perfusion will promote lung inflation and color change to pink/white.
5. Dissect out the lungs by gently tugging on the trachea while snipping away the connective tissue; leave lungs intact.
6. Place lungs in a 15 mL tube filled with cold saline solution and keep on ice (left lobes will be used for histology; right lobes will be processed for flow cytometry analysis).

3.2 Preparation of formalin-fixed, paraffin-embedded tissue sections

Materials

- Formalin (4% solution in PBS)
- Tissue cassette
- Tissue processor
- Heated paraffin embedding station
- Cold plate
- Paraffin wax
- Molds
- Microtome
- Water bath at 38 °C
- Thin tweezers or brush
- Histology glass slides
- Laboratory drying oven
- Ethanol
- Xylene

Methods

1. Place left lung lobes in an embedding cassette, keep the tissue cassette in a container with 4% formalin solution for 24 h at room temperature in a chemical hood (Note 1).

2. Place the embedding cassettes in the Tissue Processor where samples will be processed as follow (Note 2):
   - EtOH (50%) 1 h at room temperature
   - EtOH (70%) 1 h at room temperature
   - EtOH (95%) 1 h at room temperature
   - EtOH (95%) 1 h at room temperature
   - EtOH (100%) 1 h at room temperature
   - EtOH (100%) 1 h at room temperature
   - EtOH (100%) 1.5 h at room temperature
   - Xilene 1 h at room temperature
   - Xilene 1 h at room temperature
   - Xilene 1 h at 40 °C
   - Paraffin wax 1 h at 60 °C
   - Paraffin wax 1 h at 60 °C
   - Paraffin wax 1 h at 60 °C
3. Place samples in histology mold, ventral side down (Fig. 2) and surround it with additional wax to form a block (Note 3). Samples will be attached to the embedding cassettes for sectioning.
4. Make sections of the paraffin-embedded tissue block cutting five longitudinal serial sections (150 μm apart, 2–4 μm thickness) by a microtome and float them in a 38 °C water bath containing distilled water.
5. Transfer the sections onto glass slides (if you are planning to do immunohistochemistry transfer some sections on Superfrost Plus). Allow the slides to dry overnight (use a laboratory drying oven at 37 °C) and store slides at room temperature until ready for use.

   Note 1: Formalin is a suspected carcinogen. It can cause eye, skin, and respiratory tract irritation (Hazard statement H317, H350, H341, H302). It should be handled in a chemical hood.

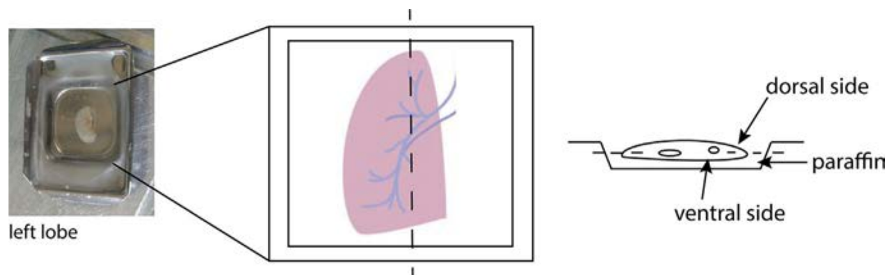

FIG. 2

Mouse lung embedded in paraffin. Ventral side down, adherent to the base of the mold. Ventral lung represents the block face.

Note 2: If you do not have a Tissue Processor, you can manually do all the passages described at point 2.
Note 3: Pay attention when you place the sample in the mold: the lateral face has to be adherent to the base of the mold to allow longitudinal cuts.

3.3 Hematoxylin and eosin (HE) staining on lung sections

Materials

- Xylene
- Ethanol (100%, 90%, 70%)
- Hematoxylin
- Eosin
- Distilled water
- Eukitt mounting medium (Sigma-Aldrich)
- Cover slides
- Optical microscope

Methods

1. Place slides containing paraffin sections in a slide holder (glass or metal).
2. Deparaffinize and rehydrate sections:
   $2 \times 5'$ Xylene
   $2 \times 1'$ 100% ethanol
   $1 \times 1'$ 95% ethanol
   $1 \times 1'$ 70% ethanol
   $1 \times 1'$ distilled water
3. Blot excess water from slide holder before going into hematoxylin.
4. $15'$ hematoxylin staining.
5. $10'$ in tap water (*to allow stain to develop*).
6. $7'$ eosin staining.
7. Wash slides 10 times in tap water.
8. $1 \times 1'$ distilled water.
9. Dehydrate and clarify sections:
   $1 \times 1'$ 70% ethanol
   $1 \times 1'$ 90% ethanol
   $2 \times 1'$ 100% ethanol (*blot excess of ethanol before going into xylene*)
   $3 \times 5'$ Xylene
10. Mount slides with Eukitt mounting medium: place a drop of mounting medium on top of the slide, taking care to avoid bubbles.
11. Angle the coverslip and let it fall gently onto the slide. Allow the Eukitt mounting medium to spread beneath the coverslip, covering all tissue.
12. Dry overnight in the hood.
13. Look the slides under optical microscopy.

