Gene Therapy as a Novel Treatment for Severe COPD

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1 INTRODUCTION

1.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

Although lung diseases are a widely prevalent cause of death and deterioration in quality of life, there is a current lack of effective treatment options. Chronic obstructive pulmonary disease (COPD) caused over 3 million deaths worldwide in 2012, over 90% of which occurred in lower to middle income countries [1]. In 2013, it was the third leading cause of death in the US, with over 149,205 deaths [2]. COPD is an umbrella term used to describe a family of diseases by which airflow through lung airways decreases due to a variety of reasons, including irritation and swelling in the walls of the airways, superfluous production of mucus leading to clogging, destruction of the walls between tiny air sacs in the lungs, and more [3]. Lung diseases characterized as COPD include emphysema, chronic bronchitis, refractory asthma, and some conditions of bronchiectasis [4]. The diseases are characterized by progressive destruction of lung parenchyma resulting in airway remodeling and

pulmonary emphysema [5]. Symptoms include severe shortness of breath, difficulty talking and low mental alertness, weight loss, and rapid heartbeat, among others [3]. The leading cause of COPD, by far, is smoking, as 9/10 cases of COPD are accounted for by smoking [3]. Additionally, over half of the people with COPD have not even been diagnosed with the condition, but still suffer COPD symptoms of breathlessness, coughing, wheezing, and more [4].

1.2 Lung Cancer, COPD, and p53-bcl2 Balance

Although lung cancer and COPD are different classes of disease, they are similar in that both are predominantly caused by smoking and both affect the delicate epithelial lung tissue. Somewhat surprisingly, they also share similarities in the way they perturb epithelial cell functioning. The destruction of pulmonary tissue observed in cases of COPD is attributed to disruption of normal proteolytic and anti-proteolytic activity, oxidative stress, abnormal apoptotic events, and more [5]. One mechanism for apoptosis is the intrinsic pathway, and features a number of promoters and inhibitors. The primary regulator of the intrinsic apoptosis pathway is p53, a tumor suppressor protein that ensures genomic integrity by stimulating DNA repair or initiating apoptosis in response to DNA damage during cellular stress [5]. Cigarette smoke introduces oxidants into the lung and causes inflamed airway cells to generate reactive oxygen species (ROS), both of which trigger the p53-mediated intrinsic apoptosis pathway, in which cell death is carried out primarily by CD8+ cytotoxic cells [5].

This pro-apoptotic effect of p53 is offset in healthy people by the anti-apoptotic effect of bcl2, a family of proteins located upstream of the apoptotic pathway that enable cellular survival by, in part, inhibition of p53-dependent apoptotic pathways [5]. Although this imbalance observed in patients with COPD may be due in part to the cellular stress caused by the increase in ROS brought on by smoking, there is also evidence that misregulation of the balance between bcl2 and p53 is brought on by direct mutations to the regulatory

sequences of the two genes. A 2004 Surgeon General's report found a causal relationship between smoking and lung cancer due to smoking exposing lung tissue to carcinogens and the somatic DNA mutations that occur as a result, with higher frequency of mutation in the region of the *p53* gene [7]. These findings will be discussed further in Section 2, and are a basis for the proposed novel gene therapy approach.

1.3 Current standards of treatment

Despite the prevalence and severity of COPD, human lung tissue cannot regenerate or repair itself beyond a microscopic level, and the current standard of treatment for severely damaged lungs is a lung transplantation procedure. However, the future of lung transplants is severely limited by an extreme shortage of lung donors available. Additionally, it's not even clear whether this expensive procedure provides a survival advantage to patients, as the 10 year post-transplant survival rate is just 10-20% [8]. There are a number of complications associated with the procedure, including infection, graft dysfunction, and *bronchiolitis obliterans* syndrome. In spite of this, the indication for lung transplant procedures has become more broad since its introduction over 50 years ago [9].

