Can immunotherapy by gene transfer tip the balance against colorectal cancer?

Summary
Gene therapy, in particular the transfer of genes encoding immunostimulatory molecules (cytokines and costimulatory molecules) as well as selectively cytotoxic enzymes and DNA vaccination, has the potential of enhancing cell mediated immune responses against tumours including those of colorectal origin. Genes can be transferred using viral vectors either to cultured tumour cells in vitro that can be returned to the patient as a “cancer vaccine”, or directly to tumour cells in vivo. Vaccination with DNA constructs expressing specific tumour antigens characteristic of colorectal neoplasia can trigger immune recognition and destruction of tumour cells. The aim is to tip the balance from protumour to antitumour mechanisms by generating a local immune response and systemic antitumour immune memory to destroy metastases. Studies in murine models, combined with human studies, show that such approaches could become an adjunct to current treatments for human colorectal cancer in the near future.

Introduction
Colorectal cancer comprises 10–15% of deaths from cancer in industrial nations, second only to lung cancer. Survival rates (40% >5 years) have remained stable over the past 20 years and so a number of treatments to supplement surgical resection and chemotherapy are under investigation, including enhancement of the immune response. This article considers gene therapy, in particular the transfer of immunomodulatory genes and selectively cytotoxic enzymes to tumour cells as well as DNA vaccination, as a means of enhancing cell mediated immunity specifically for the treatment of colorectal cancer.

The current model for colorectal tumorigenesis postulates a multi-stage progression involving an accumulation of gene mutations (APC, K-ras, p53, DNA mismatch repair genes), alterations in gene expression (c-myc, MHC) and chromosome losses, during which regulation of cell growth is disrupted. Dietary and inherited genetic factors predispose to such changes. The majority of deaths from colorectal cancer follow tumour metastasis to the liver and treatment must be aimed at controlling local regrowth after resection and distant metastases. Cell mediated immunity (mainly CD8+ cytotoxic T lymphocytes (CTL)) is potentially the most effective arm of the immune response as CTL can recognise epitopes processed and presented from any protein synthesised within the tumour cell and can kill the cell specifically and also anamnestically (memory cells). Cytokines from CD4+ helper T cells (Th) are also required to activate not only CTL, but also natural killer (NK) cells and antigen presenting cells and other inflammatory cells at the tumour site. Although lessons can be learned from gene therapy approaches against other tumours, mainly melanoma, colorectal cancers have characteristic features which require separate consideration.

The immune response to colorectal tumours and reasons for its failure
The emergence of a tumour may be the result of an inadequate immune response on two fronts: poor or lack of immunogenicity of the tumour cells and low efficiency of the immune response against the tumour. However, colorectal tumours do not develop more frequently in immunodeficient individuals, unlike some other tumours—for example, lymphomas in patients with AIDS, skin tumours in transplant recipients. This suggests that the tumour itself has immunomodulatory or immunoevasive, or both, properties.

Tumour cells often fail to present antigen due to the total loss (in around 20% of colorectal neoplasia) or reduction in expression of MHC class I molecules. Mutations in peptide transporting molecules (TAP) may also affect presentation of T cell epitopes. The genetic changes occurring during tumour development frequently lead to the expression of oncogenic and neo-antigens (tumour specific) or aberrant expression of normal or fetal antigens, which are potential targets for immune attack of the cancer cells. Antigens recognised by T cells in colorectal cancer include mutated p21 ras cell surface associated mucin and an annexin-like molecule. However, for an effective antitumour response T cell specificities may need to be directed towards subdominant or cryptic epitopes of unmutated self molecules as dominant epitopes may have induced thymic depletion or peripheral anergy of epitope specific T cells. A precedent for this is seen in melanoma where a number of self antigens are associated with protective immunity to tumours.