3.4 Microscopic evaluation of lung nodules

Materials

- Slide scanner (VS120 DotSlide BX61 virtual slide microscope, Olympus Optical).
- Image analysis software (image-Pro-Premiere software, Media Cybernetics).

Methods

1. Using the slide scanner, scan all the slides (five serial sections/each lung) (Fig. 3).
2. Using an image analysis software manually trace the perimeter of lesions to obtain number and area of nodules.

Notes

- If you haven't a slide scanner, you can count lung nodules under an optical microscope.
- If you have a camera connected to the microscope you can take a picture and use an image analysis software to measure tumor area.

3.5 Lung processing for flow cytometry acquisition and analysis

Materials

- Scissors
- Tweezers
- Petri dishes
- Six-well plates
- 90-well U bottom plates
- 50 mL plastic tubes

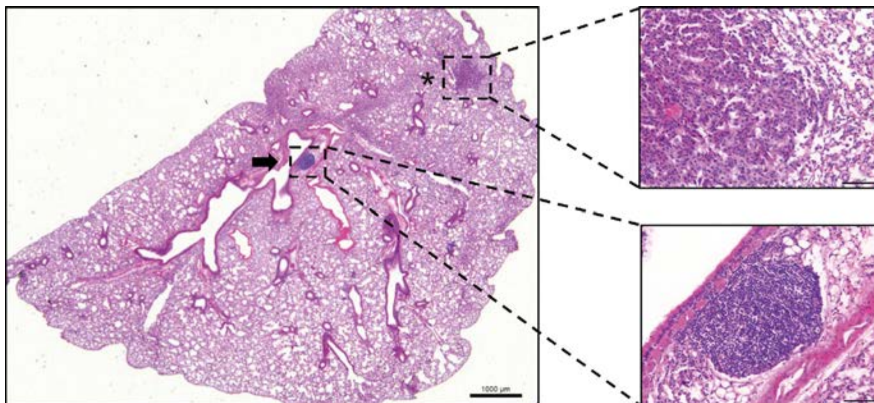

FIG. 3

Mouse left lung (HE staining, 12.5 ×—scale bar = 1000 μm). A representative image of a mouse left lung. (*) indicates an adenoma and (black arrow) a peribronchiolar/perivascular lymphoid aggregate.

- 70 µm cell strainers
- 0.2 µm strainers
- Plastic Pasteur pipettes
- Insulin syringes
- Ice
- RPMI
- Collagenase D (type IV, Clostridium Histolyticum-Sigma)
- DNase I (from bovine pancreas grade 2, Roche)
- PBS 1 × (w/o calcium and magnesium)
- Fetal Bovine Serum (FBS)
- EDTA (Invitrogen)
- Cell incubator 37 °C 5% CO_2
- ACK Lysing (Lonza)
- Live Dead (LD, Invitrogen)
- FACS (fluorescence-activated cell sorting) instrument

Antibodies

Fc block (anti mouse CD16/32, clone 2.4G2); CD45-VioGreen (clone: REA737); NK1.1-APC (clone: PK136); CD11b-PE-Vio770 (clone: M1/70); Ly6G-FITC (clone 1A8); Ly6C-PE (clone REA796); CD19-VioBlue (clone REA749); F4/80-PercP-Vio770 (clone REA126); CD45RA-APC-Vio770 (clone T6D11); CD3-PE (clone 145-2C11) from Miltenyi Biotec.

Buffer preparation

- EDTA-treated buffer: add 1 mL of 0.1 M EDTA, pH = 7, every 10 mL of filtered FBS (0.2 µm strainer)
- FACS-EDTA buffer: add 5% EDTA-treated FBS to 1 × PBS
- DIGESTIUM medium: add 1 mg/mL Collagenase D (type IV, *Clostridium Histolyticum*-Sigma) and 0.02 mg/mL DNase I (Roche) to RPMI 1% FBS

Methods

1. Cut lungs in small pieces with scissors in a six-well plate and add DIGESTIUM medium (4 mL/lung) (Cossarizza et al., 2019)
2. Incubate at 37 °C in the incubator, for 30′
3. Add 2 mL of RPMI 10% FBS to each sample to block enzymes activity
4. Collect lungs via a 70 µm cell strainer into a new 50 mL tube (use a plastic Pasteur pipette cutting thin parts with scissors): add FACS-EDTA buffer to wash the filter and use a syringe plunger to obtain a single cell suspension
5. Centrifuge at 290 rcf, 4 °C, for 8′ and discard supernatant
6. Resuspend cells in 1 mL ACK lysing buffer in ice for 5′ to lyse red blood cells
7. Add 1 mL of RPMI 10% FBS (to block ACK action) and 2 mL of FACS-EDTA buffer
8. Centrifuge at 290 rcf, 4 °C, for 8′ and discard the supernatant

