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Human Lung Cancer Cells Grown on Acellular Rat Lung Matrix Create Perfusable Tumor Nodules

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Abstract

Background—Extracellular matrix allows lung cancer to form its shape and grow. Recent studies on organ reengineering for orthotopic transplantation have provided a new avenue for isolating purified native matrix to use for growing cells. Whether human lung cancer cells grown in a decellularized rat lung matrix would create perfusable human lung cancer nodules was tested.

Methods—Rat lungs were harvested and native cells were removed using sodium dodecyl sulfate and Triton X-100 in a decellularization chamber to create a decellularized rat lung matrix. Human A549, H460, or H1299 lung cancer cells were placed into the decellularized rat lung matrix and grown in a customized bioreactor with perfusion of oxygenated media for 7 to 14 days.

Results—Decellularized rat lung matrix showed preservation of matrix architecture devoid of all rat cells. All three human lung cancer cell lines grown in the bioreactor developed tumor nodules with intact vasculature. Moreover, the lung cancer cells developed a pattern of growth similar to the original human lung cancer.

Conclusions—Overall, this study shows that human lung cancer cells form perfusable tumor nodules in a customized bioreactor on a decellularized rat lung matrix created by a customized decellularization chamber. The lung cancer cells grown in the matrix had features similar to the original human lung cancer. This ex vivo model can be used potentially to gain a deeper understanding of the biologic processes involved in human lung cancer.

Lung cancer is the leading cause of cancer-related deaths in the United States. In the United States alone, lung cancer was diagnosed in 222,520 patients in 2010, and 157,300 patients died of the disease in the same year [1]. Patients with lung cancer have a poor overall 5-year survival rate. Despite more than 30 years of research to improve the medical and surgical care of patients with lung cancer, the overall 5-year survival rate for patients with lung

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cancer has improved only from 13% in 1975 to 16% in 2005 [1]. Our lack of success may be related to the limitations of in vitro and in vivo studies, which translate poorly into practice because of their lack of concordance with human studies [2].

One possible reason for the lack of concordance is the shortcomings of in vitro systems in modeling the effect of the interaction of the tumor cells with surrounding structures. A Boyden chamber, for example, is a test used to study the invasive properties of a cell [3]. It measures the ability of a cell to go through an artificial barrier, which a tumor cell will never encounter in a native environment. Similarly, while both synthetic matrices and Matrigel three-dimensional models have improved our understanding of some aspects of the interaction of cancer cells with the matrix [4], once again both of the tests use a nonphysiologic matrix, which does not truly mimic human conditions. On the other hand, although in vivo studies provide valuable data, human cancer cells grown in an immunodeficient mouse that has a background of mouse cells and lack of immune cells imposes limitations on interpretation of data derived from these studies. A new model that uses a native matrix may more closely replicate human lung cancer biology and provide a new avenue to understand this complex biology. Thus, we set out to create a new model to study human lung cancer using a native matrix.

Matrix is the structural component of the cell microenvironment. It is composed of collagens, proteoglycans, laminins, and elastin, which are the ground substances that epithelial and mesenchymal, including endothelial, cells need to grow and proliferate [5]. It provides important tumor–stromal interactions and a microenvironment that promotes systematic cell growth in the presence of surrounding growth factors, hormones, and adhesion molecules and regulates feedback mechanisms [6–8].

Recent studies on organ reengineering [9, 10] for orthotopic transplantation have provided a new avenue for isolating natural matrix to use for growing cells in a three-dimensional environment with a preserved extracellular matrix and vasculature system. Analysis of the isolated matrix shows that the composition of the lung matrix is similar among different species [11]. Moreover, Ott and colleagues [9] have shown that lung cell lines, minced lung tissues, and endothelial cells can grow by means of a combined perfusion- and respiration-based system. We hypothesized that human lung cancer cells placed into a decellularized rat lung matrix will grow perfusable tumor nodules. We tested our hypothesis by creating a decellularized rat lung matrix using a customized decellularization chamber and placing human lung cancer cell lines into the matrix and growing them in a customized bioreactor. We found that cell lines formed tumor nodules with intact vasculature.

