

An Evaluation Criteria of Lower Respiratory Tract Infection Drug Resistant Patients in Need of Alveolar Fibroblasts: Match between Carbon Nanotube Bound Fibroblast Defense and Patient Drug Resistance: NIDHI Criteria

R.Sharma*, N.Goel**, S.Kwon***

*Center of Nanomagnetism and Biotechnology, Florida State University, Tallahassee, FL 32310, USA; **Microbiology Department, Post Graduate Institute of Medical Sciences, Rohtak, India;

***Department of Biological Engineering, Utah State University, Logan, UT 84322, USA

ABSTRACT

We report first time a criterion of evaluating the need of choosing alveolar fibroblasts immune to lower respiratory tract infection (LRTI) in LRTI patients who display fatal drug resistance and specific pattern of bacterial infection. Our study was in two phases: 1. Physical characterization of cultured alveolar fibroblast cells customized with carbon nanotube coated delivery system; 2. Gluconeogenesis control and inflammatory characteristics of fibroblasts in prototype 3D lung tissue; 3. A clinical survey of patients with LRTI active infection for presence of bacteria in lungs and clinical symptoms of drug resistance. A simple criteria of “Need of Immune Fibroblast Defense in High LTRI Infection” (NIDHI) was proposed. The hypothesis was fibroblasts regulate and control: 1. gluconeogenesis, inflammatory characteristics of surviving alveolar cells; 2. Fibroblasts exhibit defense against bacteria by blocking its oxygen and glucose; 3. carbon nanotube coated antibiotics along with fibroblasts combine the overall defense. Major points in criteria were: stage 1. Alveolar cells keep intact themselves (hypoxia, apoptosis and metabolic integrity); if all these weaken or fail, stage 2 starts with necrosis, proliferation, tissue structural damage; if it progresses further, stage 3 starts with tumor, pus, abscess, debris (all visible by microscopy)-drug response and resistance starts. The results showed: 1. the microbiology lab data provided a need of matched fibroblast characteristics to enhance the remained alveolar tissue defense and reduce further bacterial drug resistance; 2. initially cytokine induced release is common in response to bacteria/ allergy/ or probably virus injury to alveolar cells. Possibly resistant fibroblast can be helpful in initial phase of disease by enhanced vascularization of lung but later phase of disease remains unknown. Unknown fate of fibroblasts were: 1. They experience difficulty (not known) to wrestle with bacteria (unclear which type and drug resistance); 2. Fibroblasts will circumvent in tissue with dying alveolar cells (not known). Major issues of criteria were: 1. What difficulties can be there in transferring engineered tissue in human beings?; 2. who are the patients who can be benefited best by this technique? The possible clinical role of customized fibroblasts in clinical research was done by using 3 D artificial lung model (that will simulate patients in stage 2 or 3)

enhancing vascularization and use in patients is highlighted. It needs to: step 1: make up of metabolic integrity loss of alveolar cells; step 2: overpowering on bacteria by killing their gluconeogenesis/oxygen supply + saving own resources; step 3: boosting energy supply with help of phagocyte/immune defense to remained alveolar cells; step 4: Match with patients and finally clinical trial. In conclusion, the choice of fibroblasts in LRTI with drug resistance is ideal but a challenge to achieve combined defense by enhanced fibroblast immunity and more controlled antibiotic release at the site. *Key words: fibroblasts, lower respiratory infection, carbon nanotube, drug resistance*

