Pergamon

Oral Oncol, Eur J Cancer, Vol. 30B, No. 3, pp. 143–154, 1994

0964–1955/94 $7.00 + 0.00

Reviews

Gene Therapy—Its Potential in the Management of Oral Cancer

E.J. Shillitoe, J.-N. Lapeyre and K. Adler-Storthz

Gene therapy is an important new approach to the treatment of many diseases. This review summarises the methods that are available for developing gene therapy, and demonstrates that oral cancer is probably susceptible to these approaches.
Oral Oncol, Eur J Cancer, Vol. 30B, No. 3, pp. 143–154, 1994.

INTRODUCTION

GENE THERAPY is a new approach to the treatment and prevention of human disease. The possibility of using genetic modifications for therapeutic reasons has been discussed for over a decade, but the topic is now gaining increased attention. It is becoming possible because of the increase in understanding of the molecular basis for many diseases and because of the advances in the technology of genetic manipulation. Oral cancer is one of the diseases in which a large amount of genetic information has recently accumulated. In addition, it occurs at a site that is easily accessible for application of experimental treatments. This review surveys the field of gene therapy, and tries to identify strategies that could be effective in developing new treatments for oral cancer.

GENE THERAPY

History

Recent advances in molecular biology techniques have reached the point where specific changes can be made in the genotype of mammalian cells. This is known as "gene transfer". When gene transfer is used to make cells immune to infection the procedure is often called "intracellular immunisation" [1, 2], when it is used for the treatment of disease it is known as "gene therapy". Gene therapy has become practical within the past 2 years, with the initiation of a small number of human trials and several others pending [3]. The increasing interest in this area of research has led to the establishment of a journal in the U.S.A. that began publication in 1989 (*Human Gene Therapy*, New York, Mary Ann Liebert Inc.), with a European journal planned to be launched at the end of 1993. In the U.S.A. the requirements for approval of new trials have been formulated and published [4].

The gene therapy protocols that are approved by the NIH Recombinant DNA Committee are tabulated and published regularly (*Hum Gen Ther* 1993, **4**, 227). The first protocol was approved in 1989, and tried to show that a foreign gene could be introduced into human cells, which could then be returned to the patient. The second protocol aimed to correct the genetic defect that underlies combined immune deficiency disease. The gene for adenine deaminase was transduced to the lymphocytes of 2 patients, and the cells were reintroduced to each patient. Several other protocols for the marking of cells have been approved for use in patients with cancer. Other protocols have attempted to restore the gene for factor IX in patients with haemophilia, and to treat familial hypercholesterolaemia and various other diseases [3].

Gene therapy for cancer

There are several ways in which gene therapy for cancer is being examined. For tumours where the immune system recognises specific antigens, efforts may be made to strengthen the response. This can be done by modification of the tumour cells or the immune cells. For tumours where basic information is available on the molecular features of the malignancy, efforts may be made to suppress an appropriate molecular pathway. For other tumours it may be possible to introduce genes that encode toxins into the tumour cell. If the toxin gene is expressed only in tumour cells, the tumour growth should be prevented.

Early protocols that were aimed toward gene therapy for cancer attempted simply to show that gene transfer was feasible. They used cell marker genes to allow the tracking of the transduced cells in the patient. These experiments evidently showed that such cells survive within the patient for a reasonable time, and continue to express the marking gene. Now that some of these preliminary experiments have been performed, newer protocols have been introduced in an effort to provide improved treatment for cancer [5].

Correspondence to E.J. Shillitoe at PO Box 20068, Houston, Texas 77225, U.S.A.

E.J. Shillitoe and K. Adler-Storthz are at the Department of Microbiology, Dental Branch, University of Texas Health Science Center; and J.N. Lapeyre is at the Department of Experimental Pathology, M.D. Anderson Cancer Center, Houston, Texas 77225, U.S.A.
Received 29 Apr. 1993; accepted 5 May 1993.

MOLECULAR BIOLOGY OF ORAL CANCER

Mutagens and clastogens

The aetiology of oral cancer is complex, but it seems probable that the disease is largely due to agents that damage DNA. Tobacco is a major source of agents that are mutagenic. Epidemiological studies have shown that the relative risk for oral cancer is related to both the amount of tobacco that is smoked and the number of years of tobacco use. Persons with 1–24 pack-years of exposure to cigarettes have a risk that is some 1.8 times greater than non-smokers, while those with over 50 pack-years of exposure have a risk that is some 7.5 times greater [6]. Persons who quit smoking show a progressive reduction in the risk for oral cancer, and after 15 years the risk is the same as for non-smokers [7, 8].

Another factor that is associated with a higher risk for oral cancer is a high level of alcohol intake, particularly in persons who smoke [6]. The molecular basis for this is not clear, but could be related to the fact that alcohol reduces the efficiency of DNA repair mechanisms that might be needed after mutagens have caused damage to cells [9].

Clearly, most persons who drink and smoke do not get oral cancer, and there is probably some intrinsic susceptibility factor in persons who do develop tumours. The susceptibility might be related to some inability to repair damage to DNA, which would allow small lesions to persist and accumulate. Spitz *et al.* [8] showed that patients with oral cancer are more susceptible than healthy persons to chromosome damage when their cells are exposed to clastogens. Interestingly, heavy smokers who remain healthy into old age are relatively resistant to chromosome damage [10]. If the exact mechanism behind this susceptibility was known, it might provide a target for gene therapy. This susceptibility to mutagens in patients with oral cancer might conceivably be a disadvantage in gene therapy—if virus vectors can integrate more readily in such patients, then any side-effects of integration could occur more easily.

Herpes simplex virus

The herpes simplex virus type-1 (HSV-1) is a tumorigenic virus that is prevalent in all populations. Patients with oral cancer frequently have higher titers and different classes of antibody to HSV-1 than control subjects [11, 12] implying that the virus might be involved in the aetiology of the tumour. If the virus is prevented from killing the infected cells, they can become transformed to a malignant phenotype instead [13]. HSV-1 can also cooperate with chemical carcinogens to transform cells efficiently [14].

The molecular pathways by which HSV-1 transforms cells is unknown. However, it is notable that the virus particle acts as a mutagen. Gene mapping has shown that a mutagenic protein of the virus is encoded within the transforming region of the genome [15] which supports the possibility that transformation and mutagenesis are related activities. Patients with oral cancer have higher levels of antibody to this particular protein [16] and the protein produces changes related to transformation in cultured cells [17].

The types of mutation that HSV-1 produces can be detected by several types of assay [18, 19]. The virus can produce point mutations of various types, as well as short deletions and rearrangements [20]. DNA rearrangements are always of the non-homologous type, and the rearranged sequences are often from the multiple-repeat families of primate DNA. However some unique genomic sequences may also act as targets [21]. Since the mutations are apparently random they do not provide a suitable target for correction by gene therapy. Furthermore, since HSV-1 does not insert its DNA into tumour cell chromosomes, unlike other tumour viruses, the viral DNA does not provide a target for therapy either.

Papillomaviruses

Human papillomavirus (HPV) is frequently found in oral cancers. The proportion of oral cancers that contain HPV is estimated, using the most recent and sensitive techniques, to be around 75% [22]. This is mainly types 16 or 18. It is consistently found that patients have a higher incidence than control subjects of HPV in the oral mucosa [23, 24] although there has been no comprehensive epidemiological study using carefully matched control subjects and calculations of relative risks.

HPV can transform cells *in vitro* to a malignant phenotype in the presence of co-factors, such as chemicals or the *ras* oncogene [25]. Recently, human oral epithelial cells have been transformed by HPV-16 [26] and HPV-18 [27]. The transformation of human keratinocytes requires the high level expression of the E6 and E7 genes, and expression of these two genes is both necessary and sufficient for transformation by the virus [28]. Cells that are transformed *in vitro* by HPV-16 show continuous expression of these two genes, as well as phenotypic changes such as a higher saturation density, reduced requirement for serum, and tumour formation in mice [29]. The continual expression of E7 in particular is necessary for the cells to remain transformed and if synthesis of E7 is interrupted the cells return to the normal phenotype [25].

The presence of HPV in many oral cancers does not prove that the tumour is a result of the virus infection. Indeed, many normal oral tissues also contain HPV [30, 31]. However, the association of the disease with an oncogenic virus makes for a tempting hypothesis. Since transformation *in vitro* involves overexpression of the E6 and E7 genes of HPV-16 or -18, it would be expected that these genes are overexpressed in oral cancers, and that inhibition of these genes would block the malignant phenotype of the cells. It has not been shown if these particular genes are overexpressed in oral cancer, although that is the case in cancer of the uterine cervix [32].

Oncogenes

Oncogenes are sometimes activated in oral cancer cells. However, there are no changes that are seen consistently. One group has reported frequent overexpression of the gene for epidermal growth factor receptor [33] but another group did not [34]. Similarly, amplification of the *myc* and *ras* genes has been reported but not confirmed [35, 36]. Overexpression of the *neu* gene has been reported in a large proportion of patients with oral cancer, but in only one published study [37]. Thus although there may be changes in oncogene activity in oral cancer there are none that are reliably found in a large proportion of patients. This will limit research into using oncogene targets in gene therapy of oral cancer.