Material and Methods

All the animal experiments were carried out in accordance with all applicable laws, regulations, guidelines, and policies governing the use of laboratory animals in research. The protocols for animal experiments were approved by the Institutional Animal Care and Use Committee at the Methodist Hospital Research Institute (AUP-0910-0018).

Rat Lung Isolation

We anesthetized 6-week to 12-week-old male Sprague-Dawley rats with ketamine (100 mg/kg) and xylazine (10 mg/kg). After 5 to 10 minutes, once rats were anesthetized, we shaved the chest and abdomen, prepped the skin with povidone-iodine topical antiseptic (Betadine), and performed bilateral thoracotomy to open the thoracic cavity. We injected 2 mL of heparin (1,000 U/mL, Sagent Pharmaceuticals, Schaumburg, IL) into the right ventricle of the beating heart to prevent formation of blood clots in the lung. Next, we removed the rib cage and injected 20 mL of heparinized phosphate-buffered saline (12.5 U/mL; heparinized PBS) in the right ventricle after placing an 18-gauge needle (Cotran, Portsmouth, RI) in the left ventricle as a vent. The superior vena cava and inferior vena cava were cut, and the lungs were flushed again with 20 mL of heparinized PBS. Next, we divided the trachea at the level of the thyroid, the branches of the aorta at the arch, and the descending aorta at the level of the hemiazygos vein. The heart–lung block was then separated away from the esophagus and the rest of the rat body. We performed ventriculotomy to expose the right and left ventricles and placed a custom-made prefilled 18-gauge stainless steel needle (Cotran) through the right ventricle into the main pulmonary artery. This was secured with a 2–0 silk tie (Ethicon, San Angelo, TX). We also placed a female Luer bulkhead (Cole-Parmer, Vernon Hills, IL) in the left ventricle and secured it with a 2–0 silk tie. We flushed the pulmonary artery cannula with heparinized PBS and placed it in a 50-mL tube containing heparinized PBS.

Lung Decellularization

We designed a simple decellularization chamber (Fig 1A) to remove the native rat cells from the lung. The decellularization chamber was created from a 500-mL glass bottle (Fisher Scientific, Inc, Suwanee, GA). We drilled two holes in the cap with a 1/8-inch adapter drill bit, fitted the female Luer bulkhead into the hole, and secured it with a black nylon ring (Cole-Parmer). One of the Luer sides was connected to a small length of flexible plastic tubing (Tygon; Cole-Parmer) touching the bottom surface of the bottle for outflow. This chamber was connected to a 2-foot length of Tygon tube with a male Luer lock (Cole-Parmer), which was then connected by means of a 6-inch Masterflex roller tube (Cole-Parmer) to a female Luer bulkhead going into a beaker to collect the outflow of the bottle. All these items were autoclaved. We then connected the pulmonary artery cannula to the cap of the decellularization chamber. A pierced capped 500-mL bottle with a primary intravenous set (Hospira, Lake Forest, IL) was used to introduce different solutions through the pulmonary artery by means of a cannula at physiologic perfusion pressure (Fig 1A).

Heparinized PBS ran for 15 minutes through the pulmonary artery at a perfusion pressure of 30 mm Hg for the initial wash and then 0.1% sodium dodecyl sulfate (Fisher Scientific) in deionized water was perfused through the lung for 2 hours for decellularization. After decellularization, deionized autoclaved water was perfused through the lung scaffold for 15 minutes, followed by 1% Triton-X-100 (Fisher Scientific) in deionized water for 10 minutes. Next, we attached the tubing that was going to the beaker to the inflow Luer adapter of the bottle containing the hanging lung, and the perfusion system was connected to the Masterflex pump using PharMed BPT tubing (Cole-Parmer), Luerlock connectors, and Tygon tubing to remove the excess sodium dodecyl sulfate with autoclaved PBS containing

antibiotic (100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin; MP Biomedicals, Solon, OH). Lungs were perfused for 72 hours and frozen at -80°C, if not used immediately.