1 INTRODUCTION

Experimental access to the airways is, in general, very difficult. Post-mortem analysis of the smaller airways and direct bronchial biopsy of the upper airways (approximately generations 2-5) provides a new information. Unfortunately, supply of these tissues, and experimental control of the system are limited. Monolayer culture of the key individual cell types is alternative of information on the response of alveolar cells (e.g., epithelial, fibroblast, and smooth muscle) to external perturbations. However communication in these cells is limited due to the absence of cell-cell interactions *in vivo*. More recently, a contracting fibroblast-embedded collagen gel (an engineered fibroblast model) has provided a more dynamic information to simulate human airway (Fredriksson et al. 2002). Fibroblasts naturally contract the extracellular matrix (ECM) to close a wound, and, when placed in a collagen gel, respond in a similar fashion and contract the gel (an analogous model of LRTI). Another *in vitro* model of airway was reported culturing epithelial cells as monolayer on a membrane and fibroblasts as a monolayer at fixed distance away separated by culture media (Morishima et al. 2001). This fibroblast model isolates soluble mediators that participate in epithelial-fibroblast communication, but lacks the normal ECM and anatomical dimensions. Our model of the airway presented features: 1) It maintains the normal anatomical arrangement (orientation and dimensions) of epithelial and fibroblast cells. 2) The fibroblast was embedded in collagen I, yet remains anchored. 3) A thin (10 μ m) porous polyester membrane separates the

epithelial and fibroblast cell allowing communication between the epithelium and fibroblast, but also clean access to investigate cell-specific protein expression, following exposure to external perturbation. The model is compatible and suitable as artificial lung in use as transplanted fibroblasts in natural anatomical arrangement possibly to combat the respiratory infection and supplementing oxygenation.

In this study, we presented the LRTI criteria based on patients screening of those who need engineered fibroblasts. We tested fibroblast features suitable as engineered *via* alternatives to *in vivo* transplantation for LRTI to evaluate their feasibility as engineered human lung layers. In addition, fibroblast *in vitro* co-culture system of airway was tested for inflammatory and cytotoxic responses of cell layers alongwith the changes in physiological functions (secretion, tight junction formation, and cilia formation) measured following exposure of different concentrations and structures of CNTs.

In human lung the human airway lining cell layers play a role as a barrier to external stimuli. The airway consists of several cell layers, such as epithelial cells, fibroblasts, and smooth muscle cells, including several inflammatory cells (e.g. macrophages, neutrophils, mast cells, etc.) as shown in Figure 1. Physiological response to external perturbation is induced by each cell layers and/or the interaction between cell layers. Our *in vitro* co-culture system was established based on the interaction between two cell layers such as epithelial cells and fibroblasts shown in Figure 2.

3-D fibroblast *in vitro* model of the Human Lung

Fibroblast-embedded Collagen I gels were prepared using rat tail tendon collagen (RTTC; Collaborative, Bedford, MA). Normal human lung fibroblasts (NHLFs) were harvested upon reaching 75-80% confluency, and added (seeding density of 5×10^5 fibroblasts/mL of final volume) to an iced mixture of collagen (final concentration 2.0 mg/ml), 5X concentrated DMEM, and 10X reconstitution buffer comprised of NaHCO_3 , HEPES buffer (Gibco, Grand Island, NY) and NaOH. Aliquots of the mixture were pipetted onto the underside of a Transwell (Costar, Cambridge, MA) polyester membrane (0.4 μm pore). The collagen mixture is then allowed to “gel” (non-covalent cross-link) at 37° C in 5% CO_2 for 10-15 minutes (Figure 2A). Harvested primary human bronchial epithelial (HBE) cells (passage 2-3) were then seeded (1.5×10^5 cells/ cm^2) directly on top of the polyester membrane. The entire tissue was submerged in media for 5 days and the epithelium was allowed to attach and became confluent (Figure 2B). For the first 48 hours, the media was basal epithelial growth

medium (BEGM, Clonetics) and a low retinoic acid concentration. For days 3-5 (and days 6-21), the media was a 50:25:25 mixture of BEGM:DMEM:Hams F12 with a high retinoic acid concentration. At day 6, an air-liquid interface was established (media maintains a high retinoic acid concentration) and the epithelium was allowed to differentiate for approximately two weeks at which time it was ready for experimentation (Figure 2C).

2 NIDHI CRITERIA OF LRTI: NEED OF ENGINEERED FIBROBLASTS

2.1 Lower Respiratory Tract Infection (LRTI) And NIDHI Criteria in Indoor Patients: Criterion means characteristic systematic point-wise link between LRTI causes and therapeutic effect (drug resistance) pointing to a new dimension of transferring engineered fibroblasts in need.