Tumour suppressor genes

Tumour suppressor genes play an important role in the growth and proliferation of normal cells. If these genes are inactivated by a mutation, or if both alleles are lost, the result

can be a tumour. The two best-studied tumour suppressor genes are the RB gene and the p53 gene.

Functional loss of the RB gene has been reported in many tumours, including retinoblastoma, osteosarcoma [38–40], soft tissue sarcoma [41], breast carcinoma [42, 43], small cell lung carcinoma [44, 45], bladder carcinoma [46, 47] and hepatocellular carcinoma [48]. There appear to have been no reports on the status of the RB gene in oral cancer.

Loss of function of the p53 gene has been found in diverse tumours, including brain glioblastoma, breast carcinoma, lung carcinoma, neurofibrosarcoma, colon carcinoma, hepatocellular carcinoma, oesophageal carcinoma, bladder carcinoma, ovarian carcinoma, lymphoma and leukaemia (reviewed in [49, 50]). In oral cancer, alterations in p53 expression have been found [51–54].

There is an interesting association between the p53 and RB gene products and papillomaviruses. It appears that these genes are prevented from functioning by the E6 and E7 gene products of HPV-16 and -18 [28, 55–57]. Tumour suppressor proteins are also inactivated in HPV-negative anogenital carcinomas [58–61]. Thus any gene therapy approach that is developed for tumours with mutations in the p53 or RB genes might also be effective in tumours that contain HPV.

POTENTIAL GENETIC MODIFICATIONS OF ORAL MUCOSA

A variety of methods are available for correcting the genetic defects in oral cancer. Some involve the inactivation of specific genes, while others require the addition of new genes (Table 1).

Antisense RNA

Expression of genes can often be inhibited by RNA that is derived from the opposite strand of DNA than is used in gene expression. This "antisense RNA" can prevent activity of several oncogenes, including *myc* [62], *fos* [63] and *myb* [64] and can inhibit viruses such as HSV-1 [65] and human T-cell leukaemia virus type 1 [66]. Carcinoma cells whose malignant phenotype depends on expression of oncogenes will lose their ability to make tumours if the oncogene is inhibited in this way [67, 68]. The exact mechanisms of action of antisense molecules appear to be different in different systems. These include inhibition of translation of RNA to protein, inhibition of movement of RNA molecules within the cell, digestion of double stranded RNA molecules, prevention of splicing, and induction of interferon (reviewed in [69]).

Oral cancer cells that contain DNA of HPV are susceptible to antisense-mediated inhibition. Anti-E6 and anti-E7 oligonucleotides, in concentrations between 1 μmol/l and 5 μmol/l inhibit the growth of HPV-positive oral cancer cells but not HPV-negative cells [70]. A combination of 2.5 μmol/l of each of the antisense oligonucleotides was a more potent inhibitor of proliferation than up to 5 μmol/l of either one alone. However, oligonucleotides are not completely effective against HPV-positive cells. When the oligonucleotides are withdrawn after 3 days of culture the remaining cells recover and grow as before. Any therapy with oligonucleotides will therefore require continuous applications of the molecules.

Table 1. Tumour-associated genes that have been reported to be present, mutated or activated in oral cancer, and techniques by which they can be inhibited

Gene	Ref	Inhibitory mechanism	Ref
HPV	22	Antisense RNA/DNA	70
myc	35	Antisense RNA/DNA	62
neu	37	Ad E1A gene	159
ras	36	Antisense RNA	68
		Ribozymes	80
p53	53	Replacement by wild-type p53	88

A more effective way of providing antisense molecules to a cell would be through the continual expression of molecules from an expression vector. von Knebel Doeberitz *et al.* [71] cloned the E6 and part of the E7 region of HPV-18 in an expression vector in the reverse orientation to that in the virus. The expression vector plasmid was then introduced into a cervical cancer cell line that expresses genes of HPV-18. When the anti-sense E6/E7 was expressed the cells showed significant changes in the phenotype; the cell size and colony sizes were reduced both on plastic and in soft agar, and the rate of incorporation of [^{3}H]thymidine was reduced. Storey *et al.* [72] have shown that oligonucleotides directed to the translation initiation sites of the E6 and E7 genes of HPV-16 could be effective in inhibiting DNA synthesis in the CaSki cell line which carries DNA of HPV-16. HPV-containing oral cancer cells that have been exposed to antisense-expressing plasmids have not survived the transfection procedure, implying that they are extremely sensitive to anti-HPV gene transfer [73, 74].

Ribozymes

A mechanism by which specialised antisense molecules may operate is through an enzyme-like activity in which the target RNA is cleaved at a specific site. RNA molecules with this ability are known as "ribozymes", and a particular class that has been studied in detail is the "hammerhead ribozyme" [75–77]. Hammerhead ribozymes were originally described in the genome of small RNA pathogens of plants that replicate by a mechanism that produces long concatemers of viral RNA that must then be cut into monomers. In fact the cutting step is performed by the RNA itself. Analysis of the region immediately around the cleavage site in several RNA plant pathogens shows that they share a common secondary structure and some conserved sequences. The structure has three base paired stems which are essential, although the sequences of the bases that form the stems can vary. The loops are not necessary for cleavage, and it is possible to make single-stranded ribozymes that bind to the target strand of RNA. The only sequence that is required on the strand that is cleaved is GU although the next base should be C, A or U for optimal cleavage. Thus any RNA containing the sequence GUX (where X is C, A or U) can be targeted for cleavage by base pairing with a second RNA that is designed to form a hammerhead structure.

Several groups have published the results of *in vivo* gene suppression experiments with engineered ribozymes. Cotten and Birnstiel placed ribozyme-coding sequences into a tRNA gene. This construct was microinjected into the nuclei of frog oocytes and resulted in the destruction of the target RNA but not a control RNA [78]. Cameron and Jennings [79] cloned a ribozyme-coding sequence into the 3′ untranslated region of the firefly luciferase gene in an expression vector. Plasmids encoding the ribozyme and the target, chloramphenicol acetyl transferase (CAT) were co-electroporated into monkey COS-1 cells and the ribozyme suppressed CAT expression by up to 60%.

An example of the use of ribozymes in the inhibition of cancer cells is provided by the EJ line of bladder carcinoma cells which contains a *ras* gene with an activating mutation that consists of a change from GGU to GUU at codon 12. This mutation provides a ribozyme target which is not present in the normal *ras* gene. Kashani-Sabet *et al.* [80] showed that a ribozyme that was directed to that site inhibited the tumorigenicity of the cancer cells. The transforming region of the genome of human papilloma viruses has a number of sites that are potential targets for antisense molecules or ribozymes (Fig. 1) and therefore these molecules have potential uses in the treatment of oral cancer.

Triple-stranded DNA

Chromosomal DNA consists of two strands of base-paired nucleotides, but the double helix conformation does allow hybridisation of a third strand into the major groove, producing a locally triple-stranded molecule. This can only happen if the strands contain appropriate proportions of purines and pyrimidines, as well as having matched nucleotide sequences. The third strand can prevent transcription of RNA and so might inhibit the expression of genes that are encoded at that site [81, 82]. The chemical basis of the interaction is now well understood, and it is possible that examples of tumour inhibition will emerge in the near future.

Replacement of tumour-suppressor proteins

Since the loss of tumour-suppressor proteins can lead to a malignant phenotype, it follows that a replacement of the proteins could cause tumour cells to revert to a normal phenotype. There is now experimental evidence that this can be done. The first such study that used the Rb protein was performed by Kim *et al.* [83]. Retroviruses were constructed that contained RB cDNA and were used to infect WERI-Rb27 retinoblastoma cells and Saos2 osteosarcoma cells, both of which carry inactivated RB genes. Expression of the exogenous RB gene caused changes in cell morphology, growth rate, colony formation in soft agar, and tumorigenicity in nude mice in the two cell lines containing mutated RB genes. However, other cells that did not have mutated RB genes were not affected [83]. An important finding from this study was that the expression of the exogenous RB gene was not lethal to the cells and that the pRB was stably produced in the cells for at least 4 months in culture. The RB-reconstituted retinoblastoma cells were unable to form progressive tumours when injected into the anterior eye chambers of immunodeficient mice [84]. In a follow-up study using the same RB-containing retroviruses to infect DU145 human prostate carcinoma cells, no effect of the exogenous pRB on morphology or growth rate was detected, however, tumorigenicity in nude mice was lost in those cells that stably expressed the exogenous RB gene [85]. pRB-negative bladder carcinoma cells were transfected with an RB expression plasmid under control of the β-actin promoter, expressed the RB gene for over 1 year in culture, and were found to have altered growth properties *in vitro* and *in vivo* [86].

