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PROSPECTS

Gene Therapy for Lung Cancer

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Abstract Lung cancer patients suffer a 15% overall survival despite advances in chemotherapy, radiation therapy, and surgery. This unacceptably low survival rate is due to the usual finding of advanced disease at diagnosis. However, multimodality strategies using conventional therapies only minimally improve survival rates even in early stages of lung cancer. Attempts to improve survival in advanced disease using various combinations of platinum-based chemotherapy have demonstrated that no regimen is superior, suggesting a therapeutic plateau and the need for novel, more specific, and less toxic therapeutic strategies. Over the past three decades, the genetic etiology of cancer has been gradually delineated, albeit not yet completely. Understanding the molecular events that occur during the multistep process of bronchogenic carcinogenesis may make these tasks more surmountable. During these same three decades, techniques have been developed which allow transfer of functional genes into mammalian cells. For example, blockade of activated tumor-promoting oncogenes or replacement of inactivated tumor-suppressing or apoptosis-promoting genes can be achieved by gene therapy. This article will discuss the therapeutic implications of these molecular changes associated with bronchogenic carcinomas and will then review the status of gene therapies for treatment of lung cancer. J. Cell. Biochem. 99: 1–23, 2006.

Key words: gene therapy; lung cancer

Lung cancer is the leading cause of cancer-related death among both men and women in the United States. There are about 174,000 new lung cancer cases and 160,000 lung cancer-related deaths annually. Advanced disease is the usual finding upon diagnosis, as early detection has not been afforded by current screening methods. Overall survival for all stages combined is less than 15% despite advances in chemotherapy, radiation therapy, and surgery. Surgery offers the best chance of cure for patients with Stage I or II non-small cell lung cancer (NSCLC), yet less than 50% of patients are cured despite aggressive staging and complete resection [Mountain, 1997]. Even in patients with the earliest stage of disease presenting as solitary pulmonary nodules without lymph node involvement at resection, about 40% of these patients will develop systemic metastases. Adjuvant chemotherapy following definitive resection for Stages IB–IIB may result in an additional 4% to 15% improvement in overall 5-year survival [Non small Cell Lung Cancer Collaborative Group, 1995; The International Adjuvant Lung Cancer Trial Collaborative Group, 2004]. Treatment of patients with metastatic disease with chemotherapy provides only modest benefits, with an increased 1-year survival from 15% up to 25% with cisplatin-based chemotherapy [Non small Cell Lung Cancer Collaborative Group, 1995]. Importantly, the recent survival benefit conferred in metastatic disease by the addition of the anti-vascular endothelial growth factor (anti-VEGF) antibody, bevacizumab to systemic chemotherapy [Sandler et al., 2004], and by the use of epidermal growth factor receptor inhibitor, erlotinib [Shepherd et al., 2004] demonstrate the importance of approaches targeted to specific pathways in lung cancer.

Over the past three decades, the genetic etiology of cancer has been delineated, albeit not yet completely, and includes the identification of many oncogenes as well as tumor

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suppressor genes. Development of bronchogenic carcinoma has been suggested to occur through a series of such molecular events in which multiple genetic abnormalities accumulate within the cells and has been called "multistep carcinogenesis." Mutations in oncogenes may result in excessive activity or expression of the oncogene product. In contrast, tumor suppressor genes may be mutated or deleted resulting in decreased activity or expression of the tumor suppressor gene product. Either case could result in abnormal growth regulation resulting in the cancer phenotype. Additionally, some tumor cells may have lost their immunogenicity through alterations in their cell surface antigens, while others may have gained resistance to cytotoxic drugs. It has been estimated that between 10 and 20 genetic mutations occur during lung carcinogenesis including the 2 genetic changes required to inactivate tumor suppressor genes [Ihde and Minna, 1991; Toloza et al., 2000]. During these same three decades, techniques have been developed which allow transfer of functional genes into mammalian cells. This article will discuss the therapeutic implications of these molecular changes for the treatment of bronchogenic carcinomas and will then review the status of gene therapies for treatment of lung cancer.

MOLECULAR EVENTS IN LUNG CARCINOGENESIS

Oncogene Activation

Oncogene products serve as growth factors, growth factor receptors, and cytosolic and nuclear regulatory proteins. Increased levels of oncogene products, such as *ras*, *myc*, c-*erb*-B2, and *bcl*-2, have been associated with lung cancer (Table I). Patients with lung cancer have increased c-*myc* mRNA, which encodes a transcriptional activator, in both histologically normal and abnormal bronchial margins, but not in the majority of the actual tumors [Sundaresan et al., 1991]. These data suggest c-*myc* expression is an early event preceding detectable morphological change. This overexpression is due to oncogene amplification in less than 10% of tumors. Nevertheless, c-*myc* oncoprotein expression is increased in up to 43% of neoplasms and up to 80% of small cell lung cancers (SCLC) and may adversely affect survival [Gosney et al., 1990; Salgia and Skarin, 1998; Song, 2005a,b]. *Bcl*-2, a negative regulator of apoptosis, is expressed abnormally in some NSCLC cases [Salgia and Skarin, 1998].

TABLE I. Examples of Oncogenes Altered in Lung Cancer*

Non-small cell lung cancer	Small cell lung cancer
Ras**	myc**
Erb B-1 (EGFR)**	raf
erb B-2 (HER-2/neu)	myb
fur**	c-erb B-1 (EGFR)
myc	fms
raf	rlf
fes	
sis	
bcl-1	
bcl-2	
IGF-1	

*Over 100 oncogenes are currently known.
**Most frequently altered genes in tumors or cell lines evaluated.

The *ras* oncogene is one of the most frequently activated oncogenes in human cancers, with an incidence of up to 30% in NSCLC and up to 75% of human pancreatic cancers [Mitsudomi et al., 1991; Gibbs, 1992; Salgia and Skarin, 1998]. *K-ras* mutations and *Ha-ras* variable tandem repeats occur frequently in bronchogenic carcinoma, especially adenocarcinomas, and in the later stages of carcinogenesis, especially in smokers [Sugio et al., 1994]. *K-ras* mutations, which are guanosine-to-thymidine transversions induced by polycyclic aromatic hydrocarbon (PAH)–DNA adducts, which are increased in cigarette smokers, result in higher tumorigenicity and shorter latency periods of the *ras*-transformed bronchial epithelial cells [Phillips et al., 1988; Reddel et al., 1988; Randerath et al., 1989].

C-*erb*-B1 (also known as epidermal growth factor receptor, EGFR) and c-*erb*-B2 (also known as *HER-2/neu*) are overexpressed in NSCLC. Overexpression of both c-*erb*-B1 and c-*erb*-B2 are due to enhanced gene transcription and translation, with only a minority of tumors having oncogene amplification. C-*erb*-B1 (EGFR) overexpression has been associated with shorter survival, higher stage, cellular grade, poor cell differentiation, high metastatic rate, and high rate of cell proliferation [Vaele et al., 1993]. C-*erb*-B2 (*HER-2/neu*) overexpression in up to 25% of NSCLC cases is also a poor prognostic factor and has been associated with older age, more advanced stage, distant metastases, shortened survival, and chemoresistance [Kern et al., 1994; Salgia and Skarin, 1998].

In addition, EGF and/or transforming growth factor-alpha (TGF-alpha) are generally overexpressed with EGFR by NSCLC, which suggests an autocrine loop to stimulate growth [Fontanini et al., 1995]. However, activation of EGFR by EGF or TGF-alpha appears to be dose-dependent. Lower levels of overexpression of both ligand (EGF or TGF-alpha) and EGFR stimulate growth, whereas higher concentrations of either one of the ligands or EGFR can induce apoptosis. Arrest in tumors with higher EGFR expression occurs at the G_2/M stage, whereas tumors with lower EGFR that encounter high concentrations of ligand occur at the $G_{0/1}$ stage. Nevertheless, overexpression of EGFR with either EGF or TGF-alpha is correlated with frequency of lymph node metastasis, more advanced stage, and shorter survival [Fontanini et al., 1995].

Similarly, perturbation in the expression of any insulin-like growth factor (IGF) ligands, receptors, or binding proteins seems to be implicated in lung cancer formation—IGF ligands and IGF-I receptor (IGF-IR) through their mitogenic and anti-apoptotic action, and the mannose 6-phosphate/IGF-II receptor (M6-P/IGF-IIR) possibly as a tumor suppressor [Pavelic et al., 2002]. The IGF-IR is often expressed at high levels in lung cancer and is thought to be essential for maintenance of the malignant phenotype. Moreover, IGF-I and IGF-II are potent mitogens that exert autocrine/paracrine effects on growth regulation in human lung cancer and appears to also make some tumors less immunogenic [Hochscheid et al., 2000]. Their proliferative effects are modulated by IGF-binding proteins (IGFBPs), which are ontogenetically and hormonally regulated and are found in conditioned medium of lung cancer cell lines with both inhibitory and stimulatory effects on cell [Hochscheid et al., 2000].

