

Gene Transfer for the Esophagus— an Ex Vivo Study Demonstrating Transfected Gene Expression in the Human Esophagus

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ABSTRACT

Background and Objectives: Esophageal cancer has one of the worst prognoses of all cancers, mainly due to its aggressive growth pattern and late presentation. Using radiotherapy, chemotherapy, or even surgery it is only possible to cure a small minority and so alternative strategies need to be evaluated. In this study we aim to investigate the feasibility of using liposomes to transfect genes into human esophageal biopsies at high enough levels to achieve a biological effect.

Methods: Human esophageal biopsies were transfected with constructs encoding Green Fluorescent Protein or human wild type p53. Expression of Green Fluorescent Protein

within these samples was determined by FACScan analysis and confocal microscopy, whereas p53 expression was assessed by Western blotting and Reverse Transcriptase Polymerase Chain Reaction, probing for p53 and a downstream effector WAF1/p21.

Results: Confocal microscopy verified the expression of Green Fluorescent Protein in human esophageal biopsies in cells 7 to 10 layers deep. Green Fluorescent Protein expression has also been demonstrated by FACScan analysis. The successful introduction and expression of wild type p53 has been shown in normal esophageal biopsies by Western blotting and RT-PCR and in Barrett's esophageal biopsies by Western blotting alone. In addition, we have demonstrated an increase in the expression of WAF1/p21 in 50% of biopsies transfected with p53.

Conclusion: Liposome-mediated transfer

of genes into human esophageal cells is feasible and can be performed at a sufficient dose to achieve expression of downstream genes.

INTRODUCTION

Esophageal cancer is the sixth most common cancer in the world¹ and has a 5-year survival rate of less than 5%.² Patients with esophageal cancer usually present late in the progression of the disease and therefore all modalities of treatment, surgery, chemotherapy, and radiotherapy are relatively ineffective in either halting or curing the disease. For this reason new techniques for treating esophageal cancer must be explored. One possibility is gene therapy. Major obstacles to overcome in the application of gene therapy to humans include ensuring that the gene is transferred into the target cell without DNA degradation, preventing damage to non-targeted cells and limiting the application to the cells of interest. There are two main methods for gene transfer, based on viral vectors or physical methods. Retroviral vectors achieve integration of DNA into the genome of the target cell where expression of the gene product is sustained. Of the physical methods, liposomal transfer is the most promising. Synthetic cationic liposomes react spontaneously with negatively charged DNA, resulting in a complex with an overall positive charge.³ Cells actively take up this complex by a process yet to be clarified but thought to be endocytosis.^{4,5} The DNA is then transported to the nucleus where transcription takes place, resulting in transient expression of the gene product.⁶ Since DNA delivery is not by a viral vector, the transfected cells do not propagate it, thus limiting safety concerns.

Liposomes were first shown to complex with DNA to effect gene transfer in 1987, and have since been used for gene transfer into cell culture monolayers.⁴ Direct contact between the vector and the target cell over a large surface area is required for the transfection of superficial cells or monolayers. This is a major disadvantage for applica-

tions of this technique in most clinical and therapeutic settings, but it has great potential for gene transfer in the gastrointestinal tract. The human gastrointestinal tract is, in simple terms, a tube with a large surface area with a thin mucosal covering. A previous study instilled liposomes into the esophagus of rats.⁷ However, the rat esophagus is keratinized, which prevented gene transfer, except in areas where the keratin layer was damaged. Since the human esophagus does not have such a layer then there should be no physical barrier to gene transfer. This method of gene transfer could be targeted further with ease using flexible endoscopes to deliver the gene construct by direct injection into the tumor or dysplastic area.

In the human esophagus there are two situations in which replacement or reactivation of cell cycle control genes, such as p53, could be dramatically useful. These are in early dysplastic lesions within the esophagus where operative therapy is contra-indicated, or in the palliation of tumors too advanced for surgery. In this study, we set out to determine whether gene transfection using polycationic liposomes is possible in the human esophagus.