2.1.1 Strategy of patient evaluation

The evaluation is done in following manner:

1. Feasibility of most common cause of LRTI Airway obstructive disease:

(A) These two are major category of patients in hospitals:

- Infectious disease: The incidence of fungus, bacteria, virus; bacterial infection induced symptoms (characteristic fever, patient symptoms, gram test, enzymes etc.)

- Tissue metabolic damage in lungs, respiratory track: altered pO_2 , pH blood, pulmonary capacity (stage 1); poor recovery on LRTI normal alveoli and continued drug resistance (failing response symptoms with evidence of apoptosis, necrosis) (stage 2); severe lung damage with over 80% pulmonary disability and metabolic/respiratory acidosis with need of artificial lung perfusion or alveolar fibroblasts(stage 3); Poor gaseous exchange and poor glucose control.

2. Biocompatibility of dish fibroblasts to their 3D transplantation in patients (replaced by transplant or artificial lungs)\

3. Prevascularization of implanted tissue metabolically better and more perfused in LRTI.

4. Difficulties in transferring engineered tissue in human beings?

5. Symptoms of the patients who can be benefited best by transfer fibroblasts technique?

2.1.2 LRTI Infectious Disease: whether community acquired or Hospital acquired?

- What are most common pathogens (most common are bacteria) causing these disease in community?

- Treatment response in hospital: Stage 1 (signs/symptoms/ lab diagnosis) →start of first line antibacterial→fails (either no change in symptoms or worsen)→second line (Higher antibiotics/ or

liposomal/nano which ever is less expensive) → fails
→ liposomal drug delivery/nanodrug delivery → fails
→ **need of fibroblast**

2.1.3 Sequential approach of airway LRTI obstructive disease

1. Stage 1: What are the LTRI symptoms ?

- Physical signs(what are signs on clinical examination)
- What are the laboratory parameters(physiological, biochemistry, pathological, microbiological, radiological)
- First line drug treatment → First line drug treatment fails → patients progress with disease.

2. Stage 2: What are lab parameters ?

- Physical symptoms of poor prognosis, signs of respiratory failure, lab parameters of severe infection and damage by virus, bacteria etc., drug resistance(second line treatment) → second line treatment fails → patient progress is poor.

3. Stage 3: Failure of drug response, failure of gas exchange and pulmonary capability

- Physical symptoms of respiratory arrest, respiratory block signs, lab respiratory, renal panel abnormal tests → extensive lung machine treatment (if poor progress it suggests the need of fibroblast replacement either in early 3 stage or late 3 stage)

2.1.4 Fibroblast transplantation in the patients

Patients in 3rd stage or late 2nd stage will be able to accept engineered fibroblasts?

1. Immuno-compatible factors (acceptance of graft),
2. Presence of multidrug resistant bacteria in respiratory system whether these fibroblast can help to remove these bacteria from system?
3. Can fibroblast improve physiological function of lungs (increased vascularization, anastomosis)
4. Who will be the best candidate for fibroblast treatment (Patients with infectious etiology or non infectious etiology?)
5. How much is the cost for the fibroblast implantation? For infectious etiology by a multidrug resistant bacteria cost of antibiotic is about 3000/-day (for about a month or so, excluding other hospital expenses).
6. Are there any side effects of these engineered fibroblast transplants?

3 ENGINEERED FIBROBLASTS

The sole purpose of fibroblasts is to recover airway and alveolar architecture and function in close interaction among epithelium, smooth muscle, fibroblasts, and vascular cells, all within an elaborate structural matrix of lung connective tissue. The engineered fibroblasts include type I and II pneumocytes, bronchial epithelial cells, collagen I expressing cells.