Current research in the field of lung tissue engineering has set up the notion of growing lungs as a possible future superior solution to the problem of COPD that would solve the issue of lung donor shortage and greatly reduce complications associated with the transplant procedure. However, a great deal of problems remain to be solved before lung tissue engineering is a viable technology, including understanding the program of development and branching morphogenesis in human lungs, identification of human-sized scaffolds that mimic the natural lung environment and are capable of supporting normal growth and development of lung tissue, the differentiated and undifferentiated cell types and quantities that would be required to grow functioning lungs, and more [10]. While this approach has great potential to be the future standard of lung disease treatment, there is a distinct lack of promising treatment options that will be available in the near future.

1.4 Significance on basic understanding and treatment

The goal of the proposed approach is to evaluate the viability of gene therapy as a method of clinical management in severe cases of COPD, where treatment would otherwise shift to end-of-life care [11]. There are no current effective approaches for clinical management of COPD. While lung transplant procedures exist and lung tissue engineering approaches are potential solutions in the distant future, the primary impact of successfully applying a gene therapy approach to the p53-bcl2 apoptosis regulation system would be to establish a new way of halting or slowing down the progression of COPD. While disruption of the p53-bcl2 apoptosis mediation equilibrium is not necessarily the only route of pathogenesis by which smoking may cause COPD, restoring the appropriate equilibrium through a gene therapy approach may be greatly beneficial for the sake of extending life expectancy, delaying onset of symptoms, and potentially even providing a long-term cure in some cases.

It would also be a valuable source of information on the pathogenesis of COPD, in particular regarding the frequency at which p53-bcl2 activity is related to changes in environmental stimuli versus the frequency at which the change in activity is due to mutations in the regulatory sequences for the gene. This is discussed further in Section 4.

2 Rationale and Outline for Proposed Approach

2.1 Experimental Basis for the Approach

A study by Siganaki *et al.* has investigated whether abnormal apoptotic events play a role in COPD pathogenesis by comparing protein concentrations of p53 and bcl2 in 23 smokers with COPD and 20 smokers without COPD using western blot analysis. The results indicated that p53 protein levels are significantly higher in smokers with COPD as in smokers without COPD ($p = 0.038$), but showed no statistically significant difference in protein levels of bcl2 between smokers and non-smokers. Using b-actin to normalize concentrations, the protein levels of p53 were found to be doubled in COPD patients compared to non-COPD

patients.

Additionally, immunohistochemistry analysis of the lung confirmed this result, indicating that a greater proportion of lung pneumocytes stained positively for p53 in smokers with COPD (36% positive vs. 10% positive, p = 0.01), although this was not the case in alveolar macrophages or lymphocyte-like cells, and was also not the case with bcl2 stains. As the increase in pro-apoptotic p53 expression in COPD smokers is not offset by a corresponding increase in anti-apoptotic bcl2, this is thought to be the source of the increased alveolar epithelium apoptosis observed in patients with COPD [5]. These results also suggest that p53 expression is perturbed by more than just the cellular stress and inflammation that result from smoking, as the p53 increase was not observed in smokers without COPD.

The 2004 Surgeon General's report, *The Health Consequences of Smoking*, provides additional information on mutagenesis patterns associated with smoking that may further explain why p53 expression is higher in COPD cases. Once carcinogens are converted by P-450 enzymes into active forms, they are capable of covalently binding to DNA to create DNA adducts. Although cellular repair systems exist to remove DNA adducts, these systems may be overwhelmed by DNA damage, and somatic DNA mutations may develop as a result of miscoding during replication due to the adducts.