MATERIALS AND METHODS

Transfection of esophageal biopsies with fluorescently labeled liposomes

1 μ L of 1 μ M DiIC₁₈ (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, The Netherlands), a lipophilic membrane stain, was dried down under N₂ at 37°C then 20 μ L of LipofectAMINE PLUS™ (GibcoBRL Life technologies, UK) added and the sample gently mixed before 180 μ L OptiMEM (GibcoBRL Life technologies, UK) was added. For the liposome-free control, 1 μ L of 1 μ M DiIC₁₈ was dried down as above and 180 μ L OptiMEM added.

Normal human esophageal mucosal biopsies were obtained, after informed consent, at routine upper gastrointestinal endoscopy. Following excision, biopsies were immediately placed in ice-cold

Phosphate-Buffered Saline (PBS) and kept on ice until transfection was performed. The biopsies were placed on metal rafts in 60-mm organ culture dishes, with the serosal surface facing upward, away from the medium in order to facilitate application of the transfection mix to the mucosal surface of the esophageal tissue. Paired biopsies were used, using one as an internal control. Biopsies were incubated in the presence of DiIC₁₈-labeled liposomes at 37°C in 5% CO₂ for 3 hours. The control biopsies were incubated under the same conditions but DiIC₁₈ in 180µL of OptiMEM replaced the labeled liposome mixture. Following transfection, tissues were mounted and sections 5-µm thick were cut onto slides and viewed by confocal microscopy.

Transfection of normal esophageal tissue with pEGFP-N1

Tissues were washed gently in PBS then added to 200 µL of OptiMEM containing 10 mg of either pEGFP-N1 (encoding Enhanced Green Fluorescent Protein, GFP) or pcDNA3.0 (control vector; Invitrogen Life Technologies, The Netherlands) pre-complexed with LipofectAMINE PLUS or Tfx™-50 (Promega UK Ltd., UK). Following incubation at 37°C in 5% CO₂ for time points ranging from 5 minutes to 3 hours as required, the tissues were washed in PBS and set up in organ culture with RPMI1640 containing 10% FCS (Gibco BRL Life technologies, UK), incubating overnight at 37°C in 5% CO₂ to allow expression of GFP. The biopsies were then prepared for analysis. For flow cytometry, tissue was physically disaggregated in PBS and resuspended in 1.0% paraformaldehyde. In each assay, 10,000 cells were collected by FACScan and analyzed with the CellQuest program (Becton Dickinson), measuring fluorescence at 480 nm. For microscopy, tissues were frozen on cork blocks using Dichlorodifluoromethane (Cryojet, BDH Ltd., UK) and sections 5-µm thick were cut onto slides and viewed by confocal microscopy.

Transfection of tumor esophageal biopsies with pEGFP-N1

Normal and esophageal carcinoma biopsies were transfected with pEGFP-N1 or pcDNA3.0 using LipofectAMINE PLUS™ for 3 hours, then washed in PBS and set up in organ culture as before. Following overnight incubation at 37°C in 5% CO₂, samples were frozen on cork blocks using Dichlorodifluoromethane (Cryojet, BDH Ltd., UK), 5-µm sections cut, and these mounted onto slides and analysed by confocal microscopy.