A. 3.1 Cytological features of engineered alveolar fibroblasts

B. The toxic, viral or all immune destruction of the bronchiolar epithelium suggests a fibrotic deficiency in bronchiolitis obliterans. However, fibrotic reactions and scarring in response to epithelial injury can be viewed as fibroblast "fibroblast cell hyper-proliferation". They act in well-protected, innervated, and vascularized niches that provide cues regulating cell fate decisions such as proliferation, migration, and differentiation capable of abundant self-renewal and can also generate the specific cell lineages within the tissue compartment.

3.2 Measurement of Transepithelial Electrical Resistance (TER)

Human bronchial epithelial cells were grown at the interface of air and liquid. Culture media was provided from the bottom through the porous membrane. TER of human bronchial epithelial cell with fibroblasts-embedded collagen layers cultured in Transwell™ was monitored using a portable Volt ohmmeter (Millipore, Bedford, MA) attached to a dual "chopstick" or transcellular resistance measurement chamber (Millipore, Bedford, MA). Different concentrations of CNTs were exposed to the co-culture layers for 6 hours. Each of the two electrode systems contained Ag/AgCl electrode for measuring voltage and a concentric spiral of silver wire for passing current across the epithelium. Electric current could then be passed across the epithelium to measure TER (ohms.cm²). It is perceived that TER values higher than the background fluid resistance indicate a confluent airway epithelium with tight junctions.

3.3 Strategy of patient evaluation

A total of 207 patients clinically suspected of pneumonia were admitted to ICU during the study period. The clinical diagnosis of pneumonia was based on clinical, radiographic, physiological and microbiological data. Endotracheal aspirate (ETA) / bronchioalveolar lavage (BAL) / protected specimen brush (PSB) samples collected aseptically were subjected to microbiological investigations. All the samples received in laboratory were immediately plated on 5% Sheep blood agar, Mac-Conkey agar plate and Chocolate agar plate using a 4mm nichrome wire which holds .01ml of sample. Semiquantative culture was done and plates were incubated at 37⁰ C. The growth on the plates was observed after 24 hours of incubation. Culture threshold was taken as follows: 10⁵ colony forming unit (CFU)/ml for ETA, 10⁴ CFU for BAL, 10³ CFU for PSB. Growth of any organism below the threshold was assumed to be due to colonization or contamination. The isolated organisms were identified to the species level by standard microbiological methods.(ref) Antibiotic susceptibility testing of isolates was done by Kirby bauer disk diffusion method. The following panel of antimicrobials (Hi-Media Disc in mcg) were tested: amikacin(Ak)(30),

ciprofloxacin(Cf)(5), ofloxacin(Of)(5), Aztreonam(Ao)(30), netilmicin(Nt)(30), doxycycline(Do)(30), cotrimoxazole(co)(25), ceftazidime(Cz)(30), ceftizoxime(C)(30), meropenem(Mr)(10), Amoxycillin/clavulanic acid(Ac)(20/10), Ceftazidime /clavulanic acid(ccv) (30/10), Piperacillin/Tazobactam (TZP) (100/10). Zone diameter was measured and interpreted as per CLSI (Clinical and Laboratory Standards Institute) guidelines. For quality control of disc diffusion tests ATCC control strains of *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *P. aeruginosa* ATC 27853 strains were used.

4. RESULTS AND DISCUSSION

3.1 Lower Respiratory Tract Infection (LRTI) in Indoor Patients Fungal pathogens were main population in LRTI patients. Pneumonia was major player among all respiratory infections (see Table 1). However their frequency varied in different age and sex groups (see Table 2). Sputum was best source to isolate pathogens (see table 4). The patients showed variable patterns of drug resistance (Table 3) and unrecovered symptoms that suggested the need of engineered fibroblasts to replace the infected alveolar cells.

Who qualify the NIDHI criteria? 1. The patients showing drug resistance in stage 3 with poor pulmonary capacity; 2. The pneumonia and fungal pathogens in repeat sputum, aspirates; 3. Males in age group over 60 years factory laborers living in poor environments conditions; 4. Receptors with less fibroblast rejection. The frequency of isolates varied in different age group and sex.(table-1) *Pseudomonas aeruginosa* (35%) was the most frequent isolate followed by *Acinetobacter baumannii* (23.6%) and *Klebsiella pneumoniae* (13.6%) (Table-2) The resistance pattern showed that the majority of the isolate from the LRTI had increased in vitro resistance(Table-3) The unrecovered symptoms and high rate of mortality suggested the need of engineered fibroblasts to replace the infected alveolar cells.