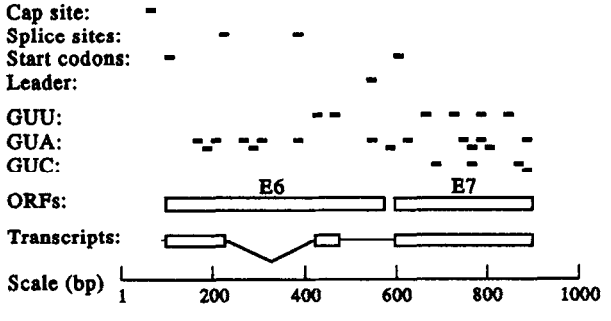

Fig. 1. Potential target sites in RNA transcripts of HPV-18 for inhibition by antisense RNA, or for cleavage by hammerhead ribozymes. The open reading frames (ORFs) that encode the E6 and E7 proteins are shown, together with the RNA transcript and its optional splice sites. The cap site, splice sites, start codons and the E7 leader site are potential targets for antisense RNA, while the GUU, GUA and GUC triplets are potential targets for ribozymes.

Replacement of the p53 gene can also revert tumour cells that have a mutation in their own p53. In studies analogous to those done with exogenous RB genes, two colon carcinoma cell lines with loss of wild-type p53 function were transfected with expression vectors containing wild-type p53 cDNA [87]. The transfected cells were 5–10-fold less efficient at forming colonies that cells transfected with a mutant p53 gene. The colonies that did form after introduction of the wild-type p53 had either deleted or rearranged p53 sequences. The cells expressing the exogenous wild-type gene did not incorporate [^{3}H]thymidine. Studies introducing wild-type p53 into prostate cancer cells produced similar results [88]. Using retroviruses to introduce p53 into Saos2 osteosarcoma cells resulted in inhibition of both colony formation in soft agar and tumorigenicity in nude mice [89]. Co-transfection of wild-type human or murine p53 sequences with HPV 16 E7 and *ras* markedly reduced transformation in baby rat kidney cells [60]. The restoration of p53 expression in a murine myeloid leukaemic cell line resulted in a rapid loss of cell viability, with features characteristic of programmed cell death or apoptosis [90].

The effective use of replacement of RB or p53 proteins in carcinoma cells deserves particular attention in the context of oral cancer, since these genes may be mutated in oral cancer, or their protein products may be affected by products of papillomaviruses.

Toxin genes and suicide phenotypes

Many potent toxins exist in nature, and the genes for several of them have been cloned. If these genes could be expressed selectively in tumour cells, it would eliminate the tumour. Several of these genes have been considered for use in gene therapy of cancer.

Diphtheria A toxin. Cell damage during natural infection by diphtheria is due to a toxin molecule that becomes endocytosed from the cell surface. However, Maxwell *et al.* [91] showed that the gene for diphtheria A chain alone was equally toxic, if it was expressed inside the cell. This has opened the door to selective cell suicide by expression of a DTA transgene. This phenomenon has been applied to problems in developmental biology for tracing lineages [92], and as an anticancer therapy strategy for induction of a suicide response [91, 93]. Diphtheria toxin is extremely lethal, which in fact might be a drawback for its potential clinical use [93].

Staphylococcal enterotoxin. It is possible that the food-poisoning toxins of staphylococci could be adapted for use in gene therapy. Five different strains of *Staphylococcus aureus* are known to secrete a cytolytic enterotoxin, called SEA when

produced by the A strain. SEA is a water soluble monomeric protein that makes pores in membranes [94]. The lipid bilayer is its primary target as binding to the membrane is accompanied by oligomerisation of the 3S monomer to a membrane-bound 12S complex. In electron micrographs, the complex appears as a ring shape formation 8 to 10 nm in diameter with a central 2–3 nm diameter pore [95]. The mode of action is analogous to immune cytolysis by complement, in that the SEA self-associates to form a pore that penetrates the lipid bilayer and permits the release of intercellular constituents. SEA toxin has thus found considerable usefulness in permeabilising cells to study mechanisms for cellular activation [96].

In the laboratory of one of us (J-N L) the SEA gene has been modified for suitable expression in cells and cloned into the pBABE series of retroviral vectors which give very high levels of constitutive expression by using the 5′ LTR as a promoter [97]. The pBABE/MSEA plasmid, when transfected into cells, was confirmed to be less toxic than the diphtheria toxin A chain constructs, which killed the cells within 2–3 days. Infectious virus that carried the SEA gene was recovered and can now be tested in oral tumour cells.

Toxic-phenotype genes. All toxin genes have the potential disadvantage that they will kill any cell that acquires the gene, unless they are expressed under the control of a tightly-regulated promoter. However, a second type of gene that can be used encodes a product that will activate a non-toxic prodrug to a toxic metabolite. The best studied example is the thymidine kinase gene of HSV-1. This enzyme, in contrast to mammalian Tk, activates nucleoside prodrugs to a monophosphorylated analog which can be further anabolised by cellular enzymes to produce cytotoxic triphosphates. The prodrug in this case is either araM or ganciclovir [98–101]. A construct that might be particularly useful is termed HyTk and is an in-frame fusion of HSV-1 Tk with the gene for hygromycin phosphotransferase [102]. This latter enzyme permits positive selection of transduced tumour cells with hygromycin. The selected cells can then be exposed to acyclovir, to kill them.

Moolten has proposed a prophylactic insertion of suicide genes in cell lineages as a general anti-cancer strategy [99]. Tissue-specific promoters are then used to drive a toxic phenotype gene if ever the lineage develops a tumour. This could be an interesting approach in patients who are at risk of developing an oral tumour, because of extensive dysplasia or a history of oral cancer.

Immune modulation

Several approaches attempt to capitalise on the assumed immune response to cancer. Lymphocytes are frequently found in human tumours, and if their cytotoxic response could be increased, this might allow them to kill more tumour cells. Thus one approach has been to remove tumour-infiltrating lymphocytes from a patient's tumour and insert the gene for tumour necrosis factor. The cells are then returned to the patient. This was the experimental plan for one of the earliest trials of human gene therapy [103].

A second way of improving the immune response to tumours could be to increase the antigenicity of the tumour cells. This was the basis of many immunotherapy experiments in the past, using non-genetic methods of enhancing antigenicity [104]. These efforts did not lead to any reliable improvements in cancer therapy, and it is not clear why the transfer of genes for new antigens should be more effective than transferring the antigens themselves. Nonetheless a number of protocols have been devised. Genes encoding HLA-B7 or cytokines are being introduced to tumour cells, either *ex vivo* or *in vivo* [105–107] and results from these efforts are awaited.

For any cancer, immune modulation can only be effective if there is an antitumour immune response that can be enhanced. In the case of oral cancer there seems to be no substantial evidence that this is so. Tumours are frequently infiltrated with lymphocytes, but there is no reliable assay for an immune response, or any evidence for a tumour-specific antigen. Thus any role for immune stimulation by means of gene transfer is very speculative.

DELIVERY SYSTEMS

An important feature of any approach to gene therapy is the method by which new DNA is introduced into cells. For oral cancer cells in culture, gene transfer to date has been carried out only with oligonucleotides which are taken in spontaneously by cells in culture, and by electroporation of plasmids into cells. These methods would be difficult to adapt to whole organisms. However, there are a variety of techniques for transduction of cells or tissues, and all of them should be considered as having some potential clinical uses.

Physical transfer systems

Direct injection. Cells in culture can be transduced by the direct transfer of DNA. This can be made relatively efficient by the use of DEAE-dextran [108], calcium phosphate [109], or electroporation [110]. For intact tissues, direct injection of plasmid DNA can be effective only for muscle [111, 112]. No other tissue has been shown to take up DNA directly in this way, and so it does not seem likely that direct injection of DNA into squamous cell carcinomas will be effective.

Lipofection. Cells will often take up compounds that are enclosed by lipid preparations that mimic a cell wall in some way. Earlier lipofection reagents had significant toxicity to cells, but more recent ones allow efficient transfer with little toxicity [113, 114]. Some *in vivo* experiments show that animals can be transfected with DNA enclosed in liposomes, either by intravenous or direct intratumour injection [115, 116]. A limited human trial is now attempting to use liposomes to mediate direct transfer to human tumours of DNA that encodes a foreign antigen [107].

Systemic oligonucleotides. It is possible to infuse high doses of oligonucleotides to animals without significant side-effects [117] and a human trial has begun using intradermal oligonucleotides for treatment of warts (Dr L. Cowsert). The current drawback of systemic oligonucleotides is the short half-life and high cost of these reagents, but they might be practical for limited local use.

DNA/ligand complexes. Certain cell types can be distinguished by their expression of unique and restricted receptor patterns, and the ligands that bind these receptors can be used as carrier molecules to target the specific cells. The asiloglycoprotein receptor is found only on liver cells, and one group [118] attached marker DNA via a polylysine chain to the asiloglycoprotein for direct delivery to hepatocytes. Others have used

overexpression of receptors for targeting marker DNA coupled with the ligand transferrin [119] or insulin [120]. One group has developed a technique for introducing toxin genes to cells that overexpress epidermal growth factor [121], which is known to be overexpresssed by some oral cancers [33].