Tumor Suppressor Gene Defects

The first isolated tumor suppressor gene, retinoblastoma (*Rb*), is a key cell-cycle regulator involved in transcription regulation that can interact with products of DNA viruses and is abnormal in up to 90% of SCLC cases (Table II). There is an inverse relationship in lung cancer cells between expression of *Rb* and *p16*INK4A, an upstream negative regulator of *Rb* [Salgia and Skarin, 1998]. Allelic loss of the short arm of human chromosome 3 (3p) also occurs relatively early in lung carcinogenesis. Lung cancer cases with decreased levels of a candidate tumor suppressor gene at 3p14.2, the fragile histidine triad (*FHIT*) gene, are associated with decreased survival [Tseng et al., 1999]. Another gene, *FUS1*, belongs to a group of candidate tumor suppressor genes at 3p21.3 that are also frequently deleted in human lung and breast cancers [Ito et al., 2004].

TABLE II. Examples of Tumor Suppressor Genes Altered in Lung Cancer*

Non-small cell lung cancer	Small cell lung cancer
*p53***	*p53***
Rb	Rb**
p16	p16
3p	3p
9p	9p
FHIT	
p21 WAF1	

*More than 10 tumor suppressor genes are currently known.
**Most frequently altered genes in tumors or cell lines evaluated.

The best-known gene with tumor suppression activity, *p53*, is located on the short arm of human chromosome 17 (17p) and encodes for a nuclear protein capable of arresting the cell cycle at G_1 and inducing apoptosis. The *p53* gene may be critical because it transactivates a variety of other genes that are important for growth inhibition and induction of apoptosis. Cytogenetic and restriction fragment-length polymorphism (RFLP) studies associated the loss of heterozygosity (LOH) in the chromosome 17p13 region with tumor cells and tissues. Loss of both *p53* alleles through point mutation, deletion, or rearrangement results in transformation of normal cells to malignant cells. Mutations of the *p53* gene, located in this region, are the most frequent abnormality (70% of genetic alterations) identified in human tumors. The mutational frequency and type varies with different tumor types (up to 50% in NSCLC and up to 80% in SCLC) and degree of differentiation and are often associated with overexpression or accumulation of the aberrant gene product, which leads to cellular proliferation and inhibition of apoptosis [Salgia and Skarin, 1998]. Mutations of the *p53* gene and the resultant altered expression and stabilization of *p53* oncoprotein appear to occur relatively late in carcinogenesis and is one of the best predictor of the progression and irreversibility of preneoplastic bronchial lesions with overwhelming morphologic changes, such as severe bronchial

epithelial dysplasia [Brambilla and Brambilla, 1997]. Moreover, dysplasias with mutant *p53* also have greater DNA indices than dysplasias with wild-type (wt) *p53* genes [Walker et al., 1994]. Mutant human *p53* cDNA transfected into non-tumorigenic human bronchial epithelial cells enhanced the colony-forming efficiency and tumorigenicity of these cells [Gerwin et al., 1992]. Additionally, mutations in *p53* may reduce the responsiveness of bronchial epithelial cells to negative growth factors, as suggested by an increased resistance to TGF-beta-1 [Gerwin et al., 1992].

Occurrence of *p53* mutations in lung cancer reveals some specificities: three hot spot codons (codon 157, 248, 273) are due to transversion at GC base pairs due to selective bulky PAH–DNA adduct formation resulting from cigarette smoke [Denissenko et al., 1996; Brambilla and Brambilla, 1997]. Missense *p53* mutations are highly concordant with *p53* stabilization and immunoreactivity. However, 20% of mutations with null phenotype (no *p53* protein) provides 20% of false negative using immunohistochemistry for evaluation of *p53* mutations in lung cancer [Brambilla and Brambilla, 1997]. Rare situations are described with wt-*p53* stabilization. Lastly, many *p53*-deficient tumors have proven to be resistant to chemotherapy and to radiation therapy [Roth et al., 2000].

Multistep Carcinogenesis

Bronchogenic carcinogenesis has been suggested to occur by successive overgrowth of cellular clones that become increasingly abnormal. For example, epidermoid bronchial carcinogenesis is thought to progress histologically from normal to basal cell hyperplasia, stratification, regular squamous metaplasia, then to mild, moderate, and severe atypical transitional and squamous dysplasia, and then to squamous cell carcinoma (SCCa) [Thoracic Oncology, ed Roth et al., 1995]. This progression has been suggested to occur through a series of events in which multiple genetic abnormalities accumulate within the cells and has been called "multistep carcinogenesis" (Fig. 1). The earliest events involve mutations, deletions, or polysomy in the cell's genome. These genetic changes may not be manifested as measurable changes in the cell's physiology or morphology, but may induce phenotypic alterations such as dysregulation of proliferation and differentiation. Additional events may then cause changes in the cell's function or appearance that ultimately lead to acquisition of local invasiveness, metastatic capacity, and ability to survive distantly. It has been estimated that between 10 and 20 genetic mutations occur during lung carcinogenesis including the 2 genetic changes required to inactivate tumor suppressor genes [Ihde and Minna, 1991; Toloza et al., 2000].

The order of acquisition of genetic abnormalities within a tumor can potentially be inferred from patterns of intracellular co-occurrence of these abnormalities within cell subpopulations from that tumor [Shackney et al., 1999]. When DNA content (diploid vs. aneuploid) and the protein levels of *p53*, *HER-2/neu*, and *ras* were correlated in NSCLC tumors, DNA content was shown to be independent of carcinogenesis, although *p53* overexpression occurred frequently even in diploid tumors. *HER-2/neu* and *ras* overexpression was also shown to occur mainly in tumors that were already overexpressing *p53*. Further, *ras* overexpression was asserted to occur mainly in tumors that overexpressed both *HER-2/neu* and *p53*. Lastly, the majority of recurrent tumors overexpressed *p53*, *HER-2/neu*, and *ras* proteins. The points along the carcinogenic evolution of tumor cells at which the various molecular events occur can thus be mapped not just by determination of the presence of a particular genetic abnormality at each step but also by analysis of the concurrent presence of multiple genetic alterations [Toloza et al., 2000].

Other investigators have proposed that some tumors originate from the dedifferentiation of bronchial epithelial cells that then redifferentiate into tumor cells. These tumor stem cells would then occupy the dividing cellular layers of the (pre) neoplastic lesions and subsequently constitute the actively dividing and invading part of the neoplasm. Bronchioalveolar, papillary, acinar, and adenoid-cystic carcinomas have been suggested to arise through this sequence of events [Ten Have-Opbroek et al., 1994]. This proposal relies on evidence of pluripotential stem cell marker expression within lung cancer tumors. However, the presence of stem cell markers may simply be due to activation and overexpression of silenced embryonic genes, such as carcinoembryonic antigen, by the same methods that oncogenes and other tumor-associated markers are activated. Alternatively, the abundance of stem cells may be due to the clonal growth of these

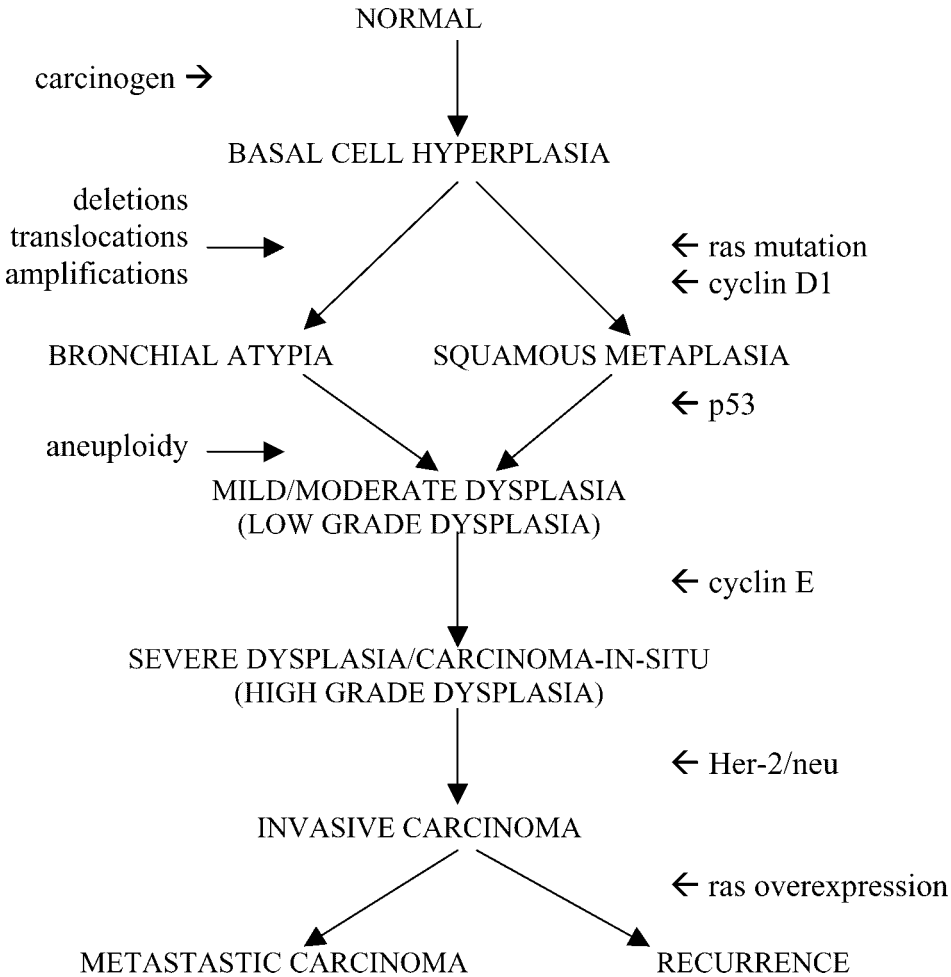

Fig. 1. Multistep carcinogenesis.

cells in the undifferentiated state or else "re-differentiated" into an abnormal phenotype. With either pathway, the phenotypic characteristics of the cells change due to accumulation of genetic abnormalities, which then confer ability for rapid growth, local invasion, metastatic capacity, and survival at distant sites.