Cell Culture

The human alveolar basal epithelial adenocarcinoma cell line A549 was supplied by Dr Kurie's laboratory (The University of Texas MD Anderson Cancer Center, Houston, TX). Lung cancer cell lines H460 and H1299 were supplied by Dr Haifa Shen's laboratory (The Methodist Hospital Research Institute, Houston, TX). These cell lines were grown in BD T175 cell culture flasks in complete medium made from Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, South Logan, UT) supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD) and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin; MP Biomedicals) at 37°C in 5% CO₂. Once cells were 85% confluent, they were washed with PBS and subjected to trypsinization using 0.25% trypsin (Cellgro, Manassas, VA) to collect the cells from flasks. Cells were washed with medium and finally suspended in 30 to 50 mL of serum-free medium. Approximately 50 million cells were used for seeding the lung matrix.

Bioreactor

A simplified, small, closed-system bioreactor was set up in an incubator for lung cell culture on the lung matrix (Fig 1B). We used a custom-designed 500-mL glass bottle with three holes in the cap fitted with a female Luer thread-style panel (Cole-Parmer), one for the pulmonary artery cannula, one for the trachea cannula, and one for circulation of medium from the bottle. Medium was constantly circulated with the help of a Masterflex pump (Cole-Parmer) through a 10-foot length of silicone oxygenator tubing wrapped around a mesh of wire solenoid (Cole-Parmer). The medium was perfused through the pulmonary artery cannula into lungs at a flow rate of 6 mL/min. For controlled flow through the pulmonary artery, it was connected to a three-way stopcock (Smith Medical, Dublin, OH). The bottle was filled with 150 mL of complete medium or serum-free medium, which was circulated through the oxygenator tubing to prevent air bubbles.

Before seeding the human lung cancer cells into the lung matrix, the trachea was cannulated using an 18-gauge needle, and the scaffold was fixed to the bioreactor bottle in a hanging position; the complete medium was perfused through the lung matrix for 30 minutes at 37°C in 5% CO₂ at a rate of 6 mL/min using a roller pump. Afterward, the 50 million cells suspended in 30 to 50 mL of medium were seeded into the lungs through the tracheal cannula using a sterile syringe fed by gravity. We placed the bioreactor in the incubator for 2 hours to allow for attachment of the cells. After 2 hours we perfused the scaffold at a flow rate of 6 mL/min. The medium in the bottle (approximately 100 to 200 mL) was changed every 1 to 2 days to make sure the nutrients were optimal for cell growth. We grew the cells on the matrix for 7 to 14 days. The lung matrix was then carefully removed from the bioreactor bottle, maintaining sterile conditions, and a lobectomy was performed under the culture hood by tying the anatomic lobe with 2-0 silk and resecting it on different days. The experiments were repeated at least three times.

DNA Extraction

DNA was extracted from three native rat lungs and three decellularized rat lungs using Qiagen DNeasy DNA isolation kit (Qiagen, Valencia, CA). Equal amounts of tissues (20 mg) were taken, minced into small pieces with a surgical blade, and digested overnight with proteinase K in AL buffer provided with the kit. After complete digestion, AL buffer and 100% ethanol were added and the mixtures loaded on columns. The mixtures were subjected to centrifugation, washed per the manufacturer's instructions, and finally eluted in 100 μ L of elution buffer. DNA concentration was quantified by using a Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Histology

Lobes of same lungs were dissected at day 0 (day of seeding cancer cells onto matrix), day 3, day 7, and day 11 or day 14 (or both) in sterile conditions under the culture hood to see the progression of tumor growth. After lobectomy, lung tissues were placed in 70% ethanol and analyzed in the Pathology Core Laboratory at The Methodist Hospital Research Institute. Briefly, the tissues were fixed in 10% formalin overnight, processed, and embedded in paraffin. Embedded tissues were cut into 4- μ m slices and mounted on slides, and the paraffin was removed; antigen retrieval was performed with antigen-unmasking solution (H-3300; Vector Laboratories, Burlingame, CA) in a steamer for 20 minutes. Slides were cooled for 20 minutes at room temperature, washed in PBS, and stained with hematoxylin and eosin, Movat Pentachrome (American MasterTech Scientific, Lodi, CA), elastin (VVG kit, Ventana Nexus, Tucson, AZ), and other markers following the standard protocol [12]. Stained slides were examined by expert board-certified pathologists, and images were captured using a microscope (Olympus, Center Valley, PA).