Patients qualifying the NIDHI criteria include 1. Lower respiratory tract infection caused by multidrug resistant *Pseudomonas* sp(resistant to ceftazidime, ofloxacin, piperacillin / Tazobactam , meropenem), 2. multidrug resistant *Acinetobacter* spp.(resistant to ceftazidime, meropenem, Amoxycillin/clavulanic acid) 3. Patients in age group of 26-50 years .4. Patients on ventilator. The above data shows that patients on ventilator and having infection with gram negative pathogens resistant to second line of antimicrobial agents are more prone to death or increased stay in hospital. So in cases of multidrug resistant *pseudomonas*, *Acinetobacter* or *Klebsiella* infection fibroblast replacement may be considered.

The art of fibroblast engineering traditionally aims at the development of tissue constructs for therapeutic

purposes, as an alternative to organ transplantation. Thompson et al 2001 showed that lung tissue engineering could potentially provide the field of lung biology with high-fidelity 3-D tissue models. What can be achieved by engineered fibroblasts: Certainly it has realistic prospects like stem cell therapy of the lung? First, we must identify lung diseases/cases/timing in which fibroblast cell and tissue damage occurs. Second, we must establish standardized sources of engineered fibroblast stem/progenitor cells and methods for their delivery to the appropriate lung sub-compartment. Once delivered, fibroblast cells must home to microscopic sites of need and integrate to serve a beneficial function. There can be adverse effects when implanted *in vivo*. Major lung diseases may pose unique challenges of resulting in hypoplasia, or repair of chronic lung disease of prematurity and advanced pulmonary emphysema. It may require neogenesis of alveolar septa without any metabolic information. Other alternative is using stem cells intrinsically "knowing" where to go and "how" to recreate alveolar septal architecture to ultimately restore higher order complex three dimensional relationships amongst alveoli, airways, and vessels. Stem cell therapy to cure cystic fibrosis is promising to colonize the airway, proliferate, and differentiate into columnar cells covering a significant portion of the airway lumen. The circulation may generate isolated, single airway basal cells.

Table 1: Resistance Pattern (%) of gram-negative bacteria (GNB) isolated from lower respiratory tract against first line and second line antibiotics

Drug	A.bau (38)	K.pneu (22)	C.fr (8)	E.coli (12)	Ent (17)	P.aer (57)
Co	79.5	90.9	100	100	100	ND*
Do	2.6	9.1	0	0	0	ND
Ak	87.2	63.6	75	41.7	72.2	61.4
Cf	89.7	95.5	100	100	100	ND
Cz	100	96.9	87.5	91.7	100	68.4
Mr	25.6	9.1	12.5	0	11.1	22.8
Ac	97.4	100	100	91.7	100	ND
Cac	97.4	76.2	100	66.7	100	ND
Ao	ND	ND	ND	ND	ND	94.7
TZP	ND	ND	ND	ND	ND	49.1
Nt	ND	ND	ND	ND	ND	70.2
Of	ND	ND	ND	ND	ND	68.4
Ck	ND	ND	ND	ND	ND	68.4

5 CONCLUSION

The present paper is an attempt to investigate a simple criterion of respiratory track infection patient selection who may need of the engineered fibroblasts (can avoid the stem cell hazards). The engineered fibroblasts are may be good alternative for regenerate 3D lung architecture in future by transfusion.

REFERENCES

- Fredriksson K. Inflamm Res. 2002 May;51(5):245-51.
- Morishima Y. Am J Respir Cell Mol Biol. 2001 Jan;24(1):1-11.
- Thompson HG. Pulm Pharmacol Ther. 2007;20(2):141-8.