CANCER GENE THERAPY STRATEGIES

The design of any successful cancer gene therapy protocols requires the identification of the correct combination of three factors for each type of cancer: the gene to be transferred, the gene delivery method, and the target cells to be modified. To choose a particular component, several technical issues must be considered and include the efficiency of transduction, the specificity of cell targeting and gene expression, the duration of gene expression, the level of gene expression, the effect on normal cells, and the ability to establish systemic immunity. While the overall goal is to eradicate all malignant cells, low transduction efficiency may be acceptable if either a local bystander effect or systemic immunity can be achieved. The specificity of cell targeting and gene expression is important when the transferred genes would be toxic to normal cells, especially during systemic administration. A high level of gene expression may be more important for secreted gene products than for ones that remain within the tumor cells. Transient gene expression may also be acceptable if the duration of gene expression exceeded the time period required to kill all tumor cells. Toxicity to normal cells may be avoided with the use of tumor-specific promoters. Finally, achievement of systemic tumor immunity, for example, by stimulation with cytokine-producing tumor cells, may produce a more durable

anti-tumor effect than strategies with antisense constructs or with suicide genes.

Choice of Target Cells

The possible target cells include not only the tumor cells and the immune cells but also surrounding normal tissue. Gene therapy of tumor cells could result in correction of their abnormal growth and re-establishment of apoptosis, or in increased drug or radio-sensitivity of the tumor cells. Gene modification of tumor cells could also enhance their immunogenicity. Immune cells, such as dendritic cells (DCs), could be gene-modified to have increase their capacity to induce lung cancer-specific T-cells.

Choice of Delivery Methods

Various physical and biological methods are available to deliver foreign genes into these target cells. Which delivery method one chooses depends generally on the local, regional, or systemic route of administration chosen or needed to reach the tumor [Woll and Hart, 1995]. Physical methods, such as calcium phosphate precipitation, electroporation, direct microinjection, and particle bombardment (or "gene gun"), may be suitable for introducing naked DNA into established cell lines in vitro, but are generally of low efficiency and are often impractical for in vivo applications. Nevertheless, gene delivery to lung tumors by aerosolization of adenoviral (Ad) vectors incorporated into calcium phosphate precipitates resulted in much greater expression in tumors than in normal lung tissue [Lee et al., 2002].

Liposomes are composed of monocationic or polycationic lipids that envelope the DNA and allow for much more highly efficient DNA entry upon fusion with the cell membrane both in vitro and in vivo. Transgene expression using an improved liposomal formulation (extruded DOTAP:cholesterol (DOTAP:Chol)–DNA complex) was significantly increased in human tumor cells in vitro, due to increased uptake of the liposomal–DNA complex by tumor cell phagocytosis, compared to normal human cells and was also greater in lung tumors than in surrounding normal tissues in vivo [Ito et al., 2003]. Nanoparticles can also be employed to mediate gene therapy. However, although DOTAP:Chol:nanoparticles complexed to DNA (DNA–nanoparticles) have been shown to be efficient vectors for systemic therapy, induction of a dose-dependent inflammatory response, due to induction of multiple signaling molecules both in vitro and in vivo that are associated with inflammation, has also been observed thereby limiting its use [Gopalan et al., 2004]. Use of small molecule inhibitors against the signaling molecules resulted in their suppression and thereby reduced inflammation without affecting transgene expression. Liposomes with improved transfection efficiencies but with reduced cytotoxicities, such as heterocyclic cationic liposomes, are actively being developed [Ilies et al., 2005].

An innovative alternative for high-efficiency gene transfer into specific cells exploits the affinity of certain ligands for cell surface receptors. The gene, conjugated to the ligand to make a ligand–DNA complex, enters the cell upon internalization of the ligand–receptor complex. However, the internalized conjugates tend to be trapped within the endosomes and are rapidly degraded. More recently, polylysine–DNA conjugates that also incorporate Ad capsid proteins, which have endosomolytic activity to allow the DNA to escape degradation and enter the nucleus to be expressed, resulted in fourfold higher reporter gene expression in up to 99% of cells both in vitro and in vivo [Cristiano and Roth, 1996].

Biological vectors in the form of genetically modified, replication-defective viruses are much more effective by exploiting their natural tropism for mammalian cells and biological life cycles to achieve gene transfer and gene expression. Retroviral vectors can infect a variety of cell types and have the advantage of being able to integrate into the target cell genome. However, because retroviral-mediated transduction might result in permanent integration of the foreign gene into the target cell, the promoter used to drive the transcription of the foreign gene must be carefully selected. Retroviral vectors, however, are generated at low titer, infect only dividing cells with low transduction efficiency, and result in variable expression levels. The addition of a selectable marker, such as the hygromycin-resistance gene, during retrovirus vector construction is useful in vitro by allowing selection of transduced cells despite the low gene transfer efficiency of retrovirus vectors. The ability for retroviruses to transfer genes only into dividing cells, such as tumor cells, may protect surrounding quiescent normal cells when used in vivo, although the proportion of actively dividing tumor cells within a given

tumor during retroviral vector administration would likely be small. However, factors may inhibit retroviral transduction such as soluble chondroitin sulfate-proteoglycans/glycosaminoglycans (CS-PG/GAGs) in malignant pleural effusions (MPE) [Batra et al., 2000].

Due to the limitations of retrovirus vectors, replication-defective recombinant Ad vectors have been developed that can be produced at high titer (10^{10}–10^{12} pfu/ml), can infect non-dividing cells at high efficiencies (up to 100%), and result in high expression levels. Deletion of the *E1a* and *E1b* genes, and often also the *E3* gene, to make replication-defective Ad vectors requires that these genes be provided in *trans* by the 293 producer line during vector production but allows up to 7.5 kb of new DNA to be introduced into the recombinant vectors. Ad vectors can infect a variety of cell types and have been successfully used to transduce lung cancer cell lines in vitro and tumors in vivo. However, Ad transduction efficiency is directly related to coxsackie-adenovirus receptor (CAR) expression at both the mRNA and protein level of NSCLC cells in vitro as well as in transplanted NSCLC xenografts in vivo. Xenografts generated from the NSCLC cells, that exhibited increased CAR expression and uniform transduction in vitro, also showed evidence of higher Ad-mediated gene transfer ranging from 10% to 70% after a single intra-tumoral injection into the xenografts [Qin et al., 2003]. Ad vectors are non-integrative and, therefore, result in only transient expression of the inserted gene over a few weeks to months. Moreover, Ad-transduced cells elicit an immune response in vivo that results in their destruction and the production of neutralizing anti-viral antibodies that prevent multiple vector administration. Nevertheless, these properties are quite suitable for strategies requiring transient bursts of high-level expression.

However, MPE can inhibit Ad-mediated gene transfer by inhibiting Ad binding and internalization into target cells, although this inhibition is not due to CS-PG/GAGS, hyaluronic acid, or fibronectin [Batra et al., 2000, 2003]. Further, sepharose A-mediated immunoglobulin depletion of MPE only partially reverses the block, and significant inhibition to Ad gene transfer persists at lower Ad:target cell ratios [Batra et al., 2000]. New evidence suggests that predominantly anti-Ad antibodies are responsible for this inhibition, but other non-specific factors such as albumin and specific factors such as soluble CAR found in MPE also inhibit Ad gene delivery, especially in certain contexts (e.g., within the extracellular matrix of solid tumors) where the concentrations of secreted (or shed) CAR may be high [Bernal et al., 2002].

Low gene transfer due to a combination of factors in vivo may be the most substantial hurdle in the practical application of gene therapy. Conventional E1-deleted Ad can become replication-competent into tumors when co-transduced with a vector that selectively supplies E1 in *trans*, resulting in selective production of large numbers of the E1-deleted Ad within the tumor mass, which will increase the transduction efficiency. For example, a vector that produces a mutant E1 without the ability to bind retinoblastoma but retaining viral replication competence in cancer cells with a defective p*Rb*/*p16*, used in combination with an E1-deleted Ad encoding the beta-galactosidase reporter gene (*Ad–lacZ*) increased the transduction efficiency of lacZ to 100% from 15% observed with *Ad–lacZ* alone and enabled the transfer of the *lacZ* gene using media from the cultured cells ("media-transferable bystander effect") [Lee et al., 2004].

Adeno-associated virus (AAV) vectors, small parvoviruses, which are ubiquitous but not pathogenic to humans, are able to frequently integrate multiple concatemeric copies of itself into the target cell genome. These AAV vectors are trophic for a wide variety of cell types and have been successfully used to transduce lung cancer cell lines in vitro [Shi et al., 2005]. However, the full potential of these vectors for in vitro and in vivo gene transfer has not been determined. Many current AAV vector generation methods are cumbersome and require the development of packaging cell lines and better purification techniques, which could make AAV the vector of choice in future gene therapy trials.