Results

The native heart–lung block was harvested from adult rats (Fig 2A). On perfusion with heparinized PBS through a cannulated pulmonary artery, flow exited the left ventricle without leakage, suggesting preservation of an intact vasculature. Hematoxylin and eosin staining of the native lung exhibited normally cellularized alveoli with pneumocytes and endothelial cells in the interstitial vessels (Fig 2B). Using the custom-made decellularization chamber, we were able to remove most cells in the rat lung (Fig 2C). Hematoxylin and eosin (Fig 2D) and Movat Pentachrome staining showed no rat cells present in the scaffold. Movat Pentachrome and elastin staining showed the presence of preserved matrix composed of collagen, elastin, and proteoglycans as well as an elastic fiber network of septal, axial, and pleural fibers of the airway and alveoli remaining intact. The DNA concentration of the decellularized lung was reduced to less than 5% of that of native lung (Fig 3).

All three human lung cancer cell lines (A549, H1299, and H460) engrafted onto the rat matrix in the custommade bioreactor and created perfusable tumor nodules. A549 cells grown on the scaffold produced no nodules on day 3, but by day 11 (Fig 4A) solid tumor nodules had formed. Hematoxylin and eosin staining on day 3 showed cells attaching to the matrix in airways, terminal bronchioles, alveolar ducts, and alveoli with intact vasculature (Fig 4B). The cells grew along the basement membrane of the alveoli. Hematoxylin and

eosin staining on day 11 showed most of the scaffold covered in the area of the nodule with a lack of organized growth of tumor cells along the basement membrane. The scaffold was populated with A549 cells (derived from a lung adenocarcinoma [10]) and had features similar to human bronchioloalveolar pattern carcinoma. The cells stained for the epithelial marker CK7 and the lung-specific nuclear marker TTF-1, with a high frequency of the proliferation marker Ki-67 (Fig 4C). The cells also stained for vimentin (Fig 4D), beta catenin, and E-cadherin.

H460 cells grown on the scaffold produced numerous nodules on the matrix after 7 days (Fig 5A). Hematoxylin and eosin staining of H460 cells, which were derived from the pleural fluid of a patient with large cell lung cancer, showed features of poorly differentiated non-small cell lung cancer with sheetlike growth of polygonal cells along the airways and alveoli with intact vasculature (Figs 5B, 5C). Two distinct patterns were seen in the matrix: areas of numerous mitoses to areas of apoptotic cells with pyknotic nuclei (Fig 5B).

H1299 cells also produced numerous nodules on the matrix after 7 days (Fig 6A). These cells, which were derived from a lymph node metastasis of a patient with non-small cell lung cancer, grew well on the scaffold. Hematoxylin and eosin staining showed very poorly differentiated features with malignant cells growing in a disorganized fashion with intact vasculature. The disordered growth resembled metastatic disease more than that of the other cell lines, which showed greater interaction with the matrix (Figs 6B, 6C).

Comment

Native lung extracellular matrix is a complex system that provides support to normal tissue and maintains cell–cell interactions, cell–matrix interactions, cellular differentiation, and tissue organization. Several groups have been successful recently in creating a pure matrix using cadaveric lung [9, 13–20]. Some have been successful in populating the decellularized organ with normal cells to recreate an organ for transplantation [9, 13, 15]. The bioreactor needed to develop an organ suitable for orthotopic transplantation into a rat is complex, requiring precise control of flow and pressure through the circulation and a ventilation loop through the trachea. Because our goal was to develop a three-dimensional lung cancer cell culture model system, we did not need this complexity. We created a simpler bioreactor from existing parts that has a pump and oxygenator without a ventilator loop, and found that it was adequate for growing human lung cancer cell lines to form perfusable lung cancer nodules with features similar to the original human lung cancer.