9. Resuspend cells in 1 mL of FACS-EDTA buffer and filter through 70 μm cell strainer to eliminate possible clumps
10. Count cells at 1:10 dilution
11. Seed cells (1×10^6 per well) in a 96-well U bottom plate
12. Centrifuge at 440 rcf for 3′ and discard the supernatant
13. Add Fc-blocking reagent and incubate at 4 °C for 20′
14. Centrifuge at 440 rcf for 3′ and discard supernatant
15. Prepare the antibodies mix in FACS-EDTA buffer
16. Add the antibodies mix (100 μL/sample) and incubate at 4 °C for 20′ in the dark
17. Centrifuge at 440 rcf for 3′ and discard supernatant
18. Wash with 1 × PBS (w/o calcium and magnesium)
19. Centrifuge at 440 rcf for 3′ and discard supernatant
20. Add LD and incubate at room temperature for 10′ in the dark
21. Centrifuge at 440 rcf for 3′ and discard supernatant
22. Wash with 1 × PBS (w/o calcium and magnesium)
23. Centrifuge at 440 rcf for 3′ and discard supernatant
24. Resuspend in FACS BUFFER
25. Perform FACS acquisition and analysis (Fig. 4)

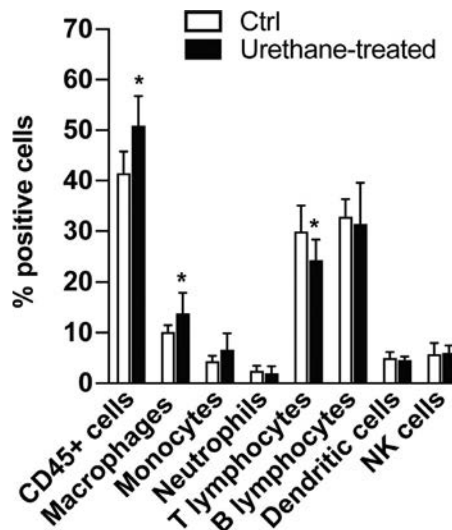

FIG. 4

FACS analysis of urethane-treated lungs. Urethan treatment induced an increase in percentage of CD45 positive cells compared with control (Ctrl) mice (51.07 ± 2.3, $n = 7$ and 41.58 ± 1.7, $n = 6$, respectively; $P = 0.008$ by Student's t-test). Characterization of leukocytes showed an increase of macrophages and reduction of T cells.

4 Conclusions

Urethane-induced lung cancer is an experimental model widely used in different genetically engineered mouse models to identify novel genes involved in lung cancer pathogenesis. The protocol based on 10-weekly urethane doses is effective in inducing lung tumor formation also in the high resistant C57BL/6J background. The incidence of lung tumors is nearly 100%, with a relatively low tumor multiplicity (usually more than 2 nodules/mouse) but in a reproducible manner. Histology of lung nodules shows adenomas and characterization of infiltrated leukocyte can be obtained by flow cytometry.

The high incidence of lung tumors, the little cost-effective and the relatively safety handling procedure make the urethane-induced lung carcinogenesis model a feasible protocol to study transgenes or null mutations also in low susceptible mouse strains.

Acknowledgments

This work was supported by the Italian Association for Cancer Research (AIRC) under IG 2017-ID. 20776 project (S.S.) and PRIN-2017 (S.S.). T.S. was the recipient of a fellowship from Umberto Veronesi Foundation.

References

Chen, T., Lu, L., Xu, C., Lin, X., Leung, Y. K., Ho, S. M., et al. (2017). Inhibition role of atherogenic diet on ethyl carbamate induced lung tumorigenesis in C57BL/6J mice. *Scientific Reports*, *7*(1), 4723. https://doi.org/10.1038/s41598-017-05053-1.

Cossarizza, A., Chang, H. D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., et al. (2019). Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *European Journal of Immunology*, *49*(10), 1457–1973. https://doi.org/10.1002/eji.201970107.

Del Prete, A., Sozio, F., Schioppa, T., Ponzetta, A., Vermi, W., Calza, S., et al. (2019). The atypical receptor CCRL2 is essential for lung cancer immune surveillance. *Cancer Immunology Research*, *7*(11), 1775–1788. https://doi.org/10.1158/2326-6066.CIR-19-0168.