Viral vectors

Viruses are considered as having major potential for use in gene transfer protocols although the study of viral vectors for gene therapy is at an early stage. Although several virus vectors have been used to transfer genetic information into cells or organisms it is still not known which ones are most effective in most cell types, nor the best way to promote the expression of their inserts. In particular, very little published work has addressed the question of the best vector for use in squamous carcinomas or in mucosal epithelial cells.

It seems unlikely that any unmodified virus will ever be used for gene therapy. However, attenuated viruses are used routinely as vaccines, and so it is possible that attenuated viruses could be used to insert novel genes to cells. It is possible that other modifications could be made to improve the efficacy of a virus, for example by changing its host range. The gene for the CD4 molecule has been incorporated into retroviruses and herpes simplex virus [122, 123] so as to allow them to infect lymphocytes with receptors for CD4. If any specific site on oral tumour cells is ever found and a receptor for that site becomes known, it might be possible to express the receptor on the surface of gene therapy viruses, thus targeting them to the cancer cells.

Retroviruses. Most research on viral vectors for gene therapy has been focused on the moloney murine leukaemia virus (MoMLV). This virus can cause malignant lymphomas and leukaemias in mice, but is usually regarded as non-pathogenic in primates. The virus has a small RNA genome, consisting of three open reading frames designated *gag*, *pol* and *env*, together with a packaging sequence (Ψ). These are flanked at each end by a long terminal repeat (LTR, Fig. 2). The LTR acts as a promoter of the genes downstream, and is used by the virus to integrate its genome into chromosomes of infected cells. This only occurs in replicating cells, and so retroviruses cannot be used to transfer genetic information to post-mitotic cells.

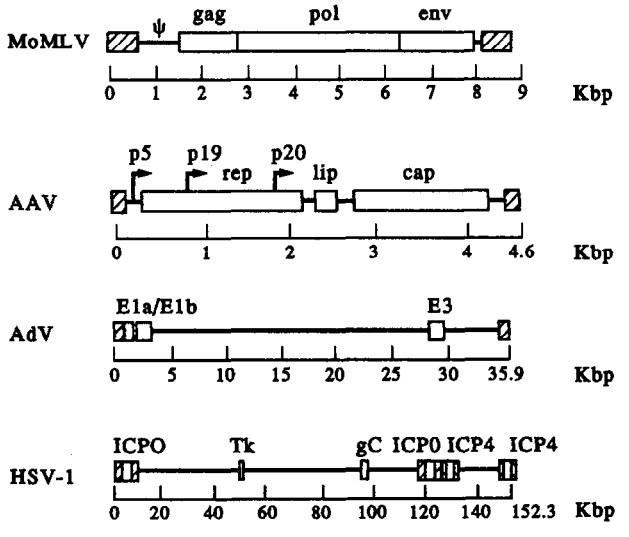

Fig. 2. Gene maps of viruses that could be used as vectors for gene therapy. Genomes are shown with features that are relevant to gene transfer, together with a scale in kilobase-pairs (Kbp). Shaded boxes indicate repeat sequences and open boxes represent open reading frames.

For gene therapy use, many variations in the MoMLV genome have been made by various researchers. These involve replacing the *gag*, *pol* and *env* genes with genes and promoters that are to be transferred to the target cells. The virus cannot replicate without these three genes, but they can be expressed by specialised cell lines that will then allow the modified virus to replicate. Various deletions and other changes are necessary so as to reduce the possibility of any recombination that might recreate the original infectious retrovirus.

The retroviral vectors that are in use for most gene therapy trials were derived from the LNL6 modification of MoMLV [124], although similar vectors have been made by other workers [97]. Another variant of retroviral vectors consists of a "double-copy" vector, in which the gene of interest is cloned into the 3' LTR of the virus, thus resulting in two copies when the virus is integrated into target cells. These vectors use a tRNA pol III promoter, and as a result they express very large amounts of RNA in the infected cell. In fact, up to 5% of the cell RNA can be derived from the inserted gene and have been used to express effective levels of anti-viral antisense transcripts in cells [125, 126].

Very few studies have attempted to transfer genetic information to oral epithelial cells. However, Garlick *et al.* [127] showed that all keratinocytes in a culture could be transfected by retroviral vectors. However, when they tried to use a more modified virus to transfer the beta-galactosidase gene, it was acquired by less than 5% of cells. This points out the unfortunate phenomenon that the most modified viruses tend to be the least infectious. This is familiar to workers with all types of virus vectors, although it is not well documented in the literature.

Safety issues are important in the use of any virus as a gene therapy vector. In the case of retroviruses, one side-effect from retroviruses could be from the viral LTR, which might promote expression of adjacent cellular genes. Another possible problem would be from interruption of cellular genes. Safety issues related to the use of retroviruses have been discussed [128], and risk calculations have been performed [129].

Adeno-associated virus. Adeno-associated virus (AAV) is widespread in the human population but is non-pathogenic, and integrates at a specific, non-essential site in chromosome 19 [130, 131]. The viral genome is extremely small, having only two open reading frames, *rep* and *cap*, together with another essential sequence designated *lip*. The genome has three promoters, p5, p19 and p40, and is flanked by repeat sequences (Fig. 2). As a result of its very limited genome the virus is defective and needs a helper virus before it can replicate in cells. AAV infects the majority of its target cells, and can be used to transfer genetic information to cells [132]. The cells do not have to be replicating in order to become infected. LaFace *et al.* [133] showed that AAV could transfer the *neo* marker gene to cells, and pointed out the value of AAV as an alternative to retrovirus systems. Chatterjee *et al.* [134] used AAV to transfer antisense sequences of HIV to cells, which were then protected against that virus. An important effect of AAV is that one of its genes, *rep*, has intrinsic antitumour effects [135]. Thus unmodified AAV can inhibit cell transformation by papillomaviruses [136], inhibit the mutagenic effects of herpes simplex virus [137], increase differentiation [138] and reduce the tumorigenicity in

mice of human cervical cancer cells [139]. These effects therefore might act synergistically with any inserted anti-cancer gene, to make AAV an especially useful vector in treatment of oral cancer.

Adenoviruses. Adenoviruses naturally infect mucous membranes of the respiratory tract, and therefore would seem to be ideal vectors for transfer of genetic material to this tissue. Adenoviruses have an advantage over retroviruses and AAV in that their genome is bigger and offers many sites, such as the E1 and E3 regions, into which new DNA can be cloned (Fig. 2). A further advantage of adenoviruses is that they can infect non-replicating cells. Rosenfeld *et al.* [140] have used adenoviruses to transfer the gene for alpha-1 antitrypsin to respiratory mucosa of rats. This has potential for the treatment of cystic fibrosis, and limited human trials have been approved. In one study, adenoviruses were delivered to animals by intravenous administration, and transferred functional genes to skeletal muscle and heart [141]. An interesting modification of the use of adenoviruses has been tested [142], in which an antibody was used to link a gene to the outside of an adenovirus. The virus carried the foreign gene into cells, and since adenoviruses disrupt cell endosomes, the transferred DNA was delivered directly to the cytoplasm. Adenoviruses have not been used in development of therapy for cancer, although they could probably be adapted for that purpose.

Herpes simplex virus. HSV-1 would seem to be an excellent vector for transfer of genetic material to the oral mucosa. The virus infects these tissues as its natural target, and can be grown to a high titer in the laboratory. It will infect non-replicating cells, unlike the retroviruses. However, it is a human pathogen and many modifications would be necessary before it can be used as a vector [143]. The genome is large and complex, but several suitable cloning sites are known (Fig. 2). Much of the viral DNA is dispensible [144]. This allows insertions of many kilobases of genetic material—probably more than for any other viral vector. Ho and Mocarski [145] showed that HSV-1 could be used to carry the gene for beta-galactosidase into epithelium, and that the gene was expressed at high levels. In that case the novel gene replaced the viral gene for thymidine kinase, and was under the control of a herpes immediate-early promotor. Intradermal expression of beta-galactosidase was seen by 4 h post-infection, which increased over the following 3 days. Presumably the virus would have killed the infected cells shortly afterward, but many reports show how HSV-1 can be modified to become non-lytic [146, 147].

Currently, HSV-1-based vectors are being developed for the transfer of genes to the brain for the eventual treatment of nervous system conditions. This is feasible because HSV has a promoter that is active when the virus is latent in nervous system tissue, the latency associated gene of the virus [148]. In epithelium, however, HSV does not persist. Obviously if the aim of gene therapy was to kill tumour cells then the failure to persist in epithelium would not be a disadvantage. However, for any system in which viral persistence is necessary, other vectors might be better than HSV-1.