A variety of other viral vectors are also under investigation; however, viral-mediated cancer gene therapy has been currently limited to treating localized tumors due to host-immunity against the gene delivery vector and the transgene. Therefore, there is a tremendous effort to develop and test alternate gene delivery vectors that are efficient, non-immunogenic, and applicable for systemic therapy.

Choice of Therapeutic Gene

The therapeutic gene to be transferred fall in one of six classes: anti-sense, RNAi, or ribozyme

sequences against oncogene transcripts; tumor suppressor gene replacement; suicide genes; cell surface antigens; cytokine genes; and multiple drug resistance genes. Oncogene inhibition or tumor suppressor gene replacement could correct the abnormal malignant phenotype. Suicide genes would provide transduced tumor cells with enzymatic machinery to convert otherwise non-toxic substances into toxic metabolites. Similarly, the transferred gene could render drug-resistant tumor cells more sensitive to cytotoxic drugs. Delivery of genes that encode tumor-specific antigen, MHC, adhesion, co-stimulatory, or cytokine molecules would result in better tumor/immune cell interaction and in stimulation of the immune response.

LUNG CANCER GENE THERAPY RESULTS

Oncogene Inhibition Therapy

Since oncogenes are critical in bronchogenic carcinogenesis, inhibiting these genes has become a novel way to treat lung cancer. Oncogene inhibition therapy can be performed using anti-sense, RNA interference (RNAi), or ribozyme technologies. The most common approach to inhibit endogenous oncogene expression is to introduce into tumor cells either single-strand anti-sense oligonucleotides or else plasmid or viral vectors containing cDNA constructs, which encode for single-strand anti-sense RNA molecules, that are complementary to oncogene (sense) mRNAs [Roth et al., 1992; Prins et al., 1993]. The anti-sense and sense sequences then bind, which inhibits translation and mutant protein production by blocking ribosome access to the mRNA or by bringing about ribonuclease-mediated degradation of any resultant double-stranded RNA. For example, expression of mutant *K-ras* oncogene can be eliminated by transfection of anti-sense *K-ras* RNA constructs or cDNA plasmids into human lung cancer cell lines [Zhang and Roth, 1994]. The anti-sense RNA constructs can selectively block mutant *K-ras* oncogene expression and reduce tumor growth in mice, despite the presence of five additional chromosomal deletions [Mukhopadhyay et al., 1991]. Transduction of a human lung cancer cell line, that has mutations in both endogenous *ras* alleles, with retroviral vectors encoding anti-sense *K-ras* constructs resulted in decreased colony formation in agarose and decreased tumorigenicity in *nu/nu* mice [Zhang et al., 1993]. Intratracheal instillation of anti-sense *K-ras* using this retroviral vector also prevented the growth of human lung cancer cells in up to 90% of *nu/nu* mice [Georges et al., 1993]. Similar treatment of murine lung cancer cells with an anti-sense cyclin D1 construct reduced in vitro and in vivo proliferation and tumorigenicity by inhibition of cyclin D1 oncogene expression [Schrump et al., 1996].

Alternatively, both the growth factor ligand and receptor can be targeted by inhibitory anti-sense constructs. Blockade of IGF-I and IGF-IR by anti-sense or dominant negative plasmid transfection can also suppress tumorigenicity and induce regression of established tumors [Lee et al., 2003]. Moreover, an anti-sense IGF-IR Ad construct administered intraperitoneally resulted in prolonged survival of nude mice bearing H640 lung cancer xenografts [Lee et al., 1996]. Anti-sense oligodeoxynucleotides to IGF-II significantly inhibited, by 25–60%, the in vitro growth of all six IGF-IR/IGF-II-positive lung cancer cell lines studied, although, the best results (growth inhibition of up to 80%) were achieved with concomitant anti-sense treatment against both IGF-IR and IGF-II [Pavelic et al., 2002].

RNAi, also called post-transcriptional gene silencing, is a slightly different strategy in which dsRNA molecules are transferred into a cell in which the anti-sense and sense RNA strands as well as the dsRNA molecules themselves have inhibitory effects on the exon (but not promoter or intron) sequences in mature target homologous mRNA [Fire et al., 2005]. Only a few dsRNA molecules per cell are required to produce effective interference, which attests to the remarkable potency of RNAi. The dsRNA is cleaved into 21–25-bp siRNAs by dicer, a RNA nuclease with a helicase domain and dual RNAse III motifs that specifically cleaves dsRNAs, thus producing multiple "effector" molecules from a single dsRNA molecule [Hamilton and Baulcombe, 1999; Bernstein et al., 2001]. The siRNA–dicer complex can further recruit additional components to form an RNA-induced silencing complex (RISC) in which the unwound siRNA base pairs with complementary mRNA and guides the RNAi machinery to target mRNA resulting in the effective cleavage of the target mRNA, at sites 21–25 nucleotides apart only within the region of identity with the dsRNA, and subsequent catalytic degradation of potentially multiple target mRNAs [Hammond et al., 2000; Zamore et al., 2000; Pham et al., 2004]. In

addition, RNA-dependent RNA polymerases have also been found to effect the RNAi response and may result in increased viral susceptibility [Hutvagner and Zamore, 2002]. For example, increase of Skp-2, which is a protein involved in the degradation of cell-cycle regulators including p27Kip1, *p21*, and c-*myc*, is one of the important mechanisms for dysregulation of cell cycles in various cancers [Sumimoto et al., 2005]. HIV-lentiviral-mediated RNAi against Skp-2, in a human SCLC cell line with increased Skp-2, resulted in inhibition of in vitro growth of cancer cells with increased Skp-2 through the increase of p27Kip1 and *p21*, but no significant effect on the growth of cells without high Skp-2 expression. Furthermore, intra-tumoral administration of Ad siRNA vector against Skp-2 efficiently inhibited growth of established subcutaneous SCLC tumor on NOD/SCID mice [Sumimoto et al., 2005].

Alternatively, reduction or blockade of oncogene expression can involve the use of ribozymes, which are RNA molecules catalytically capable of site-specific cleavage of mRNA, such as oncogene transcripts. For example, an Ad vector with a hammerhead ribozyme transgene, that is specific for the *K-ras* codon 12 mutant sequence GUU, was constructed that had in vivo anti-tumor effects against NSCLC xenografts expressing the relevant *K-ras* mutation GTT in athymic mice [Zhang et al., 2000a,b].

Lastly, an oncogene can also be inhibited by the transfer of a different gene whose expression blocks the activated oncogene's expression, activity, or signaling. The Ad-type-5 early region 1A (*E1A*) gene product can suppress *HER-2/neu*-mediated malignant transformation by inhibition of *HER-2/neu* expression. Intravenous tail injections of an Ad E1A construct in a mouse model inhibited the intratracheal growth of *HER-2/neu*-overexpressing lung cancer cells [Chang et al., 1996]. Aerosolized delivery of Ad-MKK4 (KR), an Ad vector expressing dominant-negative mutant mitogen-activated protein kinase kinase 4 (MKK4), can block *ras*-dependent signaling in *K-ras*(LA1) mice, which develop lung adenocarcinomas through activation of a latent allele carrying mutant *K-ras*(G12D) [Lee et al., 2002]. MKK4 was activated in the lungs of *K-ras*(LA1) mice, and aerosolized treatment with Ad-MKK4 (KR) decreased c-Jun-NH(2)-terminal kinase but not extracellular signal-regulated kinase activity, providing evidence that *ras* was selectively inhibited [Lee et al., 2002].

Dominant negative inhibition can result from the competition with normal growth factor receptor for ligand by defective receptor, for example, with defective subunits, or alternatively by a soluble receptor or binding protein. The transduction of either Ad-IGF-IR/950, which encodes a defective receptor with an intact alpha subunit but a defective beta subunit lacking the tyrosine kinase domain, or Ad-IGF-IR/482, which encodes the soluble extracellular domain, could effectively block IGF-I-induced Akt kinase activation and blunt the growth-stimulatory effect of IGF-I on human lung cancer cell lines [Lee et al., 2003]. Intra-tumoral injection of Ad-IGF-IR/482 vector also showed significant growth suppression in established lung cancer xenografts. Similarly, exogenous IGFBP-3 stably transfected into NSCLC line NCI-H23 was shown to block IGF action, inhibit cell growth in vitro, and abrogated the proliferation effects of IGF-I, IGF-II, IGF-I analog Long R(3) IGF-I or insulin [Hochscheid et al., 2000]. Further, xenotransplantation in nude mice resulted in marked tumor growth after the injection of control NSCLC cells, but absent or minimal growth for the IGFBP-3-transfected NSCLC cells. Unfortunately, these approaches do not result in elimination of non-transduced tumor cells nor do they convey systemic immunity. Nevertheless, the first gene therapy clinical trial specifically against lung cancer and involving oncogene, namely *K-ras*, modification was approved by the NIH Recombinant DNA Advisory Committee (RAC) and enrolled its first patient in 1995 [Roth, 1996a].