Dwyer-Nield, L. D., McQuillan, J., Hill-Baskin, A., Radcliffe, R. A., You, M., Nadeau, J. H., et al. (2010). Epistatic interactions govern chemically-induced lung tumor susceptibility and Kras mutation site in murine C57BL/6J-ChrA/J chromosome substitution strains. *International Journal of Cancer*, *126*(1), 125–132. https://doi.org/10.1002/ijc.24743.

Forkert, P. G. (2010). Mechanisms of lung tumorigenesis by ethyl carbamate and vinyl carbamate. *Drug Metabolism Reviews*, *42*(2), 355–378. https://doi.org/10.3109/03602531003611915.

Gowd, V., Su, H., Karlovsky, P., & Chen, W. (2018). Ethyl carbamate: An emerging food and environmental toxicant. *Food Chemistry*, *248*, 312–321. https://doi.org/10.1016/j.foodchem.2017.12.072.

Gurley, K. E., Moser, R. D., & Kemp, C. J. (2015). Induction of lung tumors in mice with urethane. *Cold Spring Harbor Protocols, 2015*(9), pdb.prot077446. https://doi.org/10.1101/pdb.prot077446.

Kemp, C. J. (2015). Animal models of chemical carcinogenesis: Driving breakthroughs in cancer research for 100 years. *Cold Spring Harbor Protocols*, *2015*(10), 865–874. https://doi.org/10.1101/pdb.top069906.

Li, S., MacAlpine, D. M., & Counter, C. M. (2020). Capturing the primordial Kras mutation initiating urethane carcinogenesis. *Nature Communications*, *11*(1), 1800. https://doi.org/10.1038/s41467-020-15660-8.

Lin, L., Festing, M. F., Devereux, T. R., Crist, K. A., Christiansen, S. C., Wang, Y., et al. (1998). Additional evidence that the K-ras protooncogene is a candidate for the major mouse pulmonary adenoma susceptibility (Pas-1) gene. *Experimental Lung Research*, *24*(4), 481–497. https://doi.org/10.3109/01902149809087382.

Malkinson, A. M. (2001). Primary lung tumors in mice as an aid for understanding, preventing, and treating human adenocarcinoma of the lung. *Lung Cancer*, *32*(3), 265–279. https://doi.org/10.1016/s0169-5002(00)00232-4.

McFadden, D. G., Politi, K., Bhutkar, A., Chen, F. K., Song, X., Pirun, M., et al. (2016). Mutational landscape of EGFR-, MYC-, and Kras-driven genetically engineered mouse models of lung adenocarcinoma. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(42), E6409–E6417. https://doi.org/10.1073/pnas.1613601113.

Miller, Y. E., Dwyer-Nield, L. D., Keith, R. L., Le, M., Franklin, W. A., & Malkinson, A. M. (2003). Induction of a high incidence of lung tumors in C57BL/6 mice with multiple ethyl carbamate injections. *Cancer Letters*, *198*(2), 139–144. https://doi.org/10.1016/s0304-3835(03)00309-4.

Narayan, C., & Kumar, A. (2012). Constitutive over expression of IL-1beta, IL-6, NF-kappaB, and Stat3 is a potential cause of lung tumorgenesis in urethane (ethyl carbamate) induced Balb/c mice. *Journal of Carcinogenesis*, *11*, 9. https://doi.org/10.4103/1477-3163.98965.

Nuzum, E. O., Malkinson, A. M., & Beer, D. G. (1990). Specific Ki-ras codon 61 mutations may determine the development of urethan-induced mouse lung adenomas or adenocarcinomas. *Molecular Carcinogenesis*, *3*(5), 287–295. https://doi.org/10.1002/mc.2940030509.

Siegel, R. L., Miller, K. D., & Jemal, A. (2020). Cancer statistics, 2020. *CA: A Cancer Journal for Clinicians*, *70*(1), 7–30. https://doi.org/10.3322/caac.21590.

Titis, A. P., & Forkert, P. G. (2001). Strain-related differences in bioactivation of vinyl carbamate and formation of DNA adducts in lungs of A/J, CD-1, and C57BL/6 mice. *Toxicological Sciences*, *59*(1), 82–91. https://doi.org/10.1093/toxsci/59.1.82.

Travis, W. D., Brambilla, E., & Riely, G. J. (2013). New pathologic classification of lung cancer: Relevance for clinical practice and clinical trials. *Journal of Clinical Oncology*, *31*(8), 992–1001. https://doi.org/10.1200/JCO.2012.46.9270.

Westcott, P. M., Halliwill, K. D., To, M. D., Rashid, M., Rust, A. G., Keane, T. M., et al. (2015). The mutational landscapes of genetic and chemical models of Kras-driven lung cancer. *Nature*, *517*(7535), 489–492. https://doi.org/10.1038/nature13898.