Expression of the APC gene after transfection into a colonic cancer cell line

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Abstract
Mutations in the adenomatous polyposis coli (APC) gene cause the hereditary cancer syndrome familial adenomatous polyposis and are implicated in the early stages of sporadic colorectal carcinogenesis. APC is therefore a promising candidate for use in prophylactic gene therapy of intestinal tissues at high risk of becoming malignant. The aim of the study was to discover if functional full length APC gene can be introduced into somatic gut epithelial cells and to define the optimum conditions for such transfer. Copies of the normal APC gene were introduced into SW480 cells, a colonic epithelial cell line with an APC gene mutation, using plasmid DNA combined with liposomes. Reverse transcriptase polymerase chain reaction and restriction enzyme digestion allowed the endogenous gene to be distinguished from the transgene. It was shown that the normal APC gene is expressed at high levels for 72 hours after transfection and disappears within one week. This study shows that short-term expression of normal APC gene can be achieved after transfection with liposome-DNA complexes at sufficiently high levels to permit assessment of biological effects.

Keywords: familial adenomatous polyposis, adenomatous polyposis coli, gene therapy, lipofection, reverse transcriptase polymerase chain reaction, restriction enzyme digestion.

The introduction of genetic material into somatic cells has been proposed to correct single gene disorders such as severe combined immune deficiency/adenosine deaminase deficiency, type III hypercholesterolaemia/low density lipoprotein receptor mutation, and cystic fibrosis/cystic fibrosis transmembrane conductance regulator defects. In the treatment of these single gene disorders replacement of the defective gene either is neutral or confers a selective advantage to the cells that express the gene. In gene therapy for cancer, however, any genetic modifications that reverse the malignant process could lead to negative selection pressure followed by overgrowth of residual unmodified cancer cells. It would therefore be preferable if tumour suppressor genes could be introduced into normal tissue at a high risk of becoming malignant to prevent transformation, rather than reverse the neoplastic process.

The adenomatous polyposis coli (APC) gene is mutated in the germline of patients with the hereditary disease familial adenomatous polyposis. This putative tumour suppressor gene has also been implicated in the pathogenesis of sporadic colorectal cancers. APC mutations are thought to occur early in the development of colorectal carcinoma, making this gene a promising candidate for use in preventative gene therapy.

The APC sequence is a comparatively large gene containing an open reading frame of 2843 amino acids. Given the size constraints of existing gene delivery vehicles, liposomes are the most promising vectors to transport such large sequences of DNA into cells. The main drawbacks of liposomal gene delivery are inefficiency of transfection and the need for repeated treatment. The absence of a size constraint on the DNA that can enter the cell and low toxicity are advantages, however, which have to be balanced against these drawbacks. Whatever treatment is attempted, it is unclear whether even such familial adenomatous polyposis whether every cell has to be transfected, or whether there is a possibility of bystander effects, which can act on surrounding cells when only a proportion have taken up and expressed a DNA sequence.

Methods

Cell lines
CaCO2 and SW480 are established cell lines derived from colorectal tumours. SW480 has a point mutation at codon 1338 of its single APC allele resulting in a change from glutamine (CAG) to a stop codon (TAG). This mutation abolishes the normal PsI restriction site.

Gene transfer
DNA was transfected in combination with liposomes. The AP gene construct contains full length AP, from nucleotides -22 to +8583, cloned into the CMV-Neo Bam vector. Plasmid DNA was prepared by standard methods using the Qiagen procedure for plasmid purification. The liposome used was the commercially available LipofectAMINE (Gibco BRL). DNA and liposomes were complexed together using OptiMem 1 Reduced Serum Medium (Gibco BRL) as the diluent. Ratios of DNA:LipofectAMINE of 1:2 to 1:12 by weight were used. Transfection was performed using doses of 1 and 5 μg of DNA per well, each of which contained cells at 50–80% confluence 24–48 hours after plating out at a
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Reverse transcriptase polymerase chain reaction (RT PCR) and restriction enzyme digestion

Extraction and purification of mRNA was performed using standard methods (QuickPrep Micro mRNA Purification Kit, Pharmacia). The mRNA was initially converted to cDNA (First-Strand cDNA Synthesis Kit, Pharmacia) prior to amplification by the PCR with Taq polymerase (Perkin-Elmer). Amplification of a 117 base pair fragment from nucleotides 3985 to 4102 was performed using the primers 5’GTTCCAGCAGTGTCACAG and 5’GGGAGATTTCGCTCCTGA. This fragment contains a PstI site at codon 1338, which divides the amplified region into 47 and 70 base pair fragments. This PstI site is not present in the plasmid containing APC cDNA and in the CaCO2 cell line, which has the normal sequence across this region of its APC gene. This site has been lost in the SW480 cells, distinguishing between normal and abnormal sequences on the basis of a restriction enzyme digest. Both normal APC sequence and SW480 cells have a HinfI site at codon 1345, which divides the amplified 117 base pair region into 67 and 50 base pair fragments. This HinfI site provided an internal control on the digestion reaction. Amplified PCR products from transfected cells were digested with both HinfI and PstI (New England Biolabs) by incubation at 37°C for one hour. A sample of mRNA was treated in an identical manner to the other mRNA samples, but no reverse transcriptase was added to provide a control for contaminating DNA sequences.