Tumor Suppressor Gene Therapy

Introduction of a single functioning copy of a wild-type tumor suppressor gene into tumor cells, in which both endogenous alleles have been mutated or deleted, may be able to restore either cell-cycle arrest or apoptosis and thereby reverse the malignant phenotype [Roth, 1998a]. The products of the *p16* tumor suppressor gene and a truncated *Rb* gene, have been shown to suppress tumor growth in animal models [Jin et al., 1995; Xu et al., 1996]. Intra-tumoral administration of the *FUS1* gene into two human NSCLC (H1299 and A549) subcutaneous xenografts in vivo using the DOTAP:Chol liposome also resulted in inhibition of tumor

growth [Ito et al., 2004]. Furthermore, intravenous injections of DOTAP:Chol–FUS1 complex into mice bearing experimental A549 lung metastasis decreased the number of metastatic tumor nodules and resulted in prolonged compared to control animals [Ito et al., 2004]. A Phase-I trial for systemic treatment of lung cancer using DOTAP:Chol:nanoparticles and FUS1 has been undertaken [Gopalan et al., 2004].

Wild-type *p53* genes introduced into normal bronchial epithelial cells inhibit subsequent malignant transformation and tumorigenicity and exhibited minimal toxicity, which suggests a therapeutic index and avoids the need for selective tumor targeting within the lung [Willey and Harris, 1990]. Restoration of wt-*p53* gene expression using a retroviral *p53* vector inhibited cell growth and induced apoptosis in human lung cancer cells with mutated or deleted *p53* genes [Cai et al., 1993; Fujiwara et al., 1993]. Even transient *p53* expression after transduction of *p53*-negative human lung cancer cells either with an Ad vector encoding wt-*p53* or with an Ad-DNA complex carrying a *p53*-expressing plasmid resulted in induction of apoptosis in vitro and resulted in inhibition of tumorigenicity in vivo with dose-related tumor growth suppression [Zhang et al., 1994a,b; Nguyen et al., 1997]. Intratracheal instillation of the retrovirus-*p53* vector prevented the growth of established human lung cancer tumors in *nu/nu* mice [Fujiwara et al., 1994a]. In an effort to circumvent the limitations of viral vectors, efficient delivery of *p53* using an extruded DOTAP:Chol cationic liposome resulted in transgene expression in 25% of tumor cells in localized human primary lung cancers and 10% in experimental disseminated metastatic tumors, growth suppression in both primary and metastatic lung tumors, with repeated multiple treatments showing a 2.5-fold increase in gene expression and increased therapeutic efficacy compared to single treatment, and prolonged animal survival when treated with liposome-*p53* DNA complex [Ramesh et al., 2001].

Despite the presence of multiple other genetic defects in lung cancer cells, replacement of wt-*p53* into cells containing mutant or deleted *p53* is sufficient to cause apoptosis or growth arrest [Takahashi et al., 1992]. Even cell lines with active c-*Ha-ras* oncogenes display suppressed tumorigenicity when a tumor suppressor gene is introduced [Willey and Harris, 1990]. Moreover, experiments in which transduced human lung cancer cells were mixed with non-transduced lung cancer cells resulted in a slowed growth rate in the non-transduced cells [Cai et al., 1993]. This observation suggested the occurrence of a bystander effect, although the mechanism by which this effect is mediated in vitro has not been characterized. Antiangiogenesis may explain this bystander effect in vivo in part as evidenced by the inhibition of expression of VEGF and a novel antiangiogenic factor, brain-specific angiogenesis inhibitor 1, resulting in reduced neovascularization in vivo using in nu/nu mice a subcutaneous membrane-diffusion chamber system containing Ad-transduced *p53*-expressing NSCLC cells mixed with non-transduced NSCLC cells [Nishizaki et al., 1999].

These results have led to several clinical trials involving modification of tumor suppressor (namely *p53*) gene expression in advanced NSCLC, with the first clinical protocol being approved by the NIH RAC and enrolling its first patient in 1995 [Roth, 1996c]. Retroviral wt-*p53* supernatant was directly injected into endobronchial tumors under bronchoscopic guidance in four patients and CT guidance in five patients [Roth et al., 1996]. Three of seven evaluable patients showed evidence of tumor regression and one of these patients demonstrated no viable tumor 3 months after injection. Anti-tumor activity was demonstrated in six of seven evaluable post-treatment tumor biopsies by the presence of apoptotic cells. The presence of wt-*p53* was demonstrated in the biopsied tumor specimens of three patients previously documented to have *p53* mutations [Roth et al., 1996]. This preliminary trial demonstrated the feasibility and safety of gene therapy strategies in advanced NSCLC based on the restoration of wt-*p53* gene function [Roth, 1998b; Roth et al., 1999; Swisher and Roth, 2000].

The use of retroviral vector in large-scale clinical trials, however, is limited by the inability to achieve the high titers of virus ($>10^7$) needed to treat large tumors and by the cumbersome need for a packaging cell line to produce retroviral vectors on a large scale. Ad vectors can be produced at substantially higher viral titers ($>10^{11}$) than retroviral vectors and transduce lung cancer cells regardless of mitosis status. The efficacy of wt-*p53*-containing Ad vectors (Ad*p53*) was evaluated against NSCLC

in a series of NIH RAC-approved clinical trials, with the first patient being enrolled in October 1995 [Roth, 1996b]. A total of 28 patients, whose tumors had progressed on conventional treatment, were treated with Ad*p53*. Monthly injections (up to 6 months, if no progression) of Ad*p53* were given under CT or bronchoscopic guidance. The initial Ad*p53* dose of 10^6 PFU was escalated logfold to 10^{11} PFU in subsequent patients. Development of serum antibodies to Ad with multiple doses was inconsistent, and vector-related toxicity was minimal with only one patient developing a Grade 3 or higher toxicity in 84 courses of treatment [Yen et al., 2000]. Clinical responses in 25 evaluable patients included partial response in 2 patients and disease stabilization in 16 patients was durable for 2–28 months [Swisher et al., 1991, 1999]. Patients (6 of the 12) who had bronchoscopic intra-tumoral injections had significant improvement in airway obstruction, and 3 patients met the criteria for partial response [Weill et al., 2000]. Transgene wt-*p53* expression was demonstrated by reverse transcription-PCR in 12 of 26 evaluable post-treatment tumor specimens and increased apoptosis in 11 of 24 evaluable patients. Time to disease progression was enhanced by higher vector doses and evidence of apoptosis in tumor biopsy specimens [Roth et al., 1998]. A Phase I pilot trial of Ad-*p53* against SCLC and another against bronchioloalveolar cell lung carcinoma (BAC) administered by bronchoalveolar lavage have been proposed to the NIH RAC [OBA Documents, 2002].

The gene transfer of wt-*p53* may act in part by inducing downstream pro-apoptotic genes such as *Bax* and *Bak*. Another potential gene therapy strategy, therefore, involves gene transfer and overexpression of pro-apoptotic genes downstream from *p53*. This anti-tumor effect was indeed seen with Ad-mediated *bax* gene therapy both in vitro and in vivo and occurred independent of the tumor's *p53* status [Kagawa et al., 2000]. Additionally, other downstream mediators of wt-*p53* such as *p21* WAF1, which causes cell-cycle arrest at G_1, have been shown to inhibit lung cancer cells when overexpressed by Ad vectors [Joshi et al., 1998]. Intra-tumoral injection of rAd/21 significantly suppressed tumor growth and prolonged survival in rAd/21 treated mice.

Overexpression of human melanoma differentiation-associated gene-7 (mda-7) protein in human NSCLC cells in vitro by Ad-mda-7 significantly suppressed proliferation and induced G_2/M cell-cycle arrest in wt-*p53* (A549, H460) and *p53*-null (H1299) NSCLC cells, but not in normal human lung fibroblasts [Saeki et al., 2000]. Expression of *p53*, *Bax*, and *Bak* proteins was upregulated in wt-*p53* tumor cell lines, but not in *p53*-null cells, suggesting that an intact *p53* pathway was required for *Bax* and *Bak* induction. However, in all three cancer cell lines tested, activation of the caspase cascade and cleavage of poly(ADP-ribose) polymerase appeared to be independent of the *p53* mutational status [Saeki et al., 2000].

Efficient delivery of tumor suppressor gene FHIT to localized human primary lung cancers and to experimental disseminated metastases using DOTAP:Chol-cationic liposome resulted in transgene expression in 25% of tumor cells in primary tumors and 10% in disseminated tumors and growth suppression in both primary and metastatic lung tumors, with repeated multiple treatments showing a 2.5-fold increase in gene expression and increased therapeutic efficacy compared to single treatment [Ramesh et al., 2001]. Ad-mediated overexpression of FHIT also resulted in significant inhibition of human lung cancer cells both in vitro and in vivo [Ji et al., 1999]. Ad-mediated coexpression of FHIT and *p53* synergistically inhibited tumor cell proliferation in NSCLC cells in vitro and suppressed the growth of human tumor xenografts in nude mice, which corresponded with FHIT-mediated inactivation of *mdm2*, which thereby blocked the association of *mdm2* with *p53*, thus stabilizing the *p53* protein [Nishizaki et al., 2004].

