p53 Gene Replacement for Cancer

Interactions with DNA Damaging Agents

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Clinical trials of *p53* gene replacement have provided information that will be useful in the design of future gene therapy strategies. Direct intratumor injection has low toxicity and thus can be readily combined with existing treatments. Post-injection gene expression can be documented and occurs in the presence of an anti-adenovirus immune response. Importantly, this treatment can cause tumor regression or prolonged stabilization. Future research directions will include development of more efficient vectors, use of novel genes, and combined modality approaches. Unresectable tumors are a prominent problem in oncology, with proven therapies such as radiotherapy and chemotherapy controlling less than 20% of lung cancers. Based on the preclinical and clinical studies discussed, it now seems that these conventional therapies may provide renewed potential when used in conjunction with transfer of a functional *p53* gene.

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LIMITATIONS OF RADIOTHERAPY FOR CANCER

Cancer is caused by multiple genetic alterations, which together, transform a cell and its progeny into a rapidly proliferating, invasive and progenitor-like population of cells. Radiation therapy, on first analysis, seems to be an ideal treatment for unresectable cancers, since ionizing radiation damages the DNA of rapidly dividing cells. A large number of tumors, however, have proven remarkably radiation-resistant. Increasing the dose of radiation or combining it with other conventional therapies often results in unacceptable toxicity, severely limiting the effective use of radiotherapy for cancer.

Recent advances in molecular biology have revealed the actual mechanisms of radiation-induced cell death and have suggested that radiation resistance may, in part, be due to missing or malfunctioning tumor suppressor genes. Evidence suggests that replacing these non-functional copies of tumor suppressors with functional tumor suppressor genes can restore radiation sensitivity to tumor cells, without further toxicity to normal cells.

CELL DEATH, *p53* AND APOPTOSIS

Cells have evolved mechanisms to detect damaged DNA and to arrest the cell cycle, allowing for DNA repair. When repairs cannot be made, a cell is directed to destroy itself, effectively ensuring that the damaged gene will not be passed on to the cell's progeny. This process of cell suicide, called apoptosis or programmed cell death, also plays a key role in other normal cellular mechanisms. For example, during embryogenesis, apoptosis is responsible for the disappearance of early embryonic features not present in the adult. Apoptosis has also been acknowledged as a major mechanism of cell destruction in response to ionizing radiation and to DNA damaging chemotherapeutics (1–3).

Numerous genes implicated in the induction of apoptosis have been identified and studied, and it is clear that many of them are involved in cell cycle regulation, transcriptional regulation, and, in addition, that many are the same genes which, when missing or altered, contribute directly to the initiation of cancer. The gene coding for the

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tumor suppressor/transcription factor *p53*, the very molecule which is responsible for detecting DNA damage and directing a cell either to repair or destroy itself, is missing or non-functional in over 50% of tumors. In addition to limiting the cell's ability to detect and repair the original tumor causing mutation, alterations in *p53* may also cause the cell to be inherently resistant to any therapeutic effort that targets rapidly replicating DNA, specifically, ionizing radiation and chemotherapy. Mutation of this 'guardian of the genome', *p53*, has been associated with poor prognosis in patients with many types of cancers (4–7).

TUMOR SUPPRESSOR GENES

The importance of tumor suppressor genes became evident in the early 1990s. Although difficult to study at first, because their existence could be discerned only when they were missing (8, 9), tumor suppressor genes are now acknowledged as critical elements in cell cycle regulation and regulation of gene transcription. When a cell is faced with the stress of oncogene activation, hypoxia, or DNA damage, *p53* is tasked with determining whether that cell will receive the signal to simply halt at the G1 stage of the cell cycle, whether it will be signaled to attempt repair, or whether it will self-destruct via apoptosis (10). This determination depends on the fine balance between the number of pro-apoptotic signals vs. pro-survival signals a cell is receiving at any one time. Expression of many of these critical signals is regulated by the activation status of *p53*. Two groups of genes are targeted by *p53*: the 'pro-survival' or antiapoptotic, which include *bcl-2*, *bcl-X2*, *bcl-w* and *CED9*, and the 'proapoptic', including *bax*, *Bad*, and *Bid* (11). In each cell, available transcripts of the protein products of these genes form heterodimers, and the relative ratio of the proapoptotic to prosurvival proteins in these heterodimers determines whether the cell lives or is directed to undergo apoptosis.