Ex-vivo transfection of normal and Barrett's esophageal biopsies with p53

Thirteen paired biopsies were rinsed in PBS then added to 200 µL of OptiMEM containing 10 µg of either pcDNA3.0/p53 (encoding human wild type p53; kindly provided by Dr T.R. Hupp, Department of Molecular and Cellular Pathology, Ninewells Hospital, Dundee) or pcDNA3.0 pre-complexed with Tfx™-50. After incubation at 37°C in 5% CO₂ for 3 hours, the biopsies were washed in PBS and set up in organ culture with RPMI1640 containing 10% FCS and 10 µM lactacystin (Sigma, UK) to allow expression of p53. Following overnight incubation, tissues were lysed using a Urea Lysis Buffer (7 M Urea, 100 mM DTT, 0.05% Triton X-100, 25 mM NaCl and 20 mM HEPES, pH7.6). Protein concentrations were determined using the dye-protein binding method of Bradford, standardized using bovine serum albumin. Proteins were analyzed by discontinuous SDS-PAGE using a Bio-Rad Mini Protean Cell II, processed for Western blotting then probed for p53 expression using mouse monoclonal anti-p53 antibody DO1 (Moravian Biotechnologies, Czech Republic; 1/1000 dilution in PBS containing 5% non-fat milk and 0.01% Tween 20) and CM1 (rabbit polyclonal anti-p53 antibody Moravian Biotechnologies, Czech Republic; 1/1000 dilution in PBS containing 5% non-fat milk and 0.01% Tween 20). Samples were also probed for p21 expression using WAF1 (mouse monoclonal anti-p21 immunoglobulin

Table 1 Oligonucleotide primers

Oligonucleotide primers	Sequence	Predicted Amplifier size (bp)
p53 forward oligonucleotide	5'-TCAGATCCTAGCGTCGAGCC-3'	352
p53 reverse oligonucleotide	5'-GTACACAGACTTGGCTGTCCC-3'	
p21 forward oligonucleotide	5'-CCGTCAGAACCCATGCGCC-3'	392
p21 reverse oligonucleotide	5'-CCCTGAGAGTCTCCAGGTCC-3'	
β -actin forward oligonucleotide	5'-CACCCACACTGTGCCCATC-3'	652
β -actin reverse oligonucleotide	5'-CTAGAAGCATTGCGGTGG-3'	

The sequences of the oligonucleotide primers used in PCR are listed, along with the predicted size of the DNA fragment amplified using these primers.

lin, Oncogene Research Products, UK; $1/_{100}$ dilution in PBS containing 5% non-fat milk and 0.01% Tween 20).

Semi-quantitative reverse transcriptase polymerase chain reaction

Three pairs of biopsies were transfected for 3 hours with pcDNA3.0/p53 or pcDNA3.0 using TfxTM-50, then incubated overnight in organ culture as above. Total RNA was recovered (RNeasy Mini Kit, Qiagen, UK). Samples were ground under liquid nitrogen with a mortar and pestle, suspended in lysis buffer, and then homogenized by passing five times through a 22-gauge needle attached to an RNase-free syringe. One microgram of RNA from each was used for reverse transcription using random hexamers and Superscript II reverse transcriptase (Gibco BRL Life technologies). The polymerase chain reaction (PCR) mixture contained 10% of the reverse transcription reaction mix, 0.5 μ M oligonucleotide primers, 0.8 mM dNTP mix, 2.5 units of HotStarTaq (Qiagen, West Sussex, UK), and 1.5 mM MgCl₂. The sequences of the oligonucleotide primers used are shown in Table 1. The cDNAs transcribed from each sample were amplified with β -actin oligonu-

cleotides as an internal control, to verify that equal quantities of cDNA were used in each p21 and p53 amplification reaction. Thermal cycling conditions comprised an initial step of 95°C for 15 minutes, followed by 30 cycles of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes, then a final extension at 72°C for 10 minutes.

RESULTS

Confocal analysis of normal esophageal biopsy transfected with DiIC₁₈ labeled liposome

DiIC₁₈ is a red fluorescent lipophilic membrane stain that is only weakly fluorescent until incorporated into membranes, where it then it diffuses laterally to stain the entire cells. Normal biopsies were transfected with DiIC₁₈-labeled liposomes and viewed by confocal microscopy (Figure 1A). Intense red fluorescence corresponding to penetration of the labeled liposomes could be seen along the luminal edge of the biopsy and in the 4 or 5 layers beneath following a 3-hour incubation. The liposomes appeared to penetrate the biopsies by a paracellular route. Some positive staining was seen in samples incubated for only 10 minutes (data not shown), but was maximal after 3 hours. For samples incubated for