Proapoptotic genes in the tumor necrosis factor receptor superfamily, such as the *fas* gene, and the tumor necrosis factor-related apoptosis-inducing ligand, TRAIL, can also be used therapeutically [Shinoura et al., 1998; Shi et al., 2005]. The in vitro transfer of *TRAIL* gene elicited apoptosis, activation of pro-caspase-8 and cleavage of poly(ADP-ribose) polymerase [Kagawa et al., 2001]. The intra-tumoral delivery of *TRAIL* gene elicited tumor cell apoptosis and suppressed tumor growth, but no detectable toxicity in cultured normal fibroblasts nor in mouse hepatocytes after systemic gene delivery. Transduction of A549 lung adenocarcinoma cells with an AAV vector encoding just the extracellular domain of TRAIL (amino acids 114–281) induced apoptosis in these cells [Shi et al.,

2005]. When the transduced A549 cells, which now stably express TRAIL, were implanted subcutaneously or orthotopically into nude mice, the tumor-bearing mice showed significantly reduced tumor growth and prolonged survival compared to those bearing non-transduced A549 tumors with absence of detectable toxicity in normal tissues including the liver [Shi et al., 2005]. Both TRAIL- and *Bax*-expressing vectors separately elicited significant cell killing in H1299 lung cancer cells, while combined TRAIL and *Bax* therapy produced more profound cell killing in vitro without escalation of the vector doses [Huang et al., 2002]. Furthermore, co-culture of cancer cells expressing TRAIL with those expressing green fluorescent protein (GFP) resulted in apoptosis of both cells, whereas co-culture of *Bax*-expressing cells with GFP-expressing cells resulted in the cell death of the *Bax*-expressing cells only, which suggested that the transfer of the *TRAIL* gene resulted in bystander effects, although, the bystander effect is not transferable via the medium [Kagawa et al., 2001].

Alternatively, apoptosis may also be induced by suppressing inhibitors of apoptosis, such as mutant *p53* gene products or nuclear factor-kappaB (NF-kappaB). Retrovirus-mediated transduction of a hammerhead ribozyme (Rz5a) designed to cleave unspliced *p53* RNA at codon 187 near the boundary of intron 5 and exon 6 reduced the level of mutant *p53* RNA and protein in the human H226Br lung cancer cell line, which contains a homozygous *p53* mutation at codon 254 [Cai et al., 1995]. The specific cleavage of the mutant *p53* pre-mRNA by the Rz5a ribozyme significantly suppressed the growth of the H226Br cells in culture. Ad overexpression of a "super-repressor" form of the *NF-kappaB* gene sensitized lung cancers to apoptosis [Batra et al., 1999]. Perhaps combinations of this "super-repressor" with other pro-apoptotic agents will result in enhanced tumor kill without concomitant increases in normal cell toxicity.

Lastly, apoptosis pathways independent of *p53* are also being targeted. The potential effects of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumor suppressor gene on Akt downstream pathways were investigated using an aerosol containing glucosylated-conjugated polyethylenimine (glucosylated PEI) and recombinant PTEN complex delivered into *K-ras* null lung cancer model mice through a nose-only inhalation system [Kim et al., 2004]. Expression levels of proteins in the phosphatidylinositol 3′-kinase/Akt signaling pathway in PTEN-delivered mouse lung, as well as the kinase activities of both Akt and mTOR, were decreased to varying degrees, and apoptosis was detected [Kim et al., 2004].

Antiangiogenesis Gene Therapy

Angiogenesis is an absolute requirement for tumor growth beyond 2 cubic millimeters in size. Wild-type *p53* expression by tumor cells has been suggested to be antiangiogenic due to its inhibitory effect on VEGF expression and stimulatory effect on brain-specific angiogenesis inhibitor-1 expression [Nishizaki et al., 1999]. NSCLC tumors also have been found to have an imbalance in expression of angiogenic (ELR+) compared with angiostatic (ELR−) CXC chemokines that favors angiogenesis and progressive tumor growth. Increased expression of ELR(+) CXC chemokines found in NSCLC tumor samples has been found to be not counter-regulated by a concomitant increase in the expression of the angiostatic CXC chemokine monokine induced by interferon gamma (MIG), thus favoring neovascularization [Addison et al., 2000]. Overexpression of the ELR(−) CXC chemokine MIG, by gene transfer, results in the inhibition of NSCLC tumor growth and metastasis via a decrease in tumor-derived vessel density.

Suicide Gene Therapy

The herpes simplex virus thymidine kinase (*HSV-tk*) gene has been the most commonly studied suicide gene. This enzyme can specifically bind and phosphorylate nucleoside analogs such as acyclovir and ganciclovir (GCV), whereas the endogenous mammalian thymidine kinase cannot. Cells, which express *HSV-tk* and subsequently try to replicate, incorporate phosphorylated GCV, which blocks DNA synthesis and causes cell death. Tumors surrounded by non-dividing cells are ideally suited for suicide gene therapy. Human lung cancer cells have been shown to be selectively killed after transduction with retrovirus vectors carrying the *HSV-tk* gene and systemic administration of GCV [Hasegawa et al., 1993].

This delivery method would most likely result in low transduction efficiency, but a bystander effect has been shown to result in the death of surrounding non-transduced tumor cells. This phenomenon may be due to the passage of

phosphorylated GCV from the transduced cells to adjacent non-transduced tumor cells via gap junctions. With as low as 10% transduction efficiency, complete tumor regression can be achieved in animal models [Ram et al., 1993]. However, this phenomenon is limited to tumor cells in close proximity to the *HSV-tk*-expressing cells and does not result in systemic immunity. Nevertheless, a Phase I trial of Ad-vector delivery of *HSV-tk* by intra-tumoral injection followed by intravenous GCV in advanced NSCLC patients was proposed to the NIH RAC and forwarded to the FDA [Rom and Woo, 1999; OBA Documents, 2002].

Preliminary attempts to apply radioiodide therapy, effective in thyroid cancer, to lung cancer was performed by transfection of NSCLC cells with both human sodium iodide symporter (*NIS*) gene, which allows rapid internalization of iodide into cells, and thyroperoxidase (TPO), which catalyzes iodination of proteins and subsequently causes iodide retention within cells and resulted in an increase in radioiodide uptake and retention and enhanced NSCLC tumor cell apoptosis, while single gene therapy with only the *NIS* gene may have limited efficacy because of rapid efflux of radioiodide [Huang et al., 2001].

Cytokine-Mediated Tumor Vaccines

Administration of recombinant cytokines, especially when delivered directly into tumor sites, has resulted in significant anti-tumor effects. However, cytokines have systemic side-effects and short serum half-lives which limit their therapeutic index. Several investigators have attempted to enhance the host anti-tumor response and induce immunity by introducing cytokine genes into tumor cells, such as lung cancer cells [Dubinett and Kradin, 1993], or even other cells, such as fibroblasts when tumor cells could not be grown [Miller et al., 1993; Lotze et al., 1994]. However, one group has demonstrated that induction of an anti-tumor response is more effective by a cytokine-secreting tumor cell than by cytokine-secreting fibroblasts [Tsai et al., 1993].

Cytokines can be introduced into tumor cells either in vivo, in which the gene is somehow delivered directly into the tumor or systemically with the hope of targeting to the tumor, or ex vivo, in which the tumor is explanted and the gene transferred to the tumor cells in culture. In the former, the cytokine-producing transduced tumor cells would induce an inflammatory response that results in recruitment of antigen presenting cells (APCs), such as monocytes and DCs. Antigen from degraded tumor cells would then be taken up by the APCs, processed, and presented to T-cells, which could then respond to even non-transduced tumors expressing tumor antigens, so called cross-presentation. With the ex vivo approach, the irradiated transduced tumor cells could then be re-injected either close to accessible established tumors to induce bystander effect or at remote sites to act as vaccines. Either method would then lead to highly localized secretion of therapeutic levels of the cytokine, recruitment of immune cells into the tumor site, and enhancement of tumor-specific antigen presentation. These events could potentially educate the immune mediator cells and confer effective systemic immunity to the patient against any concurrent micrometastatic disease and possibly future challenges by that particular tumor type.

In attempts to achieve high concentrations of the cytokines inside the tumor without systemic toxicity, producer cells of recombinant Ad vectors encoding for human interleukin-1 (IL-1) alpha and for rat interleukin-3 (IL-3) beta were injected into NSCLC tumors, although this method resulted in limited tumor growth delay [Esandi et al., 1998]. When freeze-thawed stocks of pooled producer cells allowed intensive treatment of groups of tumor bearing rats and induced reproducible tumor responses thought to be due to local release of cytokines rather than to systemic effects. However, growth retardation also occurred in contralateral tumors, which were not injected. In addition, rats carrying established tumors and vaccinated with lysates of tumors collected from other rats treated with these "cracked" producer cells also exhibited significant tumor growth retardation [Esandi et al., 1998]. Interleukin-6 has been able to be introduced into lung cancer cells to induce an anti-tumor effect. One group [Porgador et al., 1992] reported decreased tumorigenicity of IL-6-transduced lung carcinoma cells and resulted in T-cell-dependent systemic immunity against rechallenge as well as against metastases from non-transduced parental cells. Another cytokine, interleukin-7 (IL-7) both stimulates CTL responses and downregulates tumor production of the immunosuppressive peptide TGF-beta. Lung cancer cell lines were transduced with a retroviral vector encoding for

IL-7 and exhibited a decreased proliferation of the IL7-transduced tumor cells in vitro compared to control-vector-transduced and parental tumor cells [Sharma et al., 1996]. Interleukin-7 transduction did not alter IL-7 receptor expression but was found to alter tumor cell expression of intracellular adhesion molecule 1, MHC-1, lymphocyte function-related antigen 3, very late activation antigen beta 1, and p185neu [Sharma et al., 1996]. Moreover, peripheral blood lymphocytes co-cultured with either IL-7-transduced tumor cells or tumor supernatants had enhanced cytolytic and proliferative capacities compared with co-culture with control vector-transduced or parental cells.