Apoptosis occurs when proapoptotic signals outweigh prosurvival signals. The first gene mediator of apoptosis discovered was *bax*, a pro-apoptotic gene in the *bcl-2* family (12). Another pathway to apoptosis involves FAS/APO1, the 'death receptor'. Both pathways lead *p53* to induce the caspase cascade, which is dependent on mitochondrial cytochrome C. This enzyme functions as a co-factor for ATP to activate Apaf-1 (the mammalian homologue of CED4) which, in turn, activates caspase 9, the 'initiator' of the caspase cascade.

The antiapoptotic family members (*bax*, *bad*, *bid*) inhibit the co-factor activity of cytochrome C, effectively halting the caspase cascade. The *p53* protein can transcriptionally induce *bax* and inhibit the activity of the prosurvival molecule *bcl-2*, allowing progression through the cascade. Several other targets are known to be involved at various stages of the cascade.

Elaborate cellular mechanisms have evolved to maintain barely detectable levels of *p53* in normal, unstressed cells. When *p53* does become activated, it induces expression of mdm2, an oncogene encoding a protein which, in turn, binds to and inhibits the activation of *p53*, forming an autoregulatory loop. Normally, the N-terminal transactivating domain of *p53* is bound by mdm-2 (reviewed by Burns & El-Deiry (13)), prohibiting its activation and leading to rapid *p53* degradation. The result is a very short biological half-life (20–30 min) for *p53* in the normal unstressed cell.

In the event of genotoxic stress or oncogene activation, the *p53*/mdm2 interaction becomes unstable. DNA damage causes phosphorylation of serines on *p53*, weakening its binding to mdm2. There is evidence that this destabilization is mediated by the ATM (ataxia telangiectasia-mutated) protein: cells from ataxia telangiectasia (AT) patients, who do not express the ATM protein, cannot phosphorylate serine. In addition, phosphorylation of *p53* by ATM has been demonstrated both in vitro and in vivo (14–16). Once the *p53*/mdm2 bond has been disrupted, *p53* becomes stabilized. DNA binding activity increases and *p53*, through an array of downstream signals, acts to switch other genes on or off. There is evidence that the tumor suppressor BRCA1 (hereditary breast and ovarian tumor suppressor) may also act to stabilize *p53* (13). This sequence of finely tuned biochemical events culminating in cell death is irrelevant in the absence of a functional *p53* gene. Without *p53*, induction of apoptosis via the *p53* pathway in response to ionizing radiation will not occur, resulting in radioresistant tumors.

GENE THERAPY FOR CANCER

The goal of gene therapy is to alter gene expression in such a way as to treat, cure or prevent diseases with a genetic basis. Cancer is caused by multiple genetic alterations which occur sequentially and ultimately result in a cell capable of uncontrolled proliferation, invasion of tissues, and metastases. Several of these genetic errors present potential targets for gene therapy strategies, but because the array of genetic events varies widely between cancers and between patients, it is impossible to view all such mutations as potential targets. Despite this seemingly overwhelming diversity, genetic analysis of many tumors has revealed some patterns of mutations involving various oncogenes and tumor suppressor genes. Eventually, identifying the particular genetic profile of each person's tumor might be as critical to diagnosis and treatment as identification of bacteria is now for the treatment of infectious diseases.

There are some genetic alterations that show up more frequently than others, and mutation of the *p53* gene is the most frequent abnormality found in human tumors. Many of these *p53* defective tumors have proven resistant to

radiotherapy or other DNA damaging treatment protocols. Without a functional *p53* gene, conventional DNA damaging agents will remain ineffective treatments for this population of patients.

PRECLINICAL EVIDENCE FOR *p53* GENE REPLACMENT SUCCESS

Successful gene therapy approaches must achieve three separate goals: gene delivery, gene expression and regulation of gene expression. Current approaches use several methods of delivery, including adenoviral vectors, retroviral vectors, herpes vectors and non-viral vectors. Much effort is being expended at many gene therapy research centers to identify the most appropriate vehicle for transporting genes, as each of the methods currently in use has limitations.