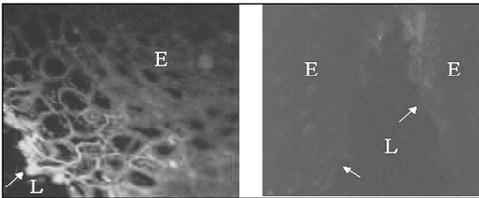


Figure 1. (A) Human esophageal biopsy transfected with DiIC₁₈-labeled liposomes. Tissue was incubated with the labeled transfection reagent for 3 hours at 37°C in 5% CO₂. Frozen sections (5 μm) were cut, mounted with mowiol and examined by confocal microscopy using a Bio-Rad MR600 system with Comos software. The epithelial surface is indicated by the arrows; L, lumen; E, epithelial cells. Original magnification: x400. (B) Control human esophageal biopsy. Tissue was incubated in the presence of the lipid dye DiIC₁₈ alone, minus transfection reagent, for 3 hours at 37°C in 5% CO₂. Frozen sections were prepared and mounted as above. The epithelial surface is indicated by the arrows; L, lumen; E, epithelial cells. Original magnification: x400.

times with labeled liposomes, no deeper penetration was observed (data not shown). No auto-fluorescence was observed in the samples incubated in the presence of DiIC₁₈ alone, i.e. without the liposomes as carriers (Figure 1B).

Transfection of human normal esophageal biopsies with pEGFP-N1

Expression of GFP in biopsies transfected with pEGFP-N1 and pcDNA3.0 using LipofectAMINE PLUS™ was determined by flow cytometry. Significant levels of GFP were detected following a 180-minute transfection (Figure 2A). GFP was also detected in 10-minute time point samples to a significant degree, but not then again until the 180-minute samples. For each time point n = 10, and in each of the 10 samples used in the 10-minute time point, a significant level of GFP expression was observed. The reason for this observation is unclear as yet and warrants further investigation. Transfection of biopsies with pEGFP-N1 using Tfx™-50 gave levels of GFP similar to those obtained under the same conditions with LipofectAMINE PLUS™ (data not shown).

Confocal microscopy of biopsies transfected with pEGFP-N1 (Figure 2 B) showed a pattern of penetration similar to that obtained with DiIC₁₈-labeled liposomes, with intense fluorescence along the luminal edge extending 4 or 5 cell layers under the surface. This was not seen in biopsies transfected with the empty vector construct pcDNA3.0 (Figure 2 C).

Biopsies from adenocarcinomas of the esophagus were transfected with pEGFP-N1 using LipofectAMINE PLUS™ and analyzed by confocal microscopy. Distribution, identical to that of normal biopsies was detected in the tumors (data not shown).

Transfection of normal and Barrett's esophageal biopsies with human wild type p53

Sixteen paired biopsies were transfected with pcDNA3.0/p53 and pcDNA3.0 using Tfx™-50. Eleven samples were analyzed by Western blotting alone and two by RT-PCR alone. Sufficient material was obtained from three patients to allow analysis by both methods.

One sample displayed an overexpression of p53 protein by Western blotting when probed with the monoclonal anti-p53 antibody DO1 (Figure 3). Blots were also probed with a β-actin antibody as a loading control. All lanes demonstrated similar β-actin levels (data not shown) confirming that the increase in p53 protein was real and not just an artifact due to unequal loading of samples.