The A549 cells (derived from human bronchial adenocarcinoma) formed nodules with cancer cells in a lepidic growth pattern characterized by tumor cells growing along preexisting alveolar structures. The tumor cells were organized to show cell–cell interaction and cell–matrix interaction, suggested by the immunohistochemistry staining. The nuclei were oval-round with prominent nucleoli, typical of moderately differentiated and well-differentiated adenocarcinoma. The cells lacked a desmoplastic stromal reaction typically seen in human lung cancer, likely attributable to a lack of mesenchymal cells. When the H460 cells (derived from pleural fluid cells of a patient with large cell lung cancer) were placed on the scaffold, they formed vascularized lung nodules like the A549 cells grown on

the matrix but the pathologic appearance was similar to human large cell lung cancer. Finally, when the H1299 cells (derived from the meta-static lymph node from a patient with non-small cell lung cancer) were placed on the scaffold, the vascularized tumor nodules formed on the matrix like the other two cell types, but the pathologic appearance was similar to tumor growth in a lymph node. These results suggest that the cancer cells retain the necessary information to form complex nodules similar to the original cancer cells.

This new ex vivo system is a significant addition to the in vitro and in vivo model systems currently available to study human lung cancer. To date, there is no system that can create perfusable lung cancer nodules with the histopathologic features of lung tumors that are similar morphologically to the original human lung cancer. The cells grow into a complex structure that is not seen in simple monolayer cell cultures. Moreover, as the tumor grows, it develops characteristic features of mitosis and apoptosis, which are difficult to appreciate with other in vitro models.

This work provides a new tool for studying lung cancer in an ex vivo environment that closely simulates the actual tumor microenvironment, having essential characteristics such as colocalization of different cell types with cell–cell interactions in the presence of extracellular matrix to provide a scaffold for mechanical stability and to regulate cell function [21]. This model can be used to improve our understanding of the tumor microenvironment and angiogenesis in lung tumor development. Using a simple decellularization chamber and bioreactor, we have shown that human lung cancer cell lines can be grown on a decellularized rat lung matrix in a manner that mimics human lung cancer.

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Fig 1. Decellularization unit and bioreactor. (A) Three customized decellularization chambers connected to a pump.(B) Four customized bioreactors inside an incubator connected to a pump and an oxygenator.

Fig 2.
Decellularized rat lung.(A) Freshly harvested intact native rat lung with heart block. (B) Hematoxylin and eosin staining of native rat lung showing cellularized alveoli with pneumocytes and endothelial cells (×20 magnification). (C) Translucent acellular lung after sodium dodecyl sulfate and Triton-X perfusion through pulmonary artery. (D) Hematoxylin and eosin staining of acellular lung lobe showing absence of any cells (×20 magnification).

Fig 3. DNA concentration of native and acellular lung. Equal amounts of tissue samples were taken for DNA extraction using Qiagen kit. DNA concentration was significantly reduced to less than 5% of native lung tissue.

Fig 4.

A549 human lung cancer cell line grown on acellular rat lung matrix. (A) Lung matrix with A549 cells on day 11 showing tumor nodules. (B) Hematoxylin and eosin staining of left upper lobe on day 3 showing cells attached to matrix in airways, terminal bronchioles, alveolar ducts, and alveoli with no cells in vasculature ($\times 20$ magnification). (C, D) Immunohistochemistry staining of lung seeded with A549 cells using Ki-67 (C) shows high proliferative index and using vimentin ($\times 20$ magnification) (D) shows presence of intermediate filaments ($\times 20$ magnification).

Fig 5. Lung matrix seeded with H460 cells. (A) Numerous tumor nodules on day 7 in lung scaffold. (B, C) Hematoxylin and eosin staining of lung section in low-power field (B) and high-power field ($\times 10$ magnification) (C) showing poorly differentiated non-small cell lung cancer with sheetlike growth along airways and alveoli ($\times 20$ magnification).

Fig 6. Lung matrix seeded with H1299 cells. (A) Numerous tumor nodules on day 7 in lung scaffold. (B, C) Hematoxylin and eosin staining of lung section in low-power field (B) and high-power field (×4 magnification) (C) showing very poorly differentiated non-small cell lung cancer cells in disorganized fashion (×20 magnification).