Preclinical studies, both in vivo and in vitro, have demonstrated that restoration of *p53* function can induce apoptosis in cancer cells. In addition, studies combining *p53* gene replacement with DNA damaging agents such as cisplatin (Platinol®) and ionizing radiation indicate synergy regarding induction of apoptosis.

Fujiwara et al. (17) observed a therapeutic effect of *p53* gene transfer in an orthotopic lung cancer model with a retroviral *p53* expression vector. High-level gene transfer was first achieved in lung cancer cells by Zhang et al. (18) using an adenoviral *p53* construct. Preclinical studies carried out in vitro in colorectal cancer cell lines (19) and pancreatic cancer cell lines (20) demonstrated suppression of cell proliferation and induction of apoptosis after transduction with *p53*. Additional studies performed in vivo in a mouse xenograft tumor model showed significant suppression of tumor growth (21). Several other lines of evidence also support the feasibility of gene transfer for *p53* gene replacement. For example, the efficacy of *p53* adenoviral gene therapy in a mouse model of human breast cancer (22) has been demonstrated, as has inhibition of human breast tumors in a preclinical model of *p53* gene transfer (23). Other studies have shown apoptosis induction in drug-resistant human breast cancer cells after adenovirus transfer. Thus, surprisingly, despite the multiplicity of genetic lesions in cancer, restoration of the function of a single tumor suppressor gene is sufficient to mediate tumor regression in vivo.

As preclinical trials began, it was believed that the inability of a vector to transduce every cell within a tumor might limit the effectiveness of gene therapy. Fujiwara et al. (17) and Cusack et al. (24) however, demonstrated in 3-dimensional cancer cell matrices and subcutaneous xenografts that therapeutic genes were likely to spread beyond the immediate intratumoral injection site.

CLINICAL APPLICATION OF *p53* GENE REPLACEMENT

Clinical trials

Non-small cell lung cancer (NSCLC) patients were administered the *p53* gene via a retroviral vector under control of the β-actin promoter (25). No vector-related toxicity was observed, and three of the nine patients demonstrated evidence of anti-tumor activity. Thus this was the first clinical trial to show that tumor suppressor gene replacement could mediate tumor regression in human cancer. Although this study demonstrated the feasibility and safety of gene therapy, the transduction efficiency of the retroviral vector was limiting. This is generally true of retroviral vectors, which are difficult to prepare at high enough titers to be useful in most gene therapy protocols.

Adenoviral vectors, unlike retroviral vectors, are capable of infecting both dividing and non-dividing cells and can be produced at high titers. These vectors do not integrate into the genome, however, so gene expression is transient. This is not necessarily a disadvantage in cancer therapy because prolonged expression is not required once tumor cell death has occurred.

A phase I clinical trial (26) of an adenoviral *p53* construct was completed in 28 patients whose NSCLC had failed to respond to conventional therapy. There were no significant toxic effects related to the vector. In addition, the transgene was expressed in spite of high serum antiadenovirus titers, gene expression levels correlated to the dose delivered, and, most importantly, there was evidence of anti-tumor activity. Two patients achieved greater than 50% reduction in tumor size and in one patient, no tumor cells were detected in post-treatment biopsies. One patient was followed-up for more than a year after cessation of therapy with no recurrence. In another patient, almost complete regression was observed in a left upper lobe endobronchial tumor which had resisted chemotherapy, radiation therapy and laser treatment (see Fig. 1).

COMBINATION THERAPY WITH *p53* AND DNA DAMAGING AGENTS

In the early 1990s, several groups (27–29) reported that overexpression of *p53* in cells transfected with *p53*-expressing plasmids could drive cells into apoptosis or growth arrest, and also illustrated the potential for *p53* gene therapy in treating the many *p53* deficient tumors. Subsequent preclinical studies suggested that *p53* gene replacement therapy in combination with conventional doses of radiation or chemotherapeutics may have a synergistic effect without the additional toxicity encountered with high doses of these conventional DNA damaging agents. The link between apoptosis and cell death caused by DNA damaging agents was established.