Vaccinia virus. Vaccinia virus has been used for many years as a vaccine against smallpox. The genome is large and can contain a number of different inserted genes. Vaccinia virus could probably be used in gene therapy procedures, but so far has been examined mostly for the development of new vaccines [149].

Table 2. Potential promoters for expression of gene therapy constructs in oral cancer tissues

Promoter	Advantage	Disadvantage	Ref
CMV early	Powerful	Constitutive Not tested	150
MoMLV	Widely used	Constitutive Not tested	103
MMTV/ RSV	Shown effective	Large—requires steroids	70
SV40 early	Widely used	Constitutive Not tested	150
tRNA	Very powerful	Constitutive Not tested	78 126
HPV URR	Very specific	Requires E2 protein of HPV	154
AAV-2 p40	Present in AAV	Not tested	160
Keratin	Tissue specific	Not tested	152
ICP-4	Shown effective	Requires other HSV functions	145

"Powerful" refers to the number of expressed molecules that the promotor drives. "Constitutive" is classified here as a disadvantage since the gene will always be expressed in all tissues that receive it. "Shown effective" means that the promoter is functional in epithelial cells, but not necessarily oral or tumour cells. "Not tested" indicates that the promotor has not been tested in oral epithelial, or similar cells.

Promoters of gene expression

The mere presence of a novel gene in a cell is no guarantee that it will be expressed. The gene must be located next to a promoter that is active in that cell (Table 2). Tissue-specific promoters can also have the advantage that if a therapeutic virus was to infect cells other than the target cell, expression of the cloned gene would not happen. Apart from tissue specificity, the level of activity is also important because different promoters lead to different levels of expression in different cell types [150, 151]. The third, and least understood facet of promoter activity, is the duration of expression. Some promoters are only active for a short time. As an example of the conflicting features of promoters, in one experiment in central nervous system tissue the use of the LTR from MoMLV as a promoter led to higher level expression than did a more tissue-specific promoter. However, the LTR shut down within 14 days, in contrast to the tissue-specific promoter which remained active for over 30 days [143].

For some types of cell, tissue-specific promoters are available. For example, α-crystalline, elastase, α-1-antitrypsin, α-fetoprotein, tryptophan pyrrolase are potentially excellent tools for targeting certain types of cancers. Epithelium at different stages of maturation expresses different cytokeratin genes [152] and the promoters of these genes could probaly be used to direct expression of novel genes [153].

In oral cancers that contain HPV, the upstream regulatory region (URR) of the HPV genome is a potential specific promoter. Many cell factors act on the URR to cause expression of the genes that are placed downstream. Many of these factors are known in cervical cancer cells, and probably have equivalents in oral cancer. In cells that carry papilloma

virus DNA the E2 protein of the virus can bind to the URR and activate or inhibit the promoter, depending on other factors. However, in tumours where HPVs are integrated, the integration frequently truncates the E2 gene, leading to a shorter gene product which cannot inhibit expression, but will only actually enhance it. The region of the URR that responds to the enhancing function of E2 is very small—only around 12 nucleotides. Ham *et al.* [154] have made synthetic promoters based on this sequence and they could probably be used in gene therapy protocols for HPV-positive oral cancers.

CLINICAL CONSIDERATIONS IN TREATMENT OF ORAL TUMOURS

Assuming that suitable genetic corrections could be applied, and suitable virus vectors and promoters were available, how could gene therapy for oral cancer be applied to the patient? The oral tissues are easily available for administration of therapy and this provides many choices for methods of administration. Techniques that could be considered include:

(i) Direct injection into a tumour. This could be done whether viral vectors, or physical transfer methods are used.

(ii) Application to tumour margins. At the time of surgery it would be possible to apply therapeutic agents to the tumour bed, to remove any remaining tumour cells.

(iii) Embolisation. Injection of therapeutic viruses or other agents into an artery that supplies blood to a tumour has been used for administration of chemotherapeutic agents.

(iv) Mouthwashes might be effective in administering therapeutic viruses to the oral mucosa. It would be important that basal layer cells become infected, which might not happen, especially as gene therapy viruses cannot replicate in tissues, but are limited to their site of entry.

For selection of patients for gene therapy, there are again several choices.

(i) Management of pre-malignant lesions. Many leukoplakias or erythroplasias become malignant and it is an important clinical problem to manage these lesions. If the genetic basis for their transformation was clear, the use of gene therapy could be applied to prevent malignant change in the lesions.

(ii) Treatment of established tumours. Most oral cancers can be removed surgically, but some have invaded vital areas where surgery is difficult, or have metastasised to multiple sites. It would be beneficial to treat these tumours in some new way. Even if gene therapy could reduce the mass of the tumour, that would make any subsequent surgery easier.

(iii) Prevention of recurrence or second primary tumours. Patients who have had a primary oral cancer are at high risk of developing a second tumour later, and it is these second tumours that are fatal. The rate of development of second primaries is higher for oral cancer than for any other cancer [155]. If the apparently normal tissues of these patients could be transduced with protective sequences using suitable gene transfer, it might reduce the risk of these second tumours. This seems to be the most likely application for gene therapy of oral cancer. Indeed, trials of chemoprevention agents have used a similar strategy, and have shown that significant data can be accumulated in a relatively short time with small groups of patients [156].

POTENTIAL RISKS OF GENE THERAPY

The potential power of modifying the genome of cells carries with it a number of possible risks. DNA that integrates into the genome may interrupt essential genes. This would modify the behaviour of cells in an unpredictable way. Conversely, genes that are close to the integration site might be activated. This again could lead to unpredictable results. Virus vectors might revert to the wild type's ability to replicate and spread in the population, but still carry their new gene sequences. This could happen if the therapeutic virus was able to recombine in some way with a wild-type strain. There is no way to recall a modified virus.

As well as these questions of biological safety there are a number of ethical issues that apply to modifications of the human genome. Manipulation of cell genomes for the sake of treating disease is becoming less controversial, but other issues stem from the ability to make changes in the germ line [157], and from the potential for trivial applications of gene transfer [158]. These considerations will be resolved slowly, as experience with the new approaches to therapy is accumulated.

CONCLUSIONS

Recent research has made very significant progress toward gene therapy of oral cancer. The molecular basis of the disease is becoming well understood, and the defects are susceptible to correction by gene transfer technology. Virus vectors and other techniques for the transfer of genes are available and being improved. These developments, together with the need for improvements in treatment, make it extremely likely that clinical trials of gene therapy will be designed and implemented very soon.

1. Baltimore D. Intracellular immunization. *Nature* 1988, **335**, 395–396.
2. Steele C, Shillitoe EJ. Oral virus infections: the potential for gene transfer in treatment and prevention. In Ciardi JE, McGhee JR, Keith J, eds. *Genetically Engineered Vaccines: Prospects for Oral Disease Prevention*. New York, Plenum Publishing Corp 1992, 35–42.
3. Miller AD. Human gene therapy comes of age. *Nature* 1992, **357**, 455–460.
4. Food and Drug Administration. Points to consider in human somatic cell therapy and gene therapy. *Hum Gene Ther* 1991, **2**, 251–256.
5. Gutierrez AA, Lemoine NR, Sikora K. Gene therapy for cancer. *Lancet* 1992, **339**, 715–721.
6. Spitz MR, Fueger JJ, Goepfert H, Hong WK, Newell GR. Squamous cell carcinoma of the upper aerodigestive tract: a case comparison analysis. *Cancer* 1988, **61**, 203–208.
7. Blot WJ, McLaughlin JK, Winn DM, *et al.* Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res* 1988, **48**, 3282–3287.
8. Spitz MR, Fueger JJ, Beddingfield NA, *et al.* Chromosome sensitivity to bleomycin-induced mutagenesis, an independent risk factor for upper aerodigestive tract cancers. *Cancer Res* 1989, **49**, 4626–4628.
9. Hsu TC, Furlong C, Spitz MR. Ethyl alcohol as a cocarcinogen with special reference to the aerodigestive tract: a cytogenetic study. *Anticancer Res* 1991, **11**, 1097–1101.
10. Hsu TC, Johnston DA, Cherry LM, *et al.* Sensitivity to genotoxic effects of bleomycin in humans: possible relationship to environmental carcinogenesis. *Int J Cancer* 1989, **43**, 403–409.
11. Shillitoe EJ, Greenspan D, Greenspan JS, Silverman S. The immunoglobulin class of antibody to herpes simplex virus in patients with oral cancer. *Cancer* 1983, **51**, 65–71.
12. Shillitoe EJ, Matney T, Conley AJ. Induction of mutations in bacteria by a fragment of DNA from herpes simplex virus type 1. *Virus Res* 1986, **6**, 181–191.