Using the same antibody for the remaining 13 patient samples, no significant difference was observed between the tissues transfected with pcDNA3.0/p53 and those treated with the control plasmid pcDNA3.0 (data not shown). In order to address the possibility of epitope masking preventing detection of p53, these samples were also probed with CM1, a polyclonal anti-p53 antibody. Again no increase in p53 levels was observed in those treated with pcDNA3.0/p53 (data not shown). Western blots were repeated probing this time with

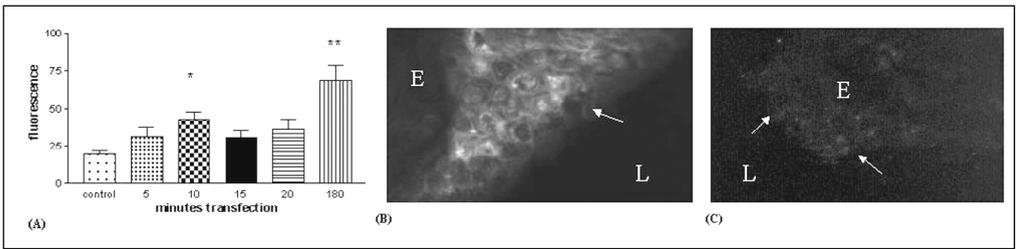


Figure 2. (A) Expression of Green Fluorescent Protein (GFP) in human esophageal biopsies. Esophageal biopsies were removed endoscopically and placed in ice-cold isotonic saline for short-term storage. They were then rinsed and placed on stainless steel mesh rafts for organ culture. After transfection with 10 μ g pEGFP-N1 using LipofectAMINE PLUSTM for the times indicated (5, 10, 15, 20, 180 min), esophageal biopsies were removed, washed, and incubated in organ culture overnight to allow expression of GFP. Biopsies were disaggregated and GFP expression analyzed by flow cytometry. Histogram shows mean \pm SEM for 10 experiments. Significance assessed by analysis of variance with Dunns' Multiple Comparison Test; * $P < 0.05$, ** $P < 0.001$. (B) Human esophageal biopsy transfected with pEGFP-N1 using LipofectAMINE PLUSTM. Following incubation for 3 hours at 37°C in the transfection mix, the biopsy was removed, washed, and cultured overnight to allow expression of GFP. Frozen sections (5 μ m) were cut, mounted, and examined by confocal microscopy using a Bio-Rad MR600 system with Comos software. The epithelial surface is indicated by the arrow; L, lumen; E, epithelial cells not expressing GFP. Original magnification: x400. (C) Human esophageal biopsy transfected with pcDNA3.0 using LipofectAMINE PLUSTM. Tissue was incubated and prepared as before. The epithelial surface is indicated by the arrow; L, lumen; E, epithelial cells not expressing GFP. Original magnification: x400.

WAF1, an anti-p21 antibody. Seven (50%) of the samples showed increased levels of p21 protein following introduction of the p53 gene as compared to their controls (Figure 4). Since p21 expression occurs downstream of p53 in the pathway to cell cycle arrest, the increase in p21 levels observed only in samples into which p53 has been introduced has established in an indirect manner, successful p53 introduction and expression.

Three of the pairs of tissues analyzed by Western blotting were taken from dysplastic areas of patients with Barrett's esophagus. One of these showed an increase in p21 protein levels in the sample transfected with p53 (Figure 4).

Five pairs of biopsies were analyzed by semi-quantitative RT-PCR, comparing levels of gene expression to that of β -actin. One sample showed p21 transcription had been initiated by p53 introduction since none was detected in the control (Figure 5A).

Two further samples demonstrated an increase in p21 expression over the background levels detected in the controls (Figure 5B). Of these three samples, two

were among the group that had also been analyzed by Western blotting and had displayed an increase in p21 protein levels.

DISCUSSION

We have shown that it is possible to transfect genes into human esophageal mucosa, and in addition we have demonstrated that it is possible to introduce a gene with therapeutic potential at sufficient dose to induce a biological response. Two transfection reagents were tested and both proved to be successful in delivering plasmid DNA into patient biopsies.