These observations suggest that cytokines, if produced at sufficiently high concentrations in tumors, induce inflammation and enhance immunogenicity, which in turn initiates a systemic immune response against similar tumors growing at distant sites. A Phase I/II trial evaluating intra-tumoral injection of IL-7 gene-modified autologous DCs for the treatment of NSCLC is currently underway [OBA Documents, 2002]. Conversely, the use of transfected cancer cells expressing interleukin-2 (IL-2) to treat limited stage SCLC and the use autologous, lethally irradiated NSCLC cells engineered by Ad-mediated gene transfer to secrete human granulocyte–macrophage colony stimulating factor (GM-CSF) in Phase I studies have been proposed to the NIH RAC and forwarded to the FDA [Cassileth et al., 1995; Dranoff and Salgia, 1998; OBA Documents, 2002]. A similar Phase I/II study, using GM-CSF gene-modified autologous tumor vaccines against early and advanced stage NSCLC, and another, using irradiated autologous lung tumor cells mixed with a GM-CSF-secreting bystander lung cell lines as a vaccine, have been proposed to the NIH RAC [OBA Documents, 2002]. A Phase II study of anti-sense TGF-beta together with the *IL-2* gene transfected into allogeneic tumor cells as a vaccine in non-curable Stage IIIB and IV NSCLC patients was proposed in 1999, although the co-transfection with the *IL-2* gene was subsequently withdrawn in a 2,000 protocol [OBA Documents, 2002]. In an alternative approach, in vivo transfer of the CD40 ligand gene to primary lung tumors to activate DCs and induce anti-tumor immunity has been proposed to the NIH RAC [OBA Documents, 2002].

Results from recent human clinical trials with cytokine gene-modified tumor cells have provided hints of efficacy. In a Phase I/II trial in patients with early and advanced stage non-small-cell lung cancer, a vaccine consisting of autologous tumor modified with an Ad vector encoding GM-CSF, resulted in complete clinical responses in 3 of 33 advanced-stage patients, two of whom had BAC [Nemunaitis et al., 2004]. The importance of technical factors such as cytokine production by the tumor was demonstrated by longer survival in patients who received vaccines secreting GM-CSF at more than 40 ng/24 h per 10(6) cells.

OVERCOMING LIMITATIONS OF GENE THERAPY

Strategies to Improve Tumor-Specific Transgene Expression

Viral vectors can be modified to target specific organs, such as the lungs, or to target specific tissues, such as lung tumors by modifying capsids proteins. Ad vectors are trophic for the lung due to expression of CAR on airway epithelial cells. However, lung cancer cells have been shown to have marked variations in CAR expression but to show relatively similar levels of fibroblast growth factor receptor (FGFR) expression [Qin et al., 2005]. One group has been able to develop FGFR-retargeted Ad vectors that could prove useful against CAR-deficient lung cancer cells [Qin et al., 2005].

Alternatively, Ad-mediated wt-*p53* transduced cells treated with 2-methoxyestradiol (2-MeOE2), which induces and stabilizes wt-*p53* protein levels in cancer cells, resulted in superinduction of wt-*p53* expression, apoptosis, and 80% growth inhibition in vitro regardless of endogenous *p53* status [Mukhopadhyay and Roth, 1998]. Systemic administration of Ad vectors expressing wt-*p53* combined with 2-MeOE2 resulted in greater than additive reduction of lung colony counts in vivo [Kataoka et al., 1998].

Besides modification of the vector in order to reduce cytotoxicity to normal cells, tissue-specific and tumor-specific promoters and enhancers can be used to control transgene expression. For example, since about 85% of SCLC tumors overexpress *myc*, the use of the *myc*–max response element (MMRE) to induce increased expression levels of transgenes, such as a proapoptotic or suicide gene, can be a

promising gene therapy strategy against SCLC [Song, 2005a,b]. Apoptin, which is derived from chicken anemia virus, has been shown to induce tumor-specific apoptosis but not in normal cells. Ad-mediated transduction of SCLC cells with the apoptin gene under control of MMRE and the SV40 promoter/enhancer resulted in significantly suppressed growth and increased apoptosis [Song, 2005b]. Similarly, the telomerase catalytic subunit, hTERT, is expressed only in cells and tissues positive for telomerase activity, such as tumor or stem cells. About 60% of SCLC tumors are telomerase-positive, and use of the hTERT promoter resulted in strong Ad-mediated luciferase expression only in telomerase-positive SCLC cells, but not in normal cells [Song, 2005a].

Combination Gene Therapy Approaches

Because lung cancers are expected to have multiple genetic abnormalities, a multimodality gene therapy approach may be more effective than one based on a single gene. To test the feasibility of such an approach, a retroviral vector was constructed containing both the *IL-7* gene and the *HSV-tk* suicide gene [Sharma et al., 1997]. When the weakly immunogenic murine alveolar cell carcinoma, L1C2, was transduced with both the *IL-6* and *HSV-tk* genes, they became significantly more sensitive to ganciclovir (GCV) in vitro than unmodified parental tumor cells. Growth of these transduced L1C2 cells in vivo in mice was also inversely related to the amount of IL-7 that these cells secreted in vitro. Furthermore, these transduced L1C2 cells regressed in mice following GCV administration. Similar results were observed with L1C2 cells transduced with both the *GM-CSF* and *HSV-tk* genes [Miller et al., 1998].

While tumor cells transduced with both *IL-7* and *HSV-tk* genes were not effective in treating established parental tumors by themselves, the administration of bone marrow-derived, in vitro-activated DCs in combination with the transduced tumor cells and GCV eradicated 80% of established parental tumors in mice [Sharma et al., 1997]. Similar results were obtained with administration of DCs in combination with tumor cells transduced with both *GM-CSF* and *HSV-tk* genes in the presence of GCV [Miller et al., 1998].

In fact, the DCs themselves could be transduced with the *IL-7* gene (DC-AdIL-7) and be delivered intra-tumorally to induce an anti-tumor response [Miller et al., 2000; Sharma et al., 2003]. Intra-tumoral DC-AdIL-7 therapy was as effective as DCs pulsed with specific tumor peptide antigens, but comparison with other intra-tumoral therapies including recombinant IL-7, AdIL-7 vector alone, unmodified DCs, IL-7-transduced fibroblasts, or DCs pulsed with tumor lysates revealed DC-AdIL-7 therapy to be superior in achieving anti-tumor responses and augmenting immunogenicity [Miller et al., 2000]. All the DC-AdIL-7-treated mice completely rejected a secondary rechallenge, whereas the AdIL-7-treated mice had sustained anti-tumor effects in only 20–25% of the mice. After complete tumor eradication, those mice treated with DC-AdIL-7 demonstrated trafficking of gene-modified DCs from the tumor to lymph nodes (where they were detected for up to 7 days after intra-tumoral injection) and spleen and also significantly greater release of splenocyte GM-CSF and IFN-gamma than did controls or AdIL-7-treated mice [Miller et al., 2000].

These results with DCs are likely due to their function as APCs. The introduction of tumor-specific antigen genes in combination with cytokine genes could possibly enhance immunogenicity against tumor cells. Thus, a Phase I/II trial of antigen specific immunotherapy in MUC-1-positive patients with advanced NSCLC using vaccinia virus vectors expressing both MUC-1 antigen and IL-2 was proposed in 1998, although results are not yet available [OBA Documents, 2002].

Synergy With Conventional Therapies

Pre-clinical studies indicate that gene therapy may have useful synergy with cytotoxic and radiation therapy. Attempts to combine cytokine administration with the insertion of a tumor-specific cell surface antigen gene to enhance the immunogenicity of tumor cells have been proposed. A clinical trial was proposed in 1996 to treat SCLC patients in partial remission or at relapse with B7-1 gene-modified autologous tumor cells as a vaccine with systemic interferon-gamma (IFN-gamma), although the protocol never initiated and was closed in 1998 [Antonia, 1998; OBA Documents, 2002]. A similar Phase I study using HLA-B7/beta 2M as a plasmid DNA/DMRIE:DOPE lipid complex by direct gene transfer in combination with recurrent low-dose subcutaneous IL-2 therapy

as an immunotherapeutic regimen in lung and head and neck cancers was also proposed in 1999, although its status is not known [OBA Documents, 2002].

Chemotherapy and radiation therapy induce G_1 arrest and apoptosis in part by upregulation of wt-*p53* following DNA damage. Cells with mutated or null *p53* genes have increased resistance to chemotherapy or radiation-therapy. Thus, restoration of wt-*p53* function in tumors may increase chemosensitivity and radiosensitivity and induce apoptosis. The administration of Ad vectors with wt-*p53* in lung cancer cells prior to chemotherapy led to enhanced apoptosis both in vivo and in vitro [Nguyen et al., 1996] and was able to markedly increase the sensitivity of these lung cancer cells to cisplatin [Fujiwara et al., 1994b]. Conversely, the treatment of cancer cells with cisplatin prior to Adv-*p53* transduction resulted in a higher level of therapeutic gene expression [Cristiano et al., 1998].