13. Duff R, Kreider JW, Levy BM, Katz M, Rapp F. Comparative pathology of cells transformed by herpes simplex virus type 1 or type 2. *J Natl Cancer Inst* 1974, **53**, 1159–1164.
14. Cornella FA, Saper CD, Christensen RE, Park NH. Effect of DMBA on oral cancer development in hamsters with latent HSV-1 infections in trigeminal ganglia. *Oral Surg Oral Med Oral Pathol* 1989, **67**, 167–171.
15. Das C, Shillitoe EJ. A protein encoded by the transforming region of herpes simplex virus type-1. (Submitted for publication)
16. Das CM, Schantz SP, Shillitoe EJ. Antibody to a mutagenic peptide of herpes simplex virus in young adult patients with cancer of the head and neck. *Oral Surg Oral Med Oral Pathol* 1993, **75**, 610–614.
17. Das C, Murrah V, Zhang S, Wong G, Gilchrist EP, Shillitoe EJ. The effect on cell phenotype of a peptide that is encoded by the transforming region of herpes simplex type-1. (Submitted for publication.)
18. Schlehofer JR, zur Hausen H. Induction of mutations within the host cell genome by partially inactivated herpes simplex virus type 1. *Virology* 1982, **122**, 471–475.
19. Pilon L, Royal A, Langelier Y. Increased mutation frequency after herpes simplex virus type 2 infection in non-permissive XC cells. *J Gen Virol* 1985, **66**, 259–265.
20. Hwang CBC, Shillitoe EJ. DNA sequence of mutations induced in cells by herpes simplex virus type-1. *Virology* 1990, **178**, 180–188.
21. Hwang CBC, Shillitoe EJ. Analysis of complex mutations induced in cells by herpes simplex virus type-1. *Virology* 1991, **181**, 620–629.
22. Woods KV, Shillitoe EJ, Spitz MR, Schantz SP, Adler-Storthz K. Analysis of human papillomavirus DNA in oral squamous cell carcinomas. *J Oral Pathol Med* 1993, **22**, 101–108.
23. Chang F, Syrjanen S, Kellokoski J, Syrjanen K. Human papillomavirus (HPV) infections and their associations with oral disease. *J Oral Pathol Med* 1991, **20**, 305–317.
24. Steele C, Shillitoe EJ. Viruses and oral cancer. *Crit Rev Oral Biol Med* 1991, **2**, 153–175.
25. Crook T, Morgenstern JP, Crawford L, Banks L. Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus EJ-ras. *EMBO J* 1989, **8**, 513–519.
26. Park NH, Min BM, Li SL, Huang MZ, Cherick HM, Doniger J. Immortalization of normal human oral keratinocytes with type 16 human papillomavirus. *Carcinogenesis* 1991, **12**, 1627–1631.
27. Park NH, Min BM, Cherrick HM, Sparkes RS. Immortalization of normal human gingival keratinocytes (NHGK) with human papillomavirus type 18 (HPV-18). *J Dent Res* 1991, **70S**, 271.
28. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989, **63**, 4417–4421.
29. Yasumoto S, Doniger J, DiPaolo JA. Differential early viral gene expression in two stages of human papillomavirus type 16 DNA-induced malignant transformation. *Mol Cell Biol* 1987, 7, 2165–2172.
30. Scully C, Cox MF, Prime SS, Maitland NJ. Papillomaviruses: the current status in relation to oral disease. *Oral Surg Oral Med Oral Pathol* 1988, **65**, 526–532.
31. Jenison SA, Yu X, Valentine JM, *et al.* Evidence of prevalent genital-type human papillomavirus infections in adults and children. *J Infect Dis* 1990, **162**, 60–69.
32. Johnson MA, Blomfield PI, Bevan IS, Woodman CBJ, Young LS. Analysis of human papillomavirus type 16 E6-E7 transcription in cervical carcinomas and normal cervical epithelium using the polymerase chain reaction. *J Gen Virol* 1990, **71**, 1473–1479.
33. Ishitoya J, Toriyama M, Oguchi N, *et al.* Gene amplification and overexpression of EGF receptor in squamous cell carcinomas of the head and neck. *Br J Cancer* 1989; **59**, 559–562.
34. Eisbruch A, Blick M, Lee JS, Sacks PG, Gutterman J. Analysis of the epidermal growth factor receptor gene in fresh human head and neck tumors. *Cancer Res* 1987, **47**, 3603–3605.
35. Spandidos DA, Lamothe A, Field JK. Multiple transcriptional activation of cellular oncogenes in human head and neck solid tumours. *Anticancer Res* 1985, **5**, 221–224.
36. Berenson JR, Yang J, Mickel RA. Frequent amplification of the *bcl*-1 locus in head and neck squamous cell carcinomas. *Oncogene* 1989, **4**, 1111–1116.
37. Hou L, Shi D, Tu S, Zhang H, Hung M, Ling D. Oral cancer progression and c-*erb*B-2/*neu* proto-oncogene expression. *Cancer Lett* 1992, **66**, 215–220.
38. Weichselbaum RM, Beckett M, Diamond A. Some retinoblastomas, osteosarcomas, and soft tissue sarcomas may share a common etiology. *Proc Natl Acad Sci USA* 1988, **85**, 2106–2109.
39. Reissman PT, Simon MA, Lee WH, Siamon DJ. Studies of the retinoblastoma gene in human sarcomas. *Oncogene* 1989, **4**, 839–843.
40. Toguchida J, Ishizaki K, Sasaki MS, *et al.* Chromosomal reorganisation for the expression of recessive mutation of retinoblastoma susceptibility gene in the development of osteosarcoma. *Cancer Res* 1988, **48**, 3939–3943.
41. Friend SH, Horowitz JM, Gerber MR, *et al.* Deletions of a DNA sequence in retinoblastomas and mesechymal tumors: organization of the sequence and its encoded protein. *Proc Natl Acad Sci USA* 1987, **84**, 9050–9063.
42. T'Ang A, Varley JM, Chakraborty S, Murphree AL, Fung YKT. Structural rearrangement of the retinoblastoma gene in human breast carcinoma. *Science* 1988, **242**, 263–266.
43. Lee EYHP, To H, Shew JY, Bookstein R, Scully P, Lee WH. Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science* 1988, **241**, 218–221.
44. Harbour JW, Li SL, Whang-Peng J, Gazdar AF, Minna JD, Kaye FJ. Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* 1988, **241**, 353–357.
45. Yokota J, Akiyama T, Fung YKT, *et al.* Altered expression of the retinoblastoma gene (RB) gene in small-cell carcinoma of the lung. *Oncogene* 1988, **3**, 471–475.
46. Horowitz JM, Yandell DW, Park SH, *et al.* Point mutational inactivation of the retinoblastoma antioncogene. *Science* 1989, **243**, 937–940.
47. Ishikawa J, Xu HJ, Hu SX, *et al.* Inactivation of the retinoblastoma gene in human bladder and renal cell carcinomas. *Cancer Res* 1991, **51**, 5736–5743.
48. Murakami Y, Hayashi K, Hirohashi S, Sekiya T. Abberations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res* 1991, **51**, 5520–5525.
49. Nigro JM, Baker SJ, Preisinger AC, *et al.* Mutations in the p53 gene occur in diverse human tumour types. *Nature* 1989, **342**, 705–708.
50. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991, **253**, 49–53.
51. Warnakulasuriya S, Johnson NW. p53 oncoprotein expression in oral carcinoma among Sri Lankan and Chinese. *J Dent Res* 1992, **72**, 707.
52. Largey J, Meltzer S, Sauk J, Norris K, Archibald D. Loss of heterozygosity involving p53 in oral cancers. *J Dent Res* 1992, **71**, 197.
53. Somers KD, Merrick MA, Lopez ME, Incognito LS, Schecter GL, Casey G. Frequent p53 mutations in head and neck cancer. *Cancer Res* 1992, **52**, 5997–6000.
54. Brachman DG, Graves D, Vokes E, *et al.* Occurrence of p53 gene deletions and human papilloma virus infection in human head and neck cancer. *Cancer Res* 1992, **52**, 4832–4836.
55. Dyson N, Howley PM, Munger K, Harlow E. Human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989, **243**, 934–936.
56. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus type 16 and 18 E6 protein with p53. *Science* 1990, **248**, 76–79.
57. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 1990, **63**, 1129–1136.
58. Scheffner M, Munger K, Byrne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci USA* 1991, **88**, 5523–5527.
59. Wrede D, Tidy JA, Crook T, Lane D, Vousden KH. Expression of RB and p53 proteins in HPV-positive and HPV-negative cervical cell lines. *Mol Carcinogen* 1991, **4**, 171–175.
60. Crook T, Fisher C, Vousden KH. Modulation of immortalizing