The process of transfection depends on liposome contact with the target cell. While this is adequate for superficial applications, akin to cell monolayers it is a handicap in multilayer tissues. We have shown that the DNA-liposome complexes penetrate intact tissues, probably by a paracellular route, leading to gene expression in cells several layers beneath the surface. This considerably extends the potential therapeutic applications of this technique, not only in surface applications but also it supports a strategy of injection directly into target tissues. This

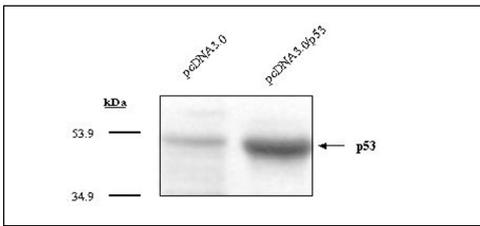


Figure 3. Transfection of p53 into a human normal esophageal biopsy. Wild type p53 DNA (pcDNA3.0/p53) was transfected into one of a pair of esophageal biopsies using the reagent Tfx™-50. As a control the other of the pair was transfected with vector alone (pcDNA3.0). Cell lysates (10 mg) were visualized by SDS-polyacrylamide gel electrophoresis, immunoblotting with a monoclonal antibody specific for p53. The presence of p53 in the pcDNA3.0/p53-transfected biopsy is indicated.

would create lakes of DNA-liposome complexes that would penetrate the surrounding tissues, allowing gene expression deep within the tissue.

Demonstration of the expression of a marker gene within tissue is all very well, but by their nature they are inert, not subject to metabolism, and will accumulate over time. From a therapeutic viewpoint we require not only expression, but also sufficient dose of expressed protein at a time point to cause a biological effect. In this study we have demonstrated that sufficient wild type p53 is produced in 50% of tissues treated to induce the expression of WAF1/p21, a gene downstream in the pathway to cell cycle arrest.

Expression of p53 is normally subject to tight regulation within cells, being targeted to the proteasome for degradation by its interaction with MDM2. Presumably p53 protein synthesized following pcDNA3.0/p53 transfection is also subject to this regulation. Therefore, it is perhaps not surprising that no elevation of p53 protein levels were observed in 12 of the 13 samples transfected with a construct encoding p53. However, it is clear that sufficient wild type p53 was produced to induce the expression of p21. From a therapeutic

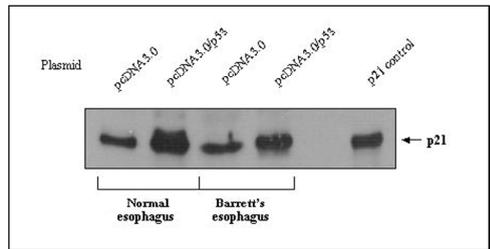


Figure 4. Induction of p21 in human normal and Barrett's esophageal biopsies following transfection with a wild type p53 gene. Paired biopsies were transfected with pcDNA3.0/p53 or pcDNA3.0 as a control. Cell proteins (10 µg) were separated by SDS-polyacrylamide gel electrophoresis then immunoblotting performed with a monoclonal antibody specific for p21. p21 control lane is included as a positive control. p21 protein is detectable in the control samples but levels are significantly higher in the p53-transfected samples.

viewpoint, this finding is important as it indicates that our transfected gene is expressed at a level sufficiently potent to exert its biological effect.

Significantly, this study has demonstrated the successful expression of p53 in dysplastic areas within a Barrett's esophagus. If the treatment was applied as an instillation into the esophagus, it could be repeated regularly as a prophylactic against dysplastic transformation.

It is vital that any gene therapy is targeted to prevent cell damage beyond the intended tissue. Liposomal DNA delivery, because it is non-replicating, is limited in its spread. In addition, we have performed further studies that have shown that the liposome-DNA complex is exquisitely pH sensitive. Thus any liposome-DNA complex directed to the esophagus that missed the target area would enter the stomach and likely be rendered ineffective and therefore safe.

The esophagus lends itself to further targeting of therapy by use of an endoscope to ensure that the liposome-DNA complex is applied to the areas most at risk. The liposome delivery could be targeted further by the endoscopic injection of pools of liposomes within a tumor.