The report found that selectivity exists between types of DNA adducts and the subsequent mutations. In particular, O^6-methylguanine adducts causing G→A mutations were observed frequently. More importantly, these types of mutations were often observed in the *p53* gene, a phenomenon that has been linked to incidences of lung cancer [7]. A study by Tang et al. uses a nuclease incision and ligation-mediated polymerase chain reaction to determine that the *p53* gene in human lung tissue is a DNA adduct hotspot, and that this is a major cause of the pattern of *p53* mutations observed in cancers [12]. In fact, the *p53* gene was found to be mutated in 50-70% of lung cancers [14]

While these studies focused on the role of mutation-induced *p53* inactivation in the role

of smoking-related lung cancer, there is also a distinct possibility that this DNA adduct→p53 mutation pattern is also responsible for over-activating *p53* expression in some smokers, and may then be the cause of the increased levels of expression seen in the COPD cases in Siganaki et al.'s study. The *bcl2* gene therapy approach described below will provide insight to what extent *p53* over-expression is mutation- or stimulus-mediated.

2.2 General Outline for the Approach and Novel Aspects

The proposed approach is to introduce more copies of the *bcl2* gene with a wild type promoter sequence in a range of concentrations to lung epithelial cells in the hopes of inducing proper regulation of cellular apoptosis via the p53-bcl2 balance. The most appropriate gene therapy approach for this scenario would be to use a recombinant adeno-associated virus (AAV) vector targeted to lung cells. An AAV is well-suited to this scenario because it is known to efficiently transduce differentiated cells, in this case lung pneumocytes [13]. Past studies aimed at gene therapy in lung cancer have used an adenovirus targeted to cancerous cell markers [14]. However, in this case an AAV is more appropriate due to its advantages in safety. Namely, AAVs are minimally immunogenic, and carry the lowest risk of inducing cellular or humoral immune responses in patients that are already in severe condition. Additionally, transgenes delivered via AAV rarely integrate into the host genome and primarily persist as extrachromosomal episomes, meaning that they have low tumorgenicity. This is extremely important, as the environment into which the vector is being introduced is already at extremely high risk of developing lung cancer due to the far greater frequency of mutations brought on by the carcinogens in tobacco smoke. Therefore, the chosen vector must be as minimally tumorgenic as possible. However, it's worth noting that as the target gene for therapy is involved in mediating apoptosis, tumorgenicity is still a concern with this treatment, and will be discussed later as part of the safety considerations of this approach. The adenovirus will be stripped of all viral genes, and the *bcl2* promoter and wild type *bcl2* gene willl be added [14]. The strategy for targeting the adenovirus

to lung tissue is explained further in Section 3.

To date, gene therapy has not been seriously explored as a treatment or clinical management option in severe or terminal cases of COPD. This approach represents a new method of treatment for patients with COPD that may be particularly relevant in cases where treatment has shifted to end-of-life care. While *p53* gene therapy for lung cancer has been shown to induce tumor regression and correct for p53 loss of function, no such approach has been attempted to correct for the *p53* over-expression observed in smokers with COPD [14]. In this case, it's necessary that the gene therapy target the *bcl2* gene to restore equilibrium, as introducing wild type copies of *p53* would not necessarily lead to inhibition of the mutated p53 protein, and would actually likely be expected to simply increase p53 production even further. An important aspect of this approach is that it would allow for further evaluation of the extent to which shortness of breath and other COPD symptoms may be due to unnecessary apoptosis of lung epithelium as a result of mutations in the *p53* gene and the resulting disruption in the apoptosis regulation system described by Siganaki *et al.* If successful, although this approach may not necessarily cure COPD symptoms, it may alleviate the progression of the disease. If this treatment is found to be successful, it could have future viability as a preventive measure to be implemented as soon as early COPD symptoms arise to minimize further deterioration of lung tissue.

3 Experimental Approach

In order to assess the efficacy of an AAV-based *bcl2* gene therapy approach aimed at increasing bcl2 protein activity to match that of p53 in patients with COPD, it will be necessary to measure p53 and bcl2 activity in cohorts of patients with severe COPD both before and after application of the treatment. As in Siganaki *et al.*'s experiment, a control cohort and a gene therapy treatment cohort will be used. The primary endpoints will be the level of p53 and bcl2 proteins as measured by western blot analysis and the proportion of en-

dothelial cells positive for p53 and bcl2 as measured by immunohistochemistry. These are the same endpoints as in the aforementioned study, so the original target study size will be comparable as well, on the order of 25 patients for each cohort.