properties of human papilloma virus type 16 E7 by p53 expression. *Virology* 1991, **65**, 505–510.
61. Yaginuma Y, Westphal H. Analysis of the p53 gene in human uterine carcinoma cell lines. *Cancer Res* 1991, **51**, 6506–6509.
62. Wickstrom EL, Bacon TA, Gonzalez A, *et al.* Human promyelocytic leukemia HL-60 cell proliferation and c-*myc* protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-*myc* mRNA. *Proc Natl Acad Sci USA* 1988, **85**, 1028–1032.
63. Nishikura K, Murray JM. Antisense RNA of proto-oncogene c-*fos* blocks renewed growth of quiescent 3T3 cells. *Mol Cell Biol* 1987, **7**, 639–649.
64. Melani C, Rivoltini L, Parmiani G, Calabretta B, Colombo M. Inhibition of proliferation by c-*myb* antisense oligodeoxynucleotides in colon adenocarcinoma cell lines that express c-*myb*. *Cancer Res* 1991, **51**, 2897–2901.
65. Smith CC, Aurelian L, Reddy MP, Miller PS, Ts'o POP. Antiviral effect of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5. *Proc Natl Acad Sci USA* 1986, **83**, 2787–2791.
66. von Ruden T, Gilboa E. Inhibition of human T-cell leukemia virus type 1 replication in primary human T cells that express antisense RNA. *J Virol* 1989, **63**, 677–682.
67. Kasid U, Pfeifer A, Brennan T, *et al.* Effect of antisense c-*raf*-1 on tumorigenicity and radiation sensitivity of a human squamous carcinoma. *Science* 1989, **243**, 1354–1356.
68. Mukhopadhyay T, Tainsky M, Cavender AC, Roth JA. Specific inhibition of K-*ras* expression and tumorigenicity of lung cancer cells by antisense RNA. *Cancer Res* 1991, **51**, 1744–1748.
69. Helene C, Toulme JJ. Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim Biophys Acta* 1990, **1049**, 99–125.
70. Steele C, Cowsert LM, Shillitoe EJ. The effects of human papillomavirus type-18-specific antisense oligonucleotides on the transformed phenotype of human carcinoma cell lines. *Cancer Res* 1993, **53**, 2330–2337.
71. von Knebel Doeberitz M, Oltersdorf T, Schwarz E, Gissman L. Correlation of modified human papilloma virus early gene expression with altered growth properties in C4-1 cervical carcinoma cells. *Cancer Res* 1988, **48**, 3780–3786.
72. Storey A, Oates D, Banks L, Crawford L, Crook T. Anti-sense phosphorothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16. *Nucl Acids Res* 1991, **19**, 4109–4114.
73. Shillitoe EJ, Steele C. Inhibition of the transformed phenotype of carcinoma cells that contain human papillomavirus. *Ann NY Acad Sci USA* 1992, **660**, 286–287.
74. Steele C. Sacks PG, Adler-Stortz K, Shillitoe EJ. Effect on cancer cells of plasmids that express antisense RNA of human papillomavirus type 18. *Cancer Res* 1992, **52**, 4706–4711.
75. Forster AC, Symons RH. Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. *Cell* 1987, **49**, 211–220.
76. Haseloff J, Gerlach WL. Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* 1988, **334**, 585–591.
77. Symons RH. Self-cleavage of RNA in the replication of small pathogens of plants and animals. *Trends Biochem Sci* 1989, **14**, 445–450.
78. Cotten M, Birnstiel ML. Ribozyme mediated destruction of RNA *in vivo*. *EMBO J* 1989, **8**, 3861–3866.
79. Cameron FH, Jennings PA. Specific gene suppression by engineered ribozymes in monkey cells. *Proc Natl Acad Sci USA* 1989, **86**, 9139–9143.
80. Kashani-Sabet M, Funato T, Tone T, *et al.* Reversal of the malignant phenotype by an anti-*ras* ribozyme. *Antisense Res Devel* 1992, **2**, 3–15.
81. Helene C. The anti-gene strategy: control of gene expression by triplex-forming-oligonucleotides. *Anticancer Drug Des* 1991, **6**, 569–584.
82. Maher LJ, Wold B, Dervan PB. Oligonucleotide-directed DNA triple-helix formation: an approach to artificial repressors? *Antisense Res Dev* 1991, **1**, 277–281.
83. Kim SJ, Lee HD, Robbins PD, Busam K, Sporn MM, Roberts AB. Regulation of the transforming growth factor beta 1 gene expression by the product of the retinoblasoma-susceptibility gene. *Proc Natl Acad Sci USA* 1991, **88**, 3052–3056.
84. Madreperia SA, Whittum-Hudson JA, Prendergast RA, Chen PL, Lee WH. Intraocular tumor suppression of retinoblastoma gene-reconstituted retinoblastoma cells. *Cancer Res* 1991, **51**, 6381–6384.
85. Bookstein R, Shew JY, Chen PL, Scully P, Lee WH. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. *Science* 1990, **247**, 712–715.
86. Takahashi R, Hashimoto T, Xu HJ, *et al.* The retinoblastoma gene functions as a growth and tumor suppressor in human bladder carcinoma cells. *Proc Natl Acad Sci USA* 1991, **88**, 5257–5261.
87. Baker SJ, Markowitz S, Fearon ER, Willson JKV, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 1990, **249**, 912–915.
88. Isaacs WB, Carter BS, Ewing CM. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. *Cancer Res* 1991, **51**, 4716–4720.
89. Chen PL, Chen Y, Bookstein R, Lee WH. Genetic mechanisms of tumor suppression by the human p53 gene. *Science* 1990, **250**, 1576–1580.
90. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 1991, **352**, 345–347.
91. Maxwell IH, Maxwell F, Glode LM. Regulated expression of a diphtheria toxin A-chain gene transfected into human cells: possible strategy for inducing cancer cell suicide. *Cancer Res* 1986, **46**, 4660–4664.
92. Palmiter RD, Behringer RR, Quaife CJ, Maxwell F, Maxwell IH, Brinster RL. Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. *Cell* 1987, **50**, 435–443.
93. Maxwell IH, Glode LM, Maxwell F. Expression of the diphtheria toxin A-chain coding sequence under the control of promoters and enhancers from immunoglobulin genes as a means of directing toxicity to B-lymphoid cells. *Cancer Res* 1991, **51**, 4299–4304.
94. Arbuthnot JP, Freer JH, Bilcliff B. Lipid-induced polymerization of staphylococcal alpha-toxin. *J Gen Microbiol* 1973, **75**, 309–316.
95. Fussle R, Bhakdi S, Sziegoleit A, Tranum-Jensen J, Kranz T, Wellensiek HJ. On the mechanism of membrane damage by Staphylococcus aureus alpha-toxin. *J Cell Biol* 1981, **91**, 83–94.
96. Ahnert-Hilger G, Bhakdi S, Gratzi M. Minimal requirements for exocytosis. *J Biol Chem* 1985, **260**, 12730–12734.
97. Morgenstern JP, Land H. Advanced mammalian gene transfer: high titer retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucl Acids Res* 1990, **18**, 3587–3596.
98. Moolten FL. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res* 1986, **46**, 5276–5281.
99. Moolten FL, Wells JM, Heyman RA, Evans RM. Lymphoma regression induced by ganciclovir in mice bearing a herpes thymidine kinase transgene. *Hum Gene Ther* 1990, **1**, 125–134.
100. Borelli E, Heymin R, Hsi M, Evans RM. Targeting of an inducible toxic phenotype in animal cells. *Proc Natl Acad Sci USA* 1988, **85**, 7572–7576.
101. Huber BE, Richards CA, Krenitsky TA. Retroviral-mediated gene therapy for the treatment of hepatocellular carcinomas: an innovative approach for cancer therapy. *Proc Natl Acad Sci USA* 1991, **88**, 8039–8043.
102. Renkawitz R. Transcriptional repression of the eukaryotes. *Trends Genet* 1990, **6**, 192–197.
103. Rosenberg SA, Aebersold P, Cornetta K, *et al.* Gene transfer into humans—immunotherapy of patients with advanced melanoma using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* 1990, **323**, 570–578.
104. Kedar E, Klein E. Cancer immunotherapy: are the results discouraging? Can they be improved? *Adv Cancer Res* 1992, **59**, 245–322.
105. Golumbek PT, Lazenby AJ, Levitsky HI, *et al.* Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science* 1991, **254**, 713–716.
106. Nabel EG, Gordon D, Yang Z, *et al.* Gene transfer *in vivo* with