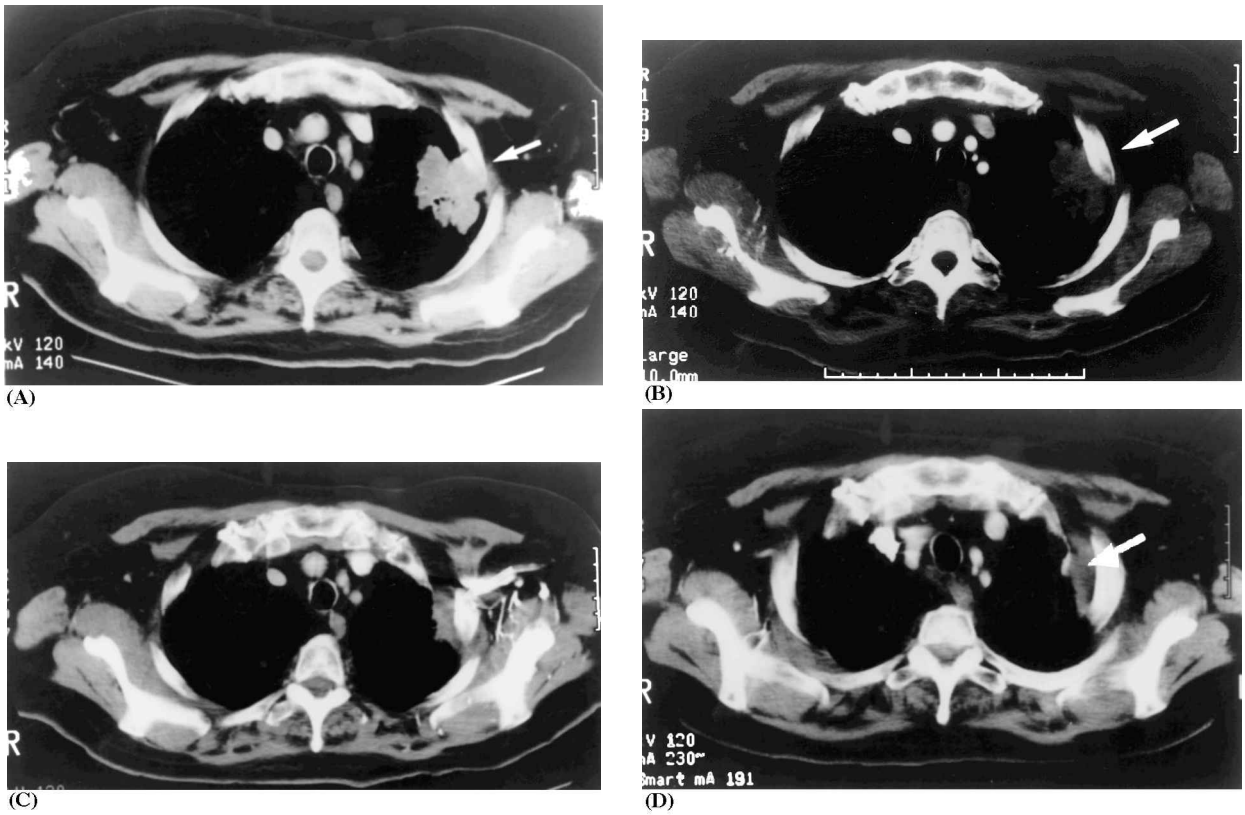

Fig. 1. Computed tomography (CT) scans of patient K following six courses of 10° plaque-forming units of Ad-*p53*, an adenovirus vector carrying the wild-type *p53* complementary DNA. A) Before treatment, arrow shows recurrent left upper lobe adenocarcinoma, which progressed after 66 Gy of external beam radiation therapy and six courses of paclitaxel and carboplatin (CT scan volume: $3 \times 4 \times 5$ cm $= 60$ cm^3). B) At one month after treatment, arrow shows tumor regression after one course of Ad-*p53* treatment (CT scan volume: $2 \times 3 \times 5$ cm $= 30$ cm^3). C) At 8 months after treatment, image shows tumor regression following six courses of Ad-*p53* gene therapy (CT scan volume: $2 \times 2 \times 3$ cm $= 12$ cm^3). D) Stable tumor 18 months after beginning treatment with Ad-*p53* (CT scan volume: $2 \times 2 \times 3$ cm $= 12$ cm^3). No viable tumor was demonstrated during the last 4 months of therapy (14 sequential percutaneous biopsies), and the patient was observed off all treatment for 12 months without evidence of tumor progression.