Results

Figure 1 shows that normal and mutated APC gene expression can be distinguished using RT PCR and restriction enzyme digestion. The no RT control (Fig 1, lane 1) is negative indicating that the products are derived from expressed gene and not from contaminating DNA sequences. CaCO2 cells express the normal APC sequence across this region of the gene to give a 117 base pair fragment, which is cut by both HinfI and PstI (Fig 1, lanes 2-5), whereas untreated SW480 cells express a mutated form of APC, which loses the PstI site at codon 1338 and produces a changed restriction enzyme digest pattern as shown (Fig 1, lanes 6-9).

Following lipofection of SW480 cells with APC plasmid DNA, cells were harvested at intervals from 24 hours and mRNA extraction, RT PCR, and restriction enzyme digestions performed. Transfected SW480 cells predominate express the normal sequence APC gene at 24 (data not shown) and 48 hours after transfection (Fig 2, lanes 1-8). The no RT control (Fig 2, lane 9) is negative, showing that the products are not derived from the plasmid DNA. At 72 hours after transfection there are roughly equal amounts of RT PCR products from mutated and normal copies of the APC gene (Fig 3). From 96 hours onwards the restriction enzyme digest pattern reverted back to the untransfected state until by one week after treatment only mutated (native SW480) APC gene was expressed and virtually no transfected normal APC sequence could be detected (Fig 4). Increasing the amount of plasmid DNA applied from one to five µg per well did not prolong expression of the transfected gene beyond 72 hours.

A DNA: LipofectAMINE ratio of 1:12 by weight produced optimum data (as shown); lower amounts of liposome were inefficient.
while higher concentrations were toxic to the cells. Reducing the time of contact between epithelial cells and the plasmid-liposome complexes from five to four hours produced similar patterns of uptake and expression. However, a shorter time of 2 hours (or less) resulted in negligible expression of the transfected gene (data not shown). All the results shown were reproducible under the same strictly controlled conditions on between four and 10 independent occasions at each time point. No change in the morphology of the transfected cells was seen at any stage.

Discussion

The treatment of severe single gene disorders by the introduction of normal genes into somatic cells is progressing to the stage of clinical trials. In cancer, gene therapy to date has focused on the introduction of genes that either kill tumour cells directly or introduce a gene producing an enzyme that metabolises a prodrug into a lethal product. The major problem in these approaches is that tumour cells must be preferentially targeted compared with normal cells, and every tumour cell must take up the transgene (in the absence of bystander effects). These requirements are similar to the problems for chemotherapy or radiotherapy.

There have been important steps forward in elucidating the genetic basis of colorectal carcinogenesis. Patients with familial adenomatous polyposis carry a germline mutation in the APC gene. These subjects develop numerous colonic polyps during adolescence and invariably progress to malignancy unless prophylactic colectomy is undertaken. The APC gene is also implicated in the pathogenesis of sporadic colorectal cancers. It seems to be one of the earliest genes to mutate in the generally accepted model of sporadic colorectal carcinogenesis. It is thought that the APC gene functions as a tumour suppressor gene and for these reasons it makes a useful candidate gene for gene therapy to prevent colorectal cancer.

The strategy of treating populations of normal cells at high risk of becoming malignant with tumour suppressor genes to prevent the early stages of carcinogenesis has not been fully explored to date, although preliminary experiments on gene transfer strategies are being undertaken. Ideally, if stem cells (or rapidly dividing precursor cells) could be targeted, this could represent a once only form of treatment for an organ or tissue population. Of the currently available gene delivery vehicles only retroviral constructs are able to transfect DNA and integrate it into the genome of dividing cells. This integration carries the risk of insertional mutagenesis, which may not be considered a major problem in a patient with a disseminated malignancy but could pose a significant risk to a young person undergoing prophylactic gene therapy. There is also a size limit of a few kilobases on the amount of DNA that can be handled by such vectors. Although adenoviruses are not as limited as retroviruses in the size of gene that can be integrated, there is some evidence that the virus may cause inflammation. Liposomes are the only available vehicles that can safely transport large sequences of DNA such as the APC gene into cells but they are inefficient in gene delivery and short lived in effect because the DNA enters the endosomes when it is taken up by the cell.

This study has shown that full length normal APC cDNA complexed with liposomes can be introduced into a colonic epithelial cell line. The transfected sequence is expressed as mRNA at high levels from 24 to 72 hours after treatment, followed by a gradual decline in expression of the transgene. By one week only endogenous gene expression can be shown by RT PCR. Changing the amount of DNA transfected did not change the level or duration of expression, indicating that the amount of DNA/liposome complexes is not the limiting factor in persistence of transgene expression. The functional significance of the normal APC gene expression that occurs from the transgene remains to be evaluated. This will require the development of surrogate end points for APC gene expression in colonic epithelium and of normal cell lines in which function can be assessed, as well as the introduction of single copies of the APC gene under normal cellular controls.

With specific regard to FAP, there are two clinical situations in which gene therapy would be a useful preventative strategy even though 100% efficiency of cell targeting is not yet feasible. First, patients after colectomy with an ileorectal anastomosis have an at risk rectal stump, and all patients are at risk of duodenal polyposis progressing to carcinoma for which no effective treatment is currently offered. Topical treatment with aspirin or sulindac, usually under the auspices of a clinical trial, is being used in such patients but the risk of malignant change is not entirely removed. Provided gene delivery to these areas can be shown to be safe then these patients have nothing to lose from prophylactic treatment with a normal APC transgene. They are already under surveillance and might be ready to undergo trials of oral or endoscopic or enema gene therapy to reduce or delay their risk of cancer developing in these sites, while accepting that their risks will not be completely abolished by these treatments.

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