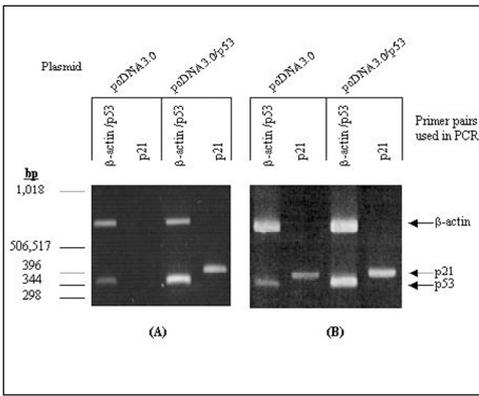


Figure 5. An increase in p21 transcription is detected by RT-PCR. Reverse transcription was performed using 1 μ g of total RNA isolated from human normal esophageal biopsies. 10% of the RT mix was used as template in PCR, amplifying each mix with oligonucleotides specific for p53, p21, and β -actin (as an internal control). Twenty percent of each PCR mix was analyzed by agarose gel electrophoresis. p53 and β -actin samples were combined prior to electrophoresis to allow easier comparison. (A) β -actin levels are similar for each sample. A slight increase in p53 transcript levels is observed. No p21 transcript is detected in the control lane (pcDNA3.0) but a strong band is visible in the p53-treated lane (pcDNA3.0/p53). (B) Again β -actin levels are similar for each sample. p53 and p21 transcripts are present in both the control and the treated samples, but levels of gene expression are noticeably higher in the p53-transfected lanes.

The ultimate targeting is to have a gene that was active only in the target tumor cell. This could lead to apoptotic death of tumor tissue with preservation of surrounding normal tissue and stroma, allowing maintenance of tissue structure. This technique, using a gene that preserved normal tissue combined with cross-sectional imaging could develop into an image guided microtherapy that might allow a radical debulking of the tumor or even an almost surgical excision. At the very least, this technique would be an important development in the palliation of cancer of the esophagus.

Further questions that need to be addressed in future studies ensuring that we can achieve sufficient dose of expressed protein from the transfected gene to achieve

the desired cellular action. This may be initiation of either apoptosis or growth arrest by transfection of p53 into a dysplastic malignant cell. However, clinical trials in esophageal cancer and dysplastic Barrett's esophagus will be required to answer these questions. It is possible to envisage further applications of these techniques throughout the GI tract to treat neoplasia of the stomach or large bowel.

In conclusion we have shown that it is possible to transfect the human esophagus with a vital cell cycle control gene. There are minimal concerns about its safety and these can be further allayed in applications in the esophagus by the endoscopic application of treatment. We feel this technique has great promise in diseases of the human gastrointestinal tract.

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REFERENCES

- 1 Ribeiro U, Posner MC, Safatale-Ribeiro AV, Reynolds JC. Risk factors for squamous cell carcinoma of the oesophagus. *Br J of Surg.* 1996; 83:1174-11
- 2 Dixon MF. Alimentary system. In: Underwood JCE, ed. *General and systemic pathology.* Edinburgh, Scotland: Churchill Livingstone; 1996:401-448.
- 3 Cevc G. *Phospholipids Handbook.* New York, NY: Marcel Dekker; 1993:855-878.
- 4 Muller WJ, Zen K, Fisher AB, Shuman H. Pathways for uptake of fluorescently labelled liposomes by alveolar type II cells in culture. *Am J Physiol.* 1995;269(pt 1):L11-L19.
- 5 Chinoy MR, Fisher A, Shuman H. Confocal imaging of time dependent internalisation and localisation of NBD-PC in intact rat lungs. *Am J Physiol.* 1994;266:L713-L721.
- 6 Felgner PL, Gadek TR, Holm M, et al. Lipofection, a highly efficient, lipid-mediated DNA transfection procedure. *Proc Natl Acad Sci USA.* 1987;84:7413-7417.
- 7 Schmid RM, Weidenbach H, Draenert GF, Liptay S, Luhrs H, and Adler G. Liposome-mediated gene transfer into the rat oesophagus. *Gut.* 1997;41:549-556.