Preclinical studies

Preclinical studies of *p53* gene therapy in combination with cisplatin (30, 31) showed in cultured NSCLC cells, as well as in human xenografts in nude mice, that sequential administration of CDDP and *p53* gene therapy resulted in enhanced expression of the *p53* gene product. In Nguyen's studies (30), pretreated cells demonstrated apoptosis in over 50% of the cells 12 h after gene transfer and in over 90% of the cells at 24 h. Cells which were not pretreated with CDDP prior to gene transfer demonstrated only 19% and 68% apoptotic cells at 12 and 24 h respectively. The in vivo studies demonstrated that systemic CDDP treatment prior to *p53* gene transfer produced at least a 55% further reduction in final tumor size when compared to mice receiving gene transfer only.

Preclinical studies of *p53* gene therapy in combination with radiotherapy indicated that delivery of *p53* to *p53*-deficient tumor cells, both in vitro and in vivo, increases their sensitivity to radiation (19). Specifically, when in vitro cultured human colorectal carcinoma cells were gamma irradiated, 55% of the tumor cells survived. Transfection of the cells with *p53* prior to irradiation, however, lowered the survival rate to 23%. Apoptosis was also increased in the pretreated cells. Furthermore, in an animal tumor model, significant tumor suppression was observed. Regrowth of tumors was delayed 2 days when tumors were treated with radiation alone, and 15 days after treatment with *p53* gene transfer alone. However, tumors of animals receiving the *p53* gene followed by radiation treatment required 37 days to reach pretreatment size.

Other recent studies have generated supporting evidence for a critical link between radiation sensitivity and the ability of a cell to induce apoptosis (32–36). Data in some tumor types, for example epithelioid tumors, have not shown a correlation between *p53* status and radiosensitivity (37–39). However, those studies where high levels of gene expressed are forced with vector transduction may result in an altered cell state quite different from cancer cells which retain low levels of wild type *p53* expression or have circumvented *p53*-mediated apoptosis during malignant progression.

Clinical studies

Based on the results of preclinical studies, Nemunaitis and colleagues (40) initiated a Phase I trial of *p53* gene transfer in sequence with cisplatin in 24 NSCLC patients with non-functional *p53* genes. Intravenous cisplatin was administered, and three days later *p53* was delivered directly into the tumor. Up to a total of six monthly courses were carried out. Seventeen patients remained stable for at least 2 months, 2 patients achieved partial responses, and 4 continued with progressive disease. One patient was unevaluable owing to progressive disease. When tumor biopsies were analyzed for apoptosis, 14% demonstrated no change, 7% showed a decrease in apoptosis and 79% demonstrated an increased number of apoptotic cells. Of note is that 75% of the patients entered in the trial had tumor progression on cisplatin- or carboplatin-containing regimens.

Phase II clinical trials of adenoviral-mediated *p53* gene transfer in conjunction with radiation therapy (41) were carried out in 17 patients with localized NSCLC. The overall response rate was 5/17 (29%); response rate at the local injected site was 9/17 (52.9%). The survival rate at one year was 56%. Post-treatment biopsies of the original tumor site were obtained 3 months following completion of treatment. In 12 cases the biopsy showed no evidence of tumor. Safety data indicated that this combination had an acceptable safety profile. Thirteen patients underwent 61 CT-guided biopsies or drug administrations. Thirteen (21%) resulted in pneumothoraxes, one of which required hospital admission. Six of the 17 patients experienced a grade 3 or 4 adverse event. These results are encouraging and are the basis for a randomized clinical trial in patients with unresectable NSCLC. This trial will compare concurrent chemotherapy and radiation therapy alone with concurrent chemotherapy and radiation therapy with intratumoral injections of adenoviral *p53*.

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