The total or partial loss of MHC class I molecules means that tumour antigens may not be presented to CTL if a particular MHC class I allele is required for peptide presentation. This provides a selective advantage for the tumour cells and a problem for the immune response. In addition, the presence of MHC class I alleles can inhibit...
the non-specific cytotoxic activities of NK cells. Thus, cer-
tain phenotypes of MHC expression can render the
tumour cells non-susceptible to direct cellular cytotoxicity
by CTL or NK cells. Loss of polarisation of the luminal
membrane of epithelial tumour cells gives rise to aberrant
expression of mucin molecules (such as MUC-1). Expression
of mucin all over the cell membrane can mask surface
immunoregulatory molecules and inhibit interaction
between tumour and immune cells. However, changes in
mucin glycosylation may make the mucin a target
for CTL activity and T cell recognition without MHC
restriction due to its repeating and possible TcR
cross-linking properties. An additional paradox in the
importance of mucin is the fact that MUC-1 expression
on adenocarcinomas (or shed from them) can cause apoptosis
of T cells, which is another way in which the tumour
may evade the immune response. A number of tumour infiltrat-
ing lymphocyte (TIL) populations have been identified in
colorectal tumours, which may have an association with
increased patient survival. Indeed, TIL cultured in vitro
and adoptively returned to patients have resulted in varying
degrees of protection in other cancers. NK cells are partic-
ularly abundant in colorectal TIL as are CD4+ T cells
which outnumber CD8+. Colorectal tumour infiltrat-
ing T cells with a limited repertoire of T cell receptor vari-
able regions suggests tumour specific clonal expansion as
does the possession of activation markers. T cells with
the 38 T cell receptor, which have been identified in
neoplastic as well as in healthy intestinal mucosa, have
specific killing activity towards epithelial-derived tumours
with a limited repertoire of T cell receptor vari-
able regions suggests tumour specific clonal expansion
as does the possession of activation markers. T cells with
the 38 T cell receptor, which have been identified in
neoplastic as well as in healthy intestinal mucosa, have
specific killing activity towards epithelial-derived tumours
in a non-MHC restricted manner and may provide
another antitumour mechanism. Furthermore, both 38 T cells
and NK cells seem to recognize heat shock proteins (hsp),
molecules which are constitutively expressed by
colorectal neoplasia (S Todryk, unpublished data). The upregulation of hsp by heating or gene
transfer could therefore be another means of improving
immune recognition of colorectal cancer.

Cell mediated immunity tends to be down regulated in
environments such as the gut in order to minimise damage
caused by excessive inflammation in response to the
barrage of antigens encountered. Indeed, colon adenomas
and carcinomas produce transforming growth factor
(TGF)-B 32 and interleukin (IL)-10, 33 cytokines known to
suppress cell mediated responses, an effect that may be
more pronounced within larger, established tumours.
Secretion by colorectal tumours of factors such as leukae-
nemia inhibitory factor and prostaglandins may also have
immunosuppressive effects. This could, in part, explain
why these tumours tend to develop and persist, despite
the presence of TIL. This suppression may also occur in gut
associated lymphoid tissue, mediated by T cells. Recent
evidence, however, has shown that colorectal tumour cells
secrete IL-7, a cytokine that can cause TIL to proliferate,
secrete tumour necrosis factor (TNF) a and lyse
autologous tumour cells. 37

The absence of costimulation (e.g. by helper cytokines or
B7 binding) during recognition of tumour cells by T cells
results in anergy of tumour specific T cells, rendering
them ineffective. Such anergy may need to be reversed in
immunotherapy. In humans with colorectal cancer the
functional suppression of T cells in the TIL and periphery
seems to coincide with alterations in the T cell receptor
signal transduction mechanism, but these may be
reversible by cytokines such as IL-2. Finally, colorectal
tumours express not only functional Fas ligand, which can
induce apoptosis in tumour infiltrating T cells bearing Fas,
but also Fas itself, which although expressed at lower levels
than in normal colon epithelium may make the tumour
cells susceptible to apoptosis. 42

**Aims of immunostimulatory gene transfer therapy against colorectal cancer**

- Induce an immunostimulatory environment in the vicinity of the tumour/vaccine
- Induce direct or cross-priming of cytotoxic and helper T cells against tumour antigens
- Overcome immunosuppression and/or T cell anergy
- Generate immune memory against tumour regrowth and metastasis

In conclusion, the fact that it is possible to detect cellular
immune responses specific for colorectal tumours in vitro, 42–44 albeit at low levels, suggests that the immune
defect could be reversed in vivo by immunotherapy.
stimulated NK cells in vitro but not tumour specific CTL. In human melanoma, exogenous IL-12 and IL-2 in combination, but not B7, yielded the best in vitro CTL responses.

In vivo infection of tumour cells with viruses targeting viruses (systemic or local administration) may also be feasible in gene therapy and avoids in vitro cell manipulation for each patient, and the production of a “personal” vaccine. Liver metastases could—for example, be targeted by perfusion of the liver with viruses via the hepatic portal vein or by intratumoral injection. Carcinoembryonic antigen (CEA) expressing tumours could be targeted by engineering proteins within the viral envelopes that bind specifically to surface CEA, or by incorporating the CEA promoter. This in vivo gene delivery approach should affect the growth of the targeted tumour and elicit protective immune responses against spread of the tumour. Alternatively, the possibility exists of using allogeneic tumour cells, with antigens in common with the patient’s tumour, which will be rapidly destroyed and these antigens released, resulting in T cell cross-priming against the antigen. Established tumours, or tumour cells ex vivo, can also be made allogeneic by transfer of allo-MHC genes. In addition to tumour cell modification, transfer of the gene for TNF-α to TIL from melanomas has been achieved, and may provide another means of enhancing cell mediated immunity against colorectal neoplasia using gene therapy.