Treating lung cancer cell lines using low-dose 5-aza-2′-deoxycytidine (DAC) causes an accumulation of procaspase-9 through mRNA upregulation without the cells undergoing apoptosis. However, when cells were treated with DAC and infected with a low dose of Ad-*p53*, a synergistic growth inhibitory effect was observed with induction of Apaf-1 and procaspase-9 expression in which cytochrome-*c* releases by Ad-*p53* triggered the mitochondrial pathway of apoptosis [Gomyo et al., 2004]. Selective blockage of caspase-9 activities completely attenuated DAC-induced enhancement of apoptosis mediated by Ad-*p53* infection, and ectopic overexpression of procaspase-9 sensitized cells to Ad-*p53*-induced apoptosis in *p53*-null cells [Gomyo et al., 2004]. In addition, DAC sensitized lung cancer cells to cisplatin and paclitaxel.

These observations led to inclusion of chemotherapy in NIH RAC-approved clinical trials involving Ad-mediated *p53* gene transfer into patients advanced NSCLC [Merritt et al., 2001; OBA Documents, 2002; Swisher and Roth, 2002a]. Results have suggested synergism between Ad-*p53* and chemotherapy with no increased side effects [Roth et al., 2001a,b; Swisher and Roth, 2002a]. Patients were intra-tumorally injected with Ad vectors containing wt-*p53* 3 days after receiving intravenous cisplatinum at a dose of 80 mg/m^2. In 24 patients, who had progression through all conventional treatments, 17 patients achieved disease stabilization and 2 patients achieved a partial response following Ad*p53* and cisplatinum. In this Phase I trial, time to disease progression was also enhanced by concomitant cisplatin therapy [Roth et al., 1998]. Another Phase II clinical study in NSCLC patients using Ad-*p53* in combination with chemotherapy for multiple cycles was proposed to the NIH RAC and forwarded to the FDA, although its current status is available [Dobbs, 1998; OBA Documents, 2002].

Ad-mediated wt-*p16*INK4a (Ad-*p16*) expression in NSCLC cell lines, all of which lacked constitutive *p16*INK4a but each of which varied in *p53* status: A549 (*p16*INK4a(−)/p*Rb*(+)/wt-*p53*), H322 (*p16*INK4a(−)/p*Rb*(+)/mt-*p53*), and H1299 (*p16*INK4a(−)/p*Rb*(+)/deleted-*p53*), resulted in enhanced radiosensitivity of A549 but not of H322 or H1299 [Kawabe et al., 2000]. The apoptosis induced by the combination therapy using Ad-*p16* plus irradiation was dependent on the endogenous *p53* status of the cancer cells. Furthermore, when wt-*p53* protein expression was restored in H1299 using Ad-*p53*, Ad-*p16* stabilized *p53* protein expression and radiosensitized the cells [Kawabe et al., 2000]. Similarly, transfer of the *p53* gene can induce radiation sensitization in previously radiation-resistant tumors, leading to the possibility of new therapeutic protocols combining gene replacement with radiation therapy [Roth et al., 2000].

A Phase II trial evaluating the clinical efficacy of combining intralesional administration of Ad-*p53* as an adjunct to radiation therapy in NSCLC patients was approved by the NIH RAC [OBA Documents, 2002]. Results have suggested synergism between Ad-*p53* and chemotherapy with no increased side effects [Roth et al., 2001a,b; Swisher and Roth, 2002a]. In one Phase II trial, 19 patients with non-metastatic NSCLC who were not eligible for chemoradiation or surgery were treated as outpatients with radiation therapy to 60 Gy over 6 weeks in conjunction with three intra-tumoral injections of Ad-*p53* on days 1, 18, and 32, and 17 of the 19 patients completed all planned radiation and Ad-*p53* gene therapy as outpatients [Swisher et al., 2003]. The most common adverse events were Grade 1 or 2 fevers (79%) and chills (53%). Computed tomography and bronchoscopic findings at the primary injected tumor revealed complete response in 1 of 19 patients (5%), partial response in 11 of 19 patients (58%),

stable disease in 3 of 19 patients (16%), progressive disease in 2 of 19 patients (11%), and not evaluable in 2 of 19 patients (11%). Three months after completion of therapy, pathologic biopsies of the primary tumor revealed no viable tumor in 12 of 19 patients (63%), viable tumor in 3 of 19 patients (16%), and not assessed in 4 of 19 patients (21%). Quantitative reverse transcription-PCR analysis of the four *p53*-related genes (*p21*/CDKN1A, *fas*, *Bak*, and *mdm2*) revealed that *Bak* expression was increased significantly 24 h after Ad-*p53* injection and levels of CDKN1A and *mdm2* expression were increased over the course of treatment [Swisher et al., 2003].

The combination of chemotherapy with docetaxel, radiation therapy, and Ad-mediated wt-*p53* gene transfer exhibited synergistic inhibitory effects on tumor cell growth in all four human NSCLC lines in vitro [Nishizaki et al., 2001]. Moreover, in mouse models with H1299 and A549 xenografts, combined treatment synergistically inhibited tumor growth in the absence of any apparent increase in toxicity, when compared with other treatment and control groups [Nishizaki et al., 2001]. A Phase II/III multi-center, open-label, randomized study to compare the effectiveness and safety of intralesional administration of Ad-*p53* in combination with docetaxel and carboplatin chemotherapy and radiotherapy in patients with locally advanced unresectable NSCLC was, therefore, proposed to the NIH RAC in 2001 and is currently ongoing [OBA Documents, 2002; Swisher and Roth, 2002b; Swisher et al., 2002].

More recently, attempts to improve the therapeutic ratio, three-dimensional conformal radiation therapy has been combined with gene therapy for radiation protection using an inhaled manganese-superoxide-dismutase (MnSOD) transgene [Greenberger et al., 1998]. Animal studies, in which mice with orthotopic lung carcinoma tumors received intratracheal transfer of MnSOD-plasmid/liposomes (MnSOD-PL) followed by irradiation, showed improved survival [Epperly et al., 2000]. The MnSOD transgene expression was demonstrated in normal lung tissue, but not in the orthotopic tumors. In addition, decreased irradiation-induction of TGF-beta1, TGF-beta2, TGF-beta3, MIF, TNF-alpha, and IL-1 was detected in normal lung tissues but not in orthotopic tumors from MnSOD-PL-injected mice [Epperly et al., 2000]. Subgroups of mice implanted with pumps to continuously replace levels of inflammatory cytokines over 7 days did not exhibit detectable alteration in the radiotherapy-mediated improved survival or the inhibition of tumor re-growth compared to irradiated control mice, although each mice that received MnSOD-PL had increased survival compared to irradiated controls [Guo et al., 2003]. A Phase I/II study of concurrent paclitaxel and carboplatin chemotherapy and thoracic radiotherapy with swallowed MnSOD-PL protection in patients with locally advanced Stage III NSCLC has been proposed to the NIH RAC [OBA Documents, 2002].

Besides improving chemotherapy and radiation therapy sensitivity, gene therapy may also improve sensitivity to photodynamic therapy (PDT) in which light-activation of a photosensitizing drug is used for tumor ablation. Lewis lung carcinoma (LLC) cells treated with a new photosensitizer, mono-L-aspartyl chlorin e6 (NPe6) and light from a diode laser were found to express increased levels of IL-2, IL-6, and TNF-alpha 6 h after NPe6-mediated-PDT [Usuda et al., 2001]. The *IL-6* gene transfected, LLC-IL-6 cells were significantly more sensitive to PDT-induced cytotoxic effects and had greater apoptosis induction than the parent LLC cells and other cytokine gene-transfected cells, suggesting IL-6 expression modulates cellular sensitivity to PDT but that IL-2 and TNF-alpha expression does not [Usuda et al., 2001]. Decreased expression of *Bcl*-2 and cytochrome-*c* was observed in both LLC cells and LLC-IL-6 cells, but *Bax* protein increased in a time-dependent manner, and the ratio of *Bax* to *Bcl*-2 rose markedly after PDT in LLC-IL-6 cells, suggesting that PDT-induced cytotoxicity results from the high ratio of *Bax* to *Bcl*-2 in the IL-6-dependent apoptotic pathway [Usuda et al., 2001].

CONCLUSIONS

The poor overall survival of lung cancer patients treated with conventional therapies (surgery, radiation therapy, and chemotherapy) mandate novel approaches to treatment. Multistep carcinogenesis implies that cancer is the result of the accumulation of multiple molecular defects. Significant strides have been attained in creating a new class of therapeutic agents that target these molecular defects to treat lung

cancers using gene therapy. Gene therapy may be simplified by identification a common pathway to carcinogenesis. More likely, the targeting of multiple defective genes may be required in order to develop an effective therapeutic strategy. Addition of gene therapy strategies to conventional therapies appears to improve their effectiveness and therefore offers a fourth dimension in the multimodality treatment of lung cancer.

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