in possibly multiple IVF treatments relative to the economic gain from avoiding unnecessary screening of high risk individuals and treatment of subsequent cancers (if these cannot be prevented). This is clearly conditioned by accuracy of the diagnosis (in cases where the mutation is inferred from linkage only), the penetrance of the gene (i.e. how many carriers develop cancer) and how life threatening the cancer is. With common mutations, such as the breast cancer gene *BRCA1*, perhaps we should be screening the thousands of couples undergoing IVF each year for infertility and offering embryo diagnosis to carriers. Although labour intensive, the biopsy and diagnostic procedures are relatively inexpensive and cost/benefit analysis in these couples may well be favourable.

Ethical issues include the morality of embryo selection for traits which are not immediately life-threatening versus the gain in cancer prevention affecting the well-being of both parents and children. The knowledge of the high probability of the development of cancer in gene carriers (quite different in magnitude to that present in most cancer prevention options) will be an important factor in individual decisions. Similar ethical issues are already being faced for late onset diseases such as Huntington's Chorea. A potential advantage of pre-implantation over later prenatal diagnosis in such diseases is that couples known to be at risk because of affected relatives, but not genotyped themselves, do not have to be told of their carrier status but simply reassured that if necessary non-carrier embryos would be selected for transfer. In addition, blood group incompatibilities, such as rhesus incompatibility, can now be identified, and may be justifiable in women who have been previously sensitised and may require repeated blood transfusions *in utero* with the associated risk of miscarriage.

Currently, there is no European consensus on the use of IVF for pre-implantation diagnosis. In the U.K., it is permitted by law and regulated by the Human Fertilisation and Embryology Authority who have, for example, banned the use of these techniques to enable couples to choose the sex of their children, except in cases of X-linked disease. In France, initial legislation outlawing pre-implantation diagnosis may now be relaxed in cases of severe inherited disease. In Germany, pre-implantation diagnosis is effectively banned altogether. Nevertheless, there is little doubt that these issues will have to be addressed in the near future.

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Gene Replacement Strategies for the Prevention and Therapy of Cancer

J.A. Roth, T. Mukhopadhyay, W.W. Zhang, T. Fujiwara and R. Georges

THE IDENTIFICATION of specific genes that contribute to the development of the cancer cell presents an opportunity to use these genes and their products as targets for treatment and perhaps prevention of the disease. The gene families implicated in carcinogenesis include dominant oncogenes and tumour suppressor genes [1, 2] and a dynamic interplay exists within the cell between dominant oncogenes and genes that constrain cell proliferation. Proto-oncogenes (normal cellular homologues of oncogenes) participate in critical cell functions, including signal transduction and transcription. Only a single mutant allele is required for malignant transformation, and primary modification to the dominant oncogenes that confer transforming ability include point mutation, amplification, chromosomal translocation, and rearrangement (Figure 1). Loss of tumour suppressor gene function may involve either mutation, deletion, or a combination of these. Some tumour suppressor genes appear

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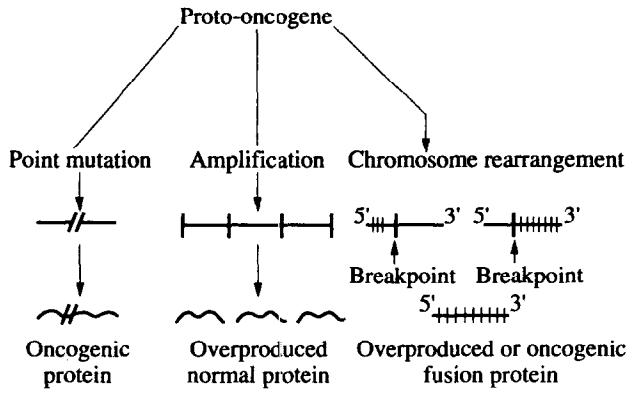

Figure 1. Mechanisms of gene activation for dominant oncogenes.

to play a role in the control of proliferation by regulation of transcription. It is possible that modification of the expression of dominant and tumour suppressor oncogenes may influence certain characteristics of cells that contribute to the malignant phenotype. Our increasing knowledge of the mechanisms involved in oncogene-mediated transformation has led to progress in developing therapeutic strategies that may alter or replace abnormal transformation-related genes in cancer cells.