DNA-liposome complexes: lack of autoimmunity and gonadal localization. *Hum Gene Ther* 1992, **3**, 649–656.
107. Nabel GJ, Chang A, Nabel EG, Plautz G. Immunotherapy of malignancy by *in vivo* gene transfer into tumors. *Hum Gene Ther* 1992, **3**, 399–410.
108. McCutchen JA, Pagano JS. Enhancement of the infectivity of SV40 deoxyribonucleic acid with diethylaminoethyl dextran. *J Natl Cancer Inst* 1968, **41**, 351–357.
109. Wigler M, Silverstein S. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* 1977, **11**, 223–232.
110. Potter H. Electroporation in biology: methods, applications, and instrumentation. *Anal Biochem* 1988, **174**, 361–373.
111. Wolff JA, Malone RW, Williams P, *et al.* Direct gene transfer into mouse muscle *in vivo*. *Science* 1990, **247**, 1465–1468.
112. Jiao S, Williams P, Berg RK, *et al.* Direct gene transfer into nonhuman primate myofibers *in vivo*. *Hum Gene Ther* 1992, **3**, 21–33.
113. Gao X, Huang L. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem Biophys Res Comm* 1991, **179**, 280–285.
114. Rose JK, Buonocore L, Whitt MA. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *Bio Techniques* 1991, **10**, 520–525.
115. Stewart MJ, Plautz GE, Del Buono L, *et al.* Gene transfer *in vivo* with DNA-liposome complexes; safety and acute toxicity in mice. *Hum Gene Ther* 1992, **3**, 267–275.
116. Yoshimura K, Rosenfeld MA, Nakamura H, Scherer EM, Pavirani A. Expression of the human cystic fibrosis transmembrane conductance regulator gene in the mouse lung after *in vivo* intratracheal plasmid-mediated gene transfer. *Nucl Acids Res* 1992, **20**, 3233–3240.
117. Agrawal S, Temsamani J, Tang JY. Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc Natl Acad Sci USA* 1991, **88**, 7595–7599.
118. Wu GY, Wu CH. Receptor mediated in vitro gene transfer by a soluble DNA carrier system. *J Biol Chem* 1987, **267**, 4429–4432.
119. Wagner E, Cotten M, Foisner R, Birnstiel ML. Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc Natl Acad Sci USA* 1991, **88**, 4255–4259.
120. Rosencranz AA, Yachmenev SV, Jans DA, *et al.* Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct. *Exp Cell Res* 1992, **199**, 323–329.
121. Pai LH, Gallo MG, FitzGerald DJ, Pastan I. Antitumor activity of a transforming growth factor alpha-Pseudomonas exotoxin fusion protein (TGF-alpha-PE40). *Cancer Res* 1991, **51**, 2808–2812.
122. Young JA, Bates P, Willert K, Varmus HE. Efficient incorporation of human CD4 protein into avian leukosis virus. *Science* 1990, **250**, 1421–1423.
123. Dolter KE, King SR, Holland TC. Incorporation of CD4 into virions by a recombinant herpes simplex virus. *Virology* 1993, **67**, 189–195.
124. Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. *BioTechniques* 1989, 7, 980–990.
125. Sullenger BA, Lee TC, Smith CA, Ungers GE, Gilboa E. Expression of chimeric tRNA-driven antisense transcipts renders NIH 3T3 cells highly resistant to Moloney murine leukemia virus replication. *Mol Cell Biol* 1990, **10**, 6512–6523.
126. Sullenger BA, Gallardo HF, Ungers GE, Gilboa E. Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. *Cell* 1990, **53**, 601–608.
127. Garlick JA, Katz AB, Fenjves ES, Taichman LB. Retrovirus-mediated transduction of cultured epidermal keratinocytes. *J Invest Dermatol* 1991, **97**, 824–829.
128. Cornetta K, Morgan RA, Anderson WF. Safety issues related to retroviral-mediated gene transfer in humans. *Hum Gene Ther* 1991, **2**, 5–14.
129. Moolten FL, Cupples LA. A model for predicting the risk of cancer consequent to retroviral gene therapy. *Hum Gene Ther* 1992, **3**, 479–486.
130. Samulski RJ, Zhu X, Xiao X, *et al.* Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J* 1991, **10**, 3941–3950.
131. Kotin RM, Siniscalco M, Samulski RJ, *et al.* Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA* 1990, **87**, 2211–2215.
132. Muzyczka N. Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr Topics Micro Immunol* 1992, **158**, 97–129.
133. LaFace D, Hermonat P, Wakeland E, Peck A. Gene transfer into hematopoietic progenitor cells mediated by an adeno-associated virus vector. *Virology* 1988, **162**, 483–486.
134. Chatterjee S, Johnson PR, Wong KK. Dual-target inhbition of HIV-1 *in vitro* by means of an adeno-associated virus antisense vector. *Science* 1992, **258**, 1485–1488.
135. Hermonat PL. Inhibition of H-*ras* expression by the adeno-associated virus Rep78 transformation suppressor gene product. *Cancer Res* 1991, **51**, 3373–3377.
136. Hermonat PL. The adeno-associated virus Rep78 gene inhibits cellular transformation induced by bovine papillomavirus. *Virology* 1989, **172**, 253–261.
137. Schlehofer JR, Heilbronn R. Infection with adeno-associated virus type 5 inhibits mutagenicity of herpes simplex virus type 1 or 4-nitroquinoline-1-oxide. *Mutat Res* 1990, **244**, 317–320.
138. Klein-Bauernschmitt P, zur Hausen H, Schlehofer JR. Induction of differentiation-associated changes in established human cells by infection with adeno-associated virus type 2. *J Virol* 1992, **66**, 4191–4200.
139. Walz C, Schlehofer JR, Flentje M, Rudat V, zur Hausen H. Adeno-associated virus sensitizes HeLa cell tumors to gamma rays. *J Virol* 1992, **66**, 5651–5657.
140. Rosenfeld MA, Siegfried W, Yoshimura K, *et al.* Adenovirus-mediated transfer of a recombinant alpha-1-antitrypsin gene to the lung epithelium *in vivo*. *Science* 1991, **252**, 431–434.
141. Stratford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. *Clin Invest* 1992, **90**, 626–630.
142. Curiel DT, Wagner E, Cotten M, *et al.* High-Efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complexes. *Hum Gene Ther* 1992, **3**, 147–154.
143. Andersen JK, Garber DA, Meaney CA, Breakefield XO. Gene transfer into mammalian central nervous system using herpes virus vectors: extended expression of bacterial lacZ in neurons using the neuron-specific enolase promoter. *J Biol Chem* 1992, **3**, 487–499.
144. Longnecker R, Roizman B. Clustering of genes dispensible for growth in culture in the S component of the HSV-1 genome. *Science* 1987, **236**, 573–576.
145. Ho DY, Mocarski ES. Beta galactosidase as a marker in the peripheral and neural tissues of the herpes simplex virus-infected mouse. *Virology* 1988, **167**, 279–283.
146. DeLuca NA, McCarthy AM, Schaffer PA. Isolation and characterization of deletion mutants of herpes simplex virus type-1 in the gene encoding immediate-early regulatory protein ICP4. *Virology* 1985, **56**, 558–570.
147. Marcy AJ, Yager DR, Coen DM. Isolation and characterization of herpes simplex virus mutants containing engineered mutations at the DNA polymerase locus. *Virology* 1990, **64**, 2208–2216.
148. Dobson AT, Sederati F, Devi RG, *et al.* Identification of the latency-associated transcript promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. *J Virol* 1989, **63**, 3844–3851.
149. Moss B. Vaccinia virus: a tool for research and vaccine development. *Science* 1991, **252**, 1662–1667.
150. Ponder KP, Dunbar RP, Wilson DR, Darlington GJ, Woo SLC. Evaluation of relative promoter strength in primary hepatocytes using optimized lipofection. *Hum Gene Ther* 1991, **2**, 41–52.
151. Ray J, Gage FH. Transfer into established and primary fibroblast cell lines: comparison of transfection methods and promters. *BioTechniques* 1992, **13**, 598–603.
152. Wilson JL, Dollard SC, Chow LT, Broker TR. Epithelial-specific gene expression during differentiation of stratified primary human keratinocyte cultures. *Cell Growth Differ* 1992, **3**, 471–483.
153. Huitfeldt HS, Heyden A, Clausen OPF, Thrane EV, Roop D, Yuspa Sh. Altered regulation of growth and expression of differentiation-associated keratins in benign mouse skin tumors. *Carcinogenesis* 1991, **12**, 2063–2067.
154. Ham J, Dostatni N, Gauthier JM, Yaniv M. The papillomavirus

E2 protein: a factor with many talents. *Trends Biochem Sci* 1991, **16**, 440–444.
155. Day GL, Blot WJ. Second primary tumors in patients with oral cancer. *Cancer* 1992, **70**, 14–19.
156. Hong WK, Lippman SM, Itri LM, *et al.* Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1990, **323**, 795–801.
157. Davis BD. Germ-line therapy: evolutionary and moral considerations. *Hum Gene Ther* 1992, **3**, 361–363.
158. Anderson WF. Uses and abuses of human gene transfer. *Hum Gene Ther* 1992, **3**, 1–2.
159. Yu D, Hamada J, Zhang H, Nicolson GL, Hung MC. Mechanisms of c-*erb*B2/*neu* oncogene-induced metastasis and repression of metastatic properties by adenovirus 5 E1A gene products. *Oncogene* 1992, **7**, 2263–2270.
160. Tratschin JD, West MHP, Sandbank T, Carter BJ. A human parvovirus, adeno-associated virus, as a eucaryotic vector: transient expression and encapsidation of the procaryotic gene for chloramphenicol aetyltransferase. *Mol Cell Biol* 1984, **4**, 2072–2081.

Acknowledgements—Original work mentioned in this review was performed with the assistance of Mr Su Zhang.