Only smokers will be included in the study, with a smoker defined as someone with 20 pack-years (packs/day * years) of smoking history. Additionally, study subjects must have COPD, as defined by the GOLD spirometric COPD classification metric, which is based on post-bronchilator forced expiratory volume [5]. The smokers with COPD will be divided into even control cohorts and treatment cohorts. A number of potential confounding variables will be measured for in both cohorts, including smoking habits, age, gender, severity of symptoms, onset of the disease, and COPD treatment history. A baseline of p53 and bcl2 activity will then be established for each subject in the study. This can be accomplished by performing a western blot for p53, bcl2, and b-actin in all patients. The b-actin is used as an internal control to normalize expression levels [5]. Additionally, an immunohistochemistry approach will be employed to establish the prevalence of bcl2 and p53 in the lung using 5μm thick sections of lung tissue and staining them using primary antibodies binding to the p53 or bcl2 proteins. The percentage of lung tissue cells staining positively for p53 and bcl2 will be calculated, and these metrics (expression level and percent of cells staining positively) will be used as the baseline for evaluating the efficacy of the gene therapy treatment approach.

No intervention will be applied to the control group. In the treatment cohort, AAV-based *bcl2* gene therapy will be carried out via a bronchoalveolar lavage procedure. This procedure enables AAVs to be delivered directly into alveolar tissue (pneumocytes) using a bronchoscope or CT guidance to direct where the fluid containing the AAVs is applied [14]. An important point to consider with this procedure is the problem of safety and FDA approval. Clinical trials have been carried out in the past applying a retroviral bronchoalveolar lavage treatment to patients with lung cancer. Although AAVs are considered to be less immuno-

genic than retroviruses and do not integrate into the host genome as retroviruses do, the subjects in this study are COPD patients, not lung cancer patients. However, past studies have indicated strongly that AAVs targeted to lung tissue via intranasal or intratracheal delivery may be used safely in murine models [16].

A goal of the study will be to attempt to elucidate a dosage-dependent effect of gene therapy on *bcl2* expression. This will be first accomplished by applying a range of AAV dosages to smoking-induced COPD murine models to get an idea of appropriate dosages. These pre-clinical trials are necessary both to obtain an idea of the appropriate dosage and the effects of dosage on transgene expression, and to establish a proof-of-concept for the proposed treatment. The murine models will continue to be monitored for a year after treatment, and will be tested monthly for a variety of metrics in addition to basic health metrics, including p53 and bcl2 activity, COPD symptoms, and presence of lung cancer. The latter metric is particularly important, as *bcl2* gene therapy may be expected to reduce apoptosis and restore the balance between p53 and bcl2 protein function, but a large risk of this is overcorrecting the balance and inhibiting proper apoptosis, leading to tumorgenesis. It will be extremely important to establish a dosage ceiling such that apoptosis will be decreased from the high levels seen in COPD patients without being decreased too much and risking development of tumors.

The baseline pre-intervention data will then be used to guide assignment of a dosage scale to patients. Specifically, COPD patients with the greatest level of p53 protein activity above normal (as measured by western blot analysis) will be given the highest dosage of AAV loaded with *bcl2* to test the hypothesis that regulation of apoptosis is at least in part a balance between p53 and bcl2 protein activity, so higher p53 concentrations call for higher bcl2 concentrations.

More specifically, the recombinant AAV used will be the AAV8 serotype tested by Wang *et al.*, containing specific pulmonary targeting capsid ligands. This AAV has been shown

to effectively bind to laminin receptors in lung tissue and achieve efficient transduction of pneumocytes [16]. Transgene expression after a single treatment with AAV8 may be expected to persist for 4 months, but will peak after approximately 1 month [16]. Therefore, treatment will consist of a bronchoalveolar lavage AAV gene therapy procedure every 3 months over the course of an entire year.