Multiple genetic abnormalities are present both in cancer cell lines and fresh tumour samples [3–6]. This is evident at the chromosomal level where multiple chromosomal abnormalities have been identified. In addition, an increasing number of oncogenes and tumour suppressor genes have been identified. It was felt that gene replacement cancer therapy would not be possible because of the difficulties associated with correcting multiple genetic abnormalities in one cell. However, several studies have shown that correction of a single genetic defect, such as the elimination of expression of a dominant oncogene or addition of a normal copy of a tumour suppressor gene to a cell reduced or eliminated critical characteristics of the malignant phenotype, such as tumorigenicity or anchorage independent growth [7–10]. We will review here our studies supporting this concept, and their application to animal models and clinical protocols.

DOMINANT ONCOGENES

Oncogenes of the *RAS* family (homologous to the rat sarcoma virus) have three primary members (*HRAS*, *KRAS* and *NRAS*), and are among the most common activated oncogenes found in human cancer [11]. The *RAS* genes code for 21-kDa proteins that are located on the inner surface of the plasma membrane, have GTPase activity, and participate in signal transduction. *RAS* oncogenes are activated by point mutations that alter the amino acid sequence of p21.

Rodenhuis and coworkers detected mutations in the 12th codon of *KRAS* in human lung tumours, using a highly sensitive technique based on amplification with the polymerase chain reaction, and detection with a panel of oligonucleotide probes [12, 13]. *KRAS* mutations were confined to adenocarcinomas of the lung, and occurred in nine of 35 such tumours. Mutations were not observed in adenocarcinomas from non-smokers. A recent study by the same group showed that *KRAS* mutations are an independent prognostic factor indicating a poor prognosis [14]. So far, current studies favour the interpretation of *KRAS* activation as a progression factor in lung cancer, occurring in approximately one-third of adenocarcinomas of patients with a heavy smoking history.

Our laboratory used anti-sense technology to investigate the effects of eliminating expression of a mutant *KRAS* oncogene in lung cancer cells [9]. A homozygous mutation at codon 61 was detected in the NCI-H460a large cell undifferentiated NSCLC cell line clone with a normal glutamine residue (CAA) substituted by histidine (CAT). An antisense (AS) KRAS construct was developed that selectively blocked the production of mutant p21. A recombinant plasmid clone was constructed using a wild-type 2-Kb *KRAS* genomic DNA segment carrying the second and third exons, with flanking intron sequences subcloned into an Apr-1-neo expression vector in AS orientation. The intron sequence used has a low degree of homology with other *RAS* genomic sequences so that specific inhibition of *KRAS* with preservation of *HRAS* and *NRAS* gene expression occurs. The 2-kb DNA insert was shown to be stably integrated into H460a cells by Southern hybridisation and northern blot analysis detected expression of AS RNA. Western blot analysis showed 95% reduction in specific K-ras p21 protein synthesis in the clones expressing the AS RNA, while H460a cells and sense *KRAS* clones showed unchanged levels of K-ras p21 protein. Expression of *RAS* genes was measured by reverse transcription-polymerase chain reaction amplification (RT-PCR). Cells expressing AS RNA showed complete loss of mRNA transcribed from the *KRAS* gene. There was no change in *HRAS* or *NRAS* expression in either AS or sense transfectants. AS transfectants showed a 3-fold reduction in growth compared to sense transfectants and parental H460a cells, but continued to grow in culture. Expression of AS K-*ras* RNA reduced the growth rate of H460a tumours in *nu/nu* mice. Although cancer cells have multiple genetic abnormalities, the reversal of a single abnormality appears to have profound effects on fundamental properties of the malignant phenotype, such as rapid proliferation and tumorigenicity. This was demonstrated when the antisense *KRAS* plasmid expression vector was transfected into H460a cells which have, in addition to a *KRAS* mutation, five chromosomal deletions (chromosomes 1, 2, 9, 12, 16).