**SUICIDE GENES**

Another form of gene therapy involves the in vitro (followed by injection) or in vivo infection of tumour cells with viruses carrying “suicide” genes which encode enzymes (e.g. herpes simplex virus thymidine kinase (tk) and Escherichia coli cytosine deaminase (CD)) that convert prodrugs (ganciclovir and 5-fluorocytosine, respectively) into toxic forms that kill the tumour cells in vivo. This inflammatory process rapidly releases antigens that stimulate memory immune responses resulting in the killing of parental tumour cells distal from the initial tumours (e.g. metastases). This approach has been successful at reducing growth of a tumour challenge in a mouse colorectal tumour model (and our unpublished data). Moreover, cotransfection of tk with GM-CSF, administered in adenovirus in vivo, was able to increase survival of mice with liver metastases. The use of suicide genes may also reduce the need for tumour cell irradiation, which could adversely affect the vaccine’s efficiency. The tumour cells would be killed when the prodrug is administered. In a comparison between tk and CD gene transfer, CD was more effective than tk at killing a human colorectal tumour line in nude mice using in vitro, or in vivo delivery (adenovirus). However, colorectal tumour lines passaged over many years may not provide the most accurate model for colorectal cancer therapy. For this reason we are currently studying tumour cells freshly isolated from patients.

**DNA VACCINATION**

The administration of genes encoding tumour associated antigens provides another potential route of immunotherapy against colorectal cancer. Antigen encoding plasmid DNA can be given in its naked form by intradermal or intramuscular routes, and by injection or “gene gun”. Alternatively, vehicles for DNA vaccination include liposomes, viral vectors and protein carriers. The prolonged antigen expression that is obtained can induce CTL and Th responses. Even though relatively few antigen specific T cell responses have been identified for colorectal tumours, as previously mentioned, there are a number of candidate proteins that could be exploited as DNA vaccines (table 1). Potential tumour specific antigens are those expressed uniquely by the tumour, or in greater abundance than normal tissue. In addition, as T cells recognise peptide epitopes of around eight to 20 amino acids, MHC restriction of peptide recognition by the heterogeneous human population may necessitate the use of larger antigenic fragments that encompass many epitopes.

**Table 1 Candidate antigens for DNA vaccination against colorectal cancer**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Class of antigen</th>
<th>T cell responses</th>
<th>Antitumour effect/association</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21 ras</td>
<td>Mutated oncogene product</td>
<td>Human CTL, Th1</td>
<td>No</td>
</tr>
<tr>
<td>p53</td>
<td>Mutated tumour suppressor</td>
<td>Murine CTL</td>
<td>No</td>
</tr>
<tr>
<td>CEA</td>
<td>Embryonic gene product</td>
<td>Human CTL</td>
<td>In mice</td>
</tr>
<tr>
<td>MUC-1</td>
<td>Epithelial mucin</td>
<td>Non-MHC CTL</td>
<td>In mice</td>
</tr>
<tr>
<td>MAGE</td>
<td>Melanoma associated antigen</td>
<td>Human CTL in melanoma</td>
<td>In melanoma</td>
</tr>
<tr>
<td>GA733</td>
<td>Surface molecule</td>
<td>Murine CTL, Th</td>
<td>In mice</td>
</tr>
<tr>
<td>Annexin-like molecules</td>
<td>Placental/structural protein</td>
<td>Th</td>
<td>No</td>
</tr>
</tbody>
</table>

Mutations in oncoproteins may be single amino acid changes, as at codons 12, 13, and 61 in p21 ras. These mutations disrupt normal ras signalling function and are not expressed in normal tissue. Human CTL that recognise a single ras mutation at residue 13 and are capable of killing tumour cells harbouring the same mutation have been isolated from a patient with colon carcinoma. Peptides from this region of ras also bind MHC class II molecules with high promiscuity, which is a desirable attribute of a vaccine. Mutations in the p53 tumour suppressor protein can give rise to multiple amino acid substitutions. Such changes mean that cell growth is unchecked and further gene mutations and chromosomal rearrangements can accumulate. Murine CTL have been raised to p53 mutated at codon 135. Carcinoembryonic antigen is a glycosylated single-chain peptide overexpressed in carcinomas of the colon, breast, stomach, pancreas, and lung. Promising CEA DNA vaccination studies in mice using naked DNA or a vaccinia virus vector are beginning to translate to human studies where CTL generated by vaccinia-CEA immunisation could lyse CEA+ tumour cells. Polymorphic epitopic mucin encoded by the MUC-1 gene is overexpressed in a number of adenocarcinomas. The mucin expression is no longer only associated with the apical surface of ductal epithelial cells and aberrant mucin glycosylation on tumour cells results in exposure of the polypeptide core and unmasking of otherwise cryptic epitopes. Immunisation with MUC-1 DNA, again naked or in vaccinia virus, has shown protection against tumour growth in mice. Although most frequently associated with melanoma, the MAGE family of genes has also been found in colorectal neoplasia and so represent another potential candidate for DNA vaccination