One month after each treatment, the primary endpoints will be again measured. The levels of p53 and bcl2 protein activities will be compared to those measured in the same patient at the prior time of measurement and to the baseline established prior to treatment. Additionally, progression of COPD will be measured each time, primarily by checking post-bronchilator forced expiratory volume, as well as by checking on the status or progression of other common COPD symptoms. If the gene therapy treatment is successful, it would be expected to see an increase in *bcl2* gene expression in proportion to the dosage of AAV applied at each 3 month interval. Additionally, halting of the progression of COPD may be observed as well, which would indicate high potential for the future use of this treatment.

4 Discussion

This approach of applying gene therapy to restore the p53-bcl2 balance in smokers with COPD has a distinct advantage of being a comparably inexpensive, possibly effective clinical management strategy for an especially widespread condition for which the treatment standard at present is quite poor. Lung transplantation procedures are the current most effective treatment option for patients with severe COPD. However, these treatments are extremely resource-intensive, as not only are they particularly expensive, but they also require lung donors, the supply of which is limited. Additionally, although lung transplants do occur, the 10 year post-operative survival rate is just 10-20% and the procedure involves a variety of complications. The standard of care is especially low, so any success achieved via this gene therapy approach would likely be a significant improvement [8].

Furthermore, in cases where lung transplants are not appropriate or the patient cannot afford such care, treatment typically shifts to end-of-life care. Thus, an enormous advantage of pursuing this type of approach is that it represents a potential way to arrest the progression of the disease where such a method did not previously exist. In addition to frequently causing death, COPD is also extremely detrimental to quality of life, with many patients reporting frequent trouble breathing, fear, social isolation, anxiety, depression, dyspnea, hypoxemia, and more [11].

This approach also has the added advantage of being based on existing technologies. Gene therapy using AAVs is well-understood and frequently used, and studies using AAVs to target lung tissue have shown successful results, with the AAV8 serotype proving most effective [16]. This is a distinct benefit over possible future solutions like lung tissue engineering, which seems to have a lot of problems in need of solving before it becomes a clinically viable solution to the problem of COPD, including identifying appropriate extracellular matrix scaffolds for lung tissue, better understanding of branching morphogenesis in the lung, and more [10].

A final advantage of the gene therapy approach is that, unlike lung transplants or lung tissue engineering solutions, it would provide valuable insight regarding the pathogenesis of COPD in smokers. Siganaki *et al.* have determined that smokers with COPD exhibit much higher p53 levels than smokers without p53, but that bcl2 levels stay roughly the same. However, a causal relationship between development of COPD and the imbalance of the p53 and bcl2 apoptosis regulators has not been conclusively established [5]. If the treatment is shown to be successful in pre-clinical and clinical trials at halting or slowing down the deterioration of lung tissue, it will be strong evidence that pathenogesis of COPD due to smoking occurs through disruption of the p53-bcl2 balance, and that restoration of this balance is enough to stop progression of the disease. If this is the case, then it may be worth considering this gene therapy treatment in patients with earlier stages of the disease

as a preventive measure to maintain quality of life before severe COPD develops.

One highly significant disadvantage of this approach relates to the relative risk associated with the treatment, and is dependent on the efficacy and safety of pre-clinical trials. In particular, the danger of manipulating *bcl2* expression in lung tissue is that it may over-suppress apoptosis and lead to the development of lung cancer, meaning that it risks shifting the p53-bcl2 balance too far in the wrong direction. It is therefore critical that the safety of the gene therapy procedure is determined beyond doubt by calculating a ceiling dosage level in the murine model and converting this to dosage recommendations in clinical trials that are low enough as to not risk increasing the risk of tumorgenesis.

This paper represents my own work in accordance with University regulations.

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