A major obstacle to direct correction of genetic lesions in cancer cells is the difficulty of efficiently delivering genetic constructs to all aberrant cells. Retroviruses have been extensively studied as delivery vehicles in gene transfer protocols [15]. Retroviral vectors have been created that lack genes essential for replication, and are capable of infecting cells and integrating as a provirus which will then express recombinant genes (Figure 2).

Because gene constructs transduced by retroviruses are integrated preferentially into dividing cells, this technique gives proliferating cancer cells a potentially selective advantage for expressing the gene construct. Retroviruses and cells modified by retroviral transduction have little acute toxicity, making multiple treatments with high-titre preparations feasible. A retroviral vector system was developed to efficiently transduce a *KRAS* antisense construct into human cancer cells [16]. The 1.8-kb *KRAS* gene fragment DNA in antisense (AS) orientation to a β-actin promoter was inserted into the retroviral vector, LNSX. The constructs were transfected into the amphotropic packaging cell line GP + envAm12, followed by alternating infection between the ecotropic packaging cell line, Ψ2, and GP + envAm12. Titres up to 9.7×10^7 CFU/ml were achieved without detectable production of replication-competent virus. The human large cell lung carcinoma cell line, H460a, was transduced with this recombinant virus, and a transduction efficiency of 95% was achieved after five to seven repeated infections. DNA PCR analysis showed that the retroviral con-

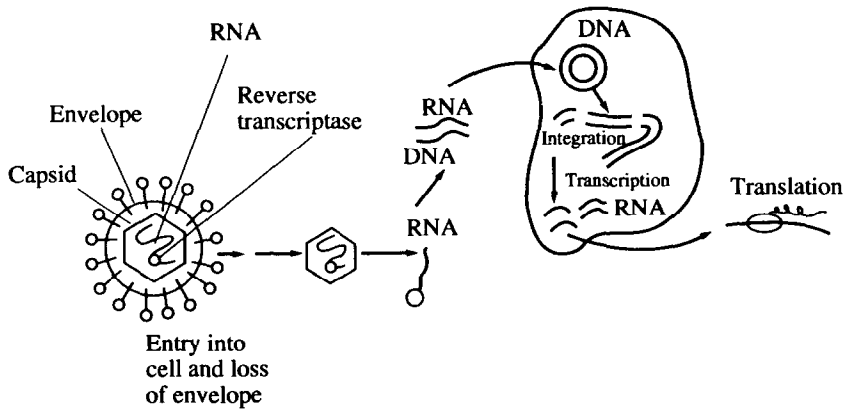

Figure 2. Transduction of cell by replication defective retrovirus.

struct was integrated into the genome of H460a cells. *KRAS* antisense RNA expression was detected in the cells by Northern analysis, slot blot hybridisation and RT-PCR. Translation of the mutated K-ras p21 protein RNA was specifically inhibited, whereas expression of other p21 species was unchanged. Proliferation of H460a cells was suppressed tenfold following transduction by LNSX-AS-KRAS, and colony formation in soft agarose was also dramatically decreased. We conclude that an antisense construct for *KRAS* can be expressed effectively in a retroviral vector, which can efficiently transduce human cancer cells.

An orthotopic human lung cancer model in *nu/nu* mice was used to study the effect of an antisense *KRAS* (As-K-*ras*) retroviral construct on tumour growth *in vivo*. Irradiated (350 cGy) *nu/nu* mice were first inoculated intratracheally with 10^5 H460a human large cell lung carcinoma cells which have a codon 61 mutation of the *KRAS* oncogene. Three days later they received intratracheal instillation of viral supernatant (5×10^6 CFU/ml) from either LNSX, LNSX-AS-KRAS, LNSX-sense(S)-KRAS producer cells or medium daily for 3 days. At autopsy, 30 days after tumour cell inoculation, 90% of the control mice had tumours whereas 87% of mice treated with the LNSX-AS-KRAS viral supernatant were free of tumours. The efficacy of the viral supernatant was dose-dependent. Intratracheal administration of retroviral LNSX-AS-KRAS supernatant thus prevents the growth of human lung cancer cells implanted orthotopically in *nu/nu* mice. These studies constitute the first reported use of retroviral supernatants to mediate anti-tumour effects, the first successful use of an antisense construct to mediate therapeutic tumour regression *in vivo*, and the first report of a therapeutic anti-tumour effect based on inhibition of oncogene expression.

TUMOUR SUPPRESSOR GENES

The *TP53* gene encodes a 375 amino acid phosphoprotein that can form complexes with a number of regulatory host proteins, SV40 large-T antigen and adenovirus E1B, and so far is the most commonly mutated gene identified in human cancers [17]. Missense mutations are most common, and, in many cases, will functionally impair the gene product p53 [18, 19]. The mechanism of p53 transformation may vary depending on the type of p53 mutation. The *TP53* gene product appears multifunctional, with major domains that can transactivate, bind proteins, bind DNA in a sequence-specific manner, and oligomerise with itself (Figure 3a, b). Abnormalities in one or more of these functions could contribute to abrogation of the tumour suppressor function of p53. Failure of mutant p53 to activate transcription of molecules essential for regulating the cell cycle, DNA repair or the untimely expression of other regulatory molecules may make the cell more susceptible to genetic instability. Certain mutations also have a dominant transforming capability. The wild-type *TP53* gene may suppress genes that contribute to uncontrolled cell growth and proliferation, or activate genes that suppress uncontrolled cell growth. Thus, absence of the wild-type p53 or inactivation of wild-type p53 may contribute to transformation. However, some studies that indicate the presence of the mutant p53 may be necessary for full expression of the transforming potential of the gene.

To assess the role of the *TP53* gene in the development of human cancer, wild-type *TP53* cDNA in either sense or antisense orientation was introduced into human non-small cell lung cancer cell lines [20]. The cell line H226b has a wild-type *TP53* gene, whereas H322a's gene has a codon 248 mutation. H226b cells transfected with the *TP53* sense gene construct grew more slowly than its parental population, and we were unable to recover any H322a sense transfected clones. Transfection with antisense *TP53* also reduced colony formation. However, some clones transfected with antisense *TP53* showed increased proliferation. Elevated levels of antisense *TP53* RNA in transfected cells reduced the levels of wild-type and mutated p53 proteins, and interestingly, although parental H322a and H226b cells form tumours in *nu/nu* mice after a long latency period, their antisense transfectants, with reduced levels of p53 proteins formed large tumours in 15 days. Functional inactivation of mutated and wild-type *TP53* by antisense RNA provides direct experimental demonstration of tumour suppressor function for this gene, and suggests that at least some *TP53* mutations have residual cell growth and tumour suppressor functions, which may be dose-dependent. Hence, introduction of a normal *TP53* gene into cancer cells would be the preferred approach, rather than the use of antisense to inactivate mutated *TP53*.

A retroviral vector-mediated system was established to allow efficient transduction of the wild-type *TP53* gene into human lung cancer cell lines H358a (deleted *TP53*) and H322a (mutant *TP53*) [21]. LNSX/*TP53* constructs incorporating *TP53* cDNA, driven by a β-actin promoter, mediated stable integration of this gene. *TP53* mRNA and protein were detected in these cell lines 6 months after transduction by northern and western blot analyses. Restoration of the wild-type *TP53* gene suppressed growth in the two transduced cell lines, but had no effect in another transduced tumour cell line, H460a, which has an endogenous wild-type *TP53* gene. A high transduction efficiency (90%) was obtained after five cycles of transduction *in*

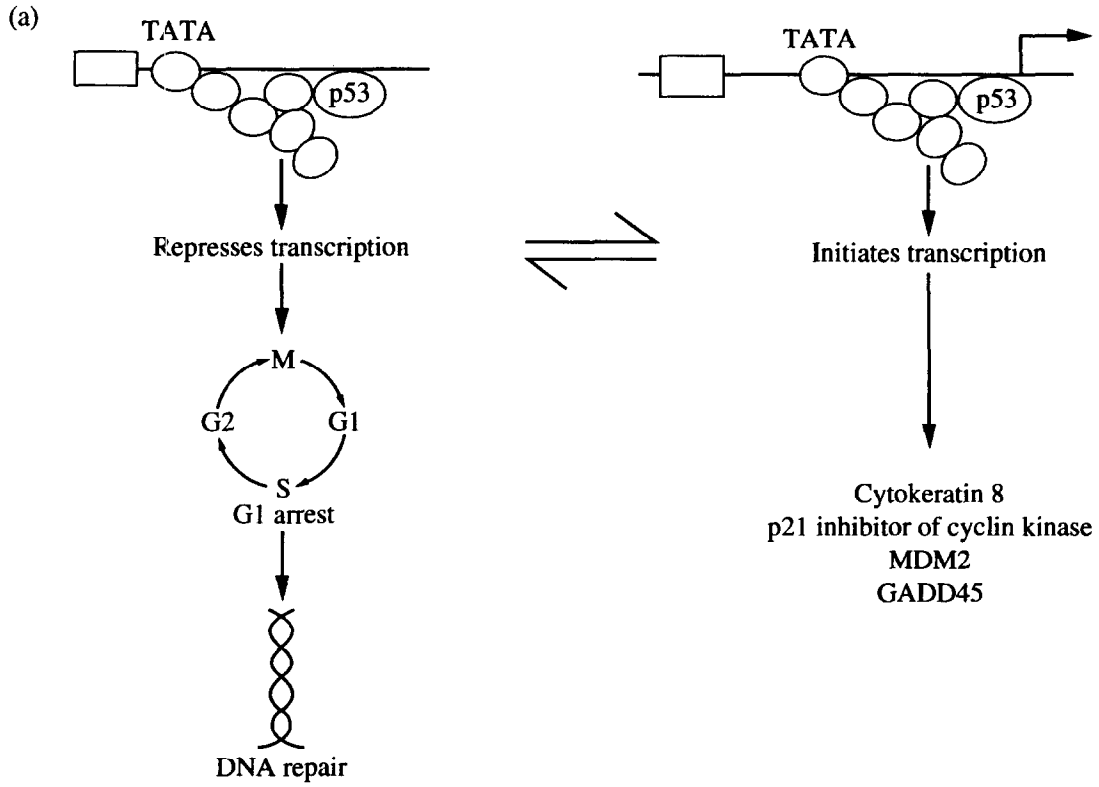

Figure 3. (a) A model of the function of wild-type *TP53* gene. The model illustrates regulation of transcription of molecules involved in cell cycle regulation and DNA repair.

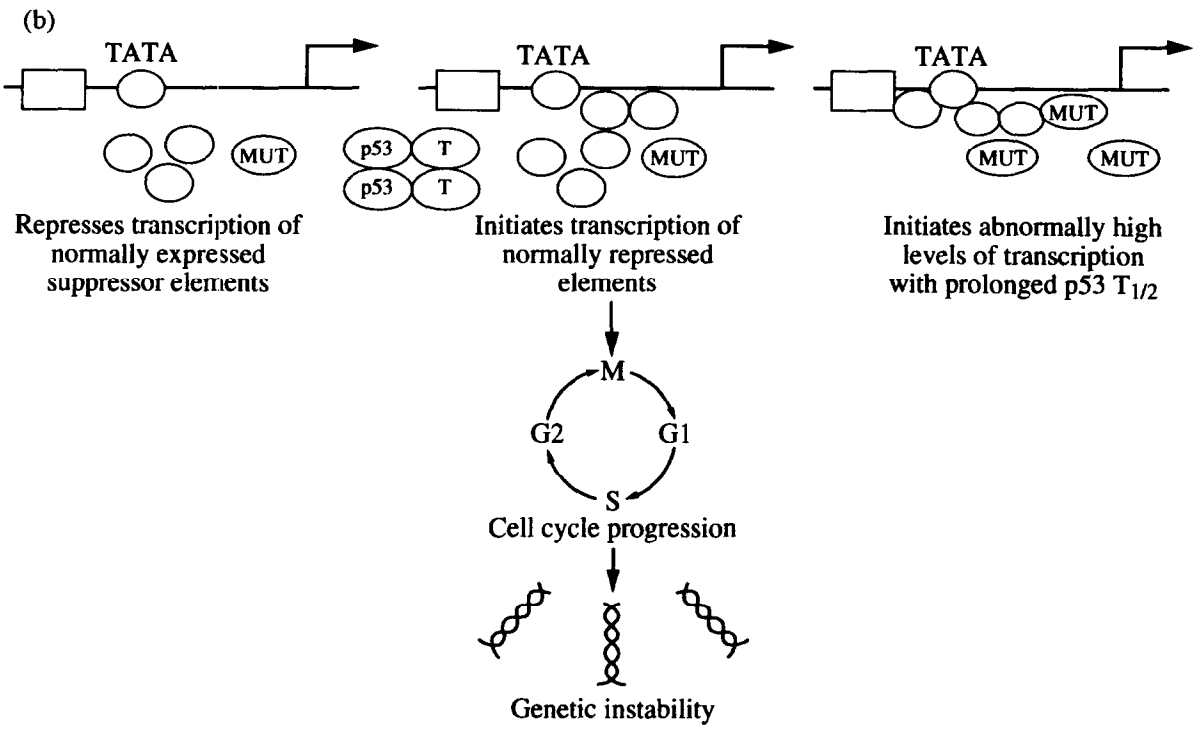

Figure 3. (b) Disruption of p53 function by mutations or deletions may lead to inappropriate gene expression or lack of expression of molecules necessary for DNA repair and genetic stability.

vitro. Mixing experiments showed that transduced cells could reduce the growth rate of nontransduced cells; this reduction may have been mediated by factors shed into the supernatant of the transduced cell cultures.

Mutations in the *TP53* tumour suppressor gene are common in human lung cancers. The wild-type form of *TP53* is dominant over the mutant, and thus restoration of wild-type *TP53* function in lung cancer cells may suppress their growth as tumours. We investigated the therapeutic efficacy of direct administration of a retroviral wild-type *TP53* expression vector (LN*TP53*B) in an orthotopic human lung cancer model. Irradiated (350 cGy) *nu/nu* mice were intratracheally inoculated with 2×10^6 H226Br cells (codon 254 mutation) and 3 days later, were treated with an intratracheal instillation of LN*TP53*B retroviral supernatant for 3 days. Infection with LN*TP53*B inhibited proliferation of H226Br cells *in vitro*. Thirty days after tumour cell inoculation, 63–80% of the control mice showed macroscopic tumours of the right mainstem bronchus. LN*TP53*B suppressed H226Br tumour formation in 62–100% of mice, and the effect was dose-dependent. These results suggest that direct administration of a retroviral vector expressing wild-type *TP53* may inhibit local growth *in vivo* of human lung cancer cells with abnormal p53 expression. We conclude that development of gene replacement treatment strategies based on the type of mutations found in target cancers is warranted, and may lead to the development of new adjunctive therapies and gene-specific prevention strategies for lung cancer.

Adenovirus vectors can transduce both dividing and non-dividing cells, and may have tropism for lung epithelium. We developed an adenovirus vector for delivery of wild-type *TP53*. The p53 expression construct, which contains human cytomegalovirus (CMV) promoter, wild-type *TP53* cDNA, and SV40 early polyadenylation signal, was inserted between the *Xba I* and *Cla I* sites of pXCJL.1 (a gift from Dr F.L. Graham). The *TP53* shuttle vector (pEC53) and the recombinant plasmid pJM17 [22] were cotransfected into 293 cells [23] by liposome-mediated transfection with DOTAP. A high level of expression of exogenous p53 was achieved in the H358 cells, which were infected by Ad5CMV-p53 at an MOI of 30 PFU/cell. When H322 or H460 cells were infected at the same MOI, the level of expression of the exogenous *TP53* gene was 3-fold higher than that of the endogenous mutated protein in H322, and 14-fold higher than that of the endogenous wild-type protein in H460 cells. The time course of the expression of the exogenous *TP53* gene after a single infection of 10 PFU/cell was studied in H358 cells. The protein expression peaked on day 3, post infection, sharply decreased after day 5, and lasted for at least 15 days. This is a critical point with respect to safety of the vector. Transient p53 expression is sufficient for mediating apoptosis. However, normal cells taking up the vector will express the exogenous *TP53* gene for only a short time. Ad5CMV-p53 inhibited the proliferation of lung cancer cells with mutated or deleted *TP53*, but only minimally affected growth of cells with wild-type *TP53*. The efficacy of Ad5CMV-p53 in inhibiting tumorigenicity was also evaluated in the mouse model of orthotopic human lung cancer [24]. H226Br cells, which originated from a squamous lung cancer that metastasised to brain, have a point mutation (ATC to GTC) at exon 7, codon 254, of the *TP53* gene. The irradiated nude mice were inoculated with 2×10^6 H226Br cells/mouse by intratracheal instillation. Three days after inoculation, each of the mice (8–10 mice per group) were treated with 0.1 ml of either Ad5CMV-p53 or the control virus, Ad5/RSV/GL2, at 5×10^7 PFU/mouse or vehicle (PBS) by intratracheal instillation once a day for 2 days. After 6 weeks, only 25% of the Ad5CMV-P53-treated mice compared with 70–80% of mice treated with vehicle or Ad5/RSV/GL2 control virus formed tumours. The average tumour size of the Ad5CMV-p53 group was significantly smaller than those of the control groups. These results indicate that Ad5CMV-p53 can prevent H226Br from forming tumours in the mouse model of orthotopic human lung cancer.

We have also examined whether Ad5CMV-p53 and cisplatinum (CDDP) given in a sequential combination could induce synergistic tumour regression *in vivo* [25]. Following 3 days of direct intratumoral injection of Ad5CMV-p53, H358a tumours subcutaneously transplanted in *nu/nu* mice showed a modest slowing of growth; Ad5CMV-p53-injected tumours, however, regressed if CDDP was administered intraperitoneally for 3 days. Histological examination revealed necrosis of tumoral tissue in the area where Ad5CMV-p53 was injected in mice treated with CDDP. *In situ* staining showed extensive areas of apoptosis. In contrast, tumours treated with CDDP alone or AD5CMV-p53 alone showed no apoptosis.

CLINICAL APPLICATIONS

These studies provide a rationale for a new clinical protocol, recently approved by the NIH Recombinant DNA Advisory Committee, to inhibit expression of mutant K-ras p21 or replace a defective *TP53* gene with intratumour injection of recombinant retrovirus expressing AS-*KRAS* or normal *TP53*, respectively. Patients with unresectable lung cancer obstructing a bronchus, which has a *KRAS* or *TP53* mutation, will have the tumour remaining after endoscopic resection directly injected with the appropriate retroviral supernatant. Toxicity, integration of the proviral DNA by tumour cells, and rate of tumour regrowth will be monitored.

Successful therapy and preventive interventions that reverse genetic lesions may be possible, using genetic constructs designed to specifically inhibit expression of dominant mutant oncogenes or replace the function of deleted or mutated tumour suppressor genes, providing that they can be delivered with high efficiency to tumour cells *in vivo*. Viral vectors have the potential for this. The aerodigestive tract is suited to this approach because high concentrations of these relatively non-toxic agents could be achieved with local installation, thus avoiding the dilutional effects of intravenous injection. Intervention to halt the progression of premalignant lesions to invasive cancer may be possible. Premalignant lesions, such as Barrett's epithelium, have tumour suppressor gene mutations [26]. Preventing the development of invasive cancers would clearly be preferable to treating established cancer. However, there is also a potential role for these agents in the treatment of patients with more advanced cancer. Local recurrence or persistence of local disease is still a major problem for many cancers such as lung, head and neck, and pancreas. Intralesional injections or adjuvant use to prevent local recurrence after surgery could also be considered. Limited metastatic disease could be injected with these agents percutaneously. If these agents are efficacious, their lack of toxicity may provide a sufficiently high therapeutic index such that they could be used as an adjuvant to surgery to treat patients with earlier stages of cancer or as a prophylactic against second primary cancers in individuals with genetic abnormalities in premalignant lesions. The high titres achievable with adenovirus vectors suggest that they could be used systemically. Vector targeting by expression of receptor ligands in the viral capsid is also possible. Although much research still needs to be done, the

possibility of specific gene targeting with a high therapeutic index makes this an exciting and promising area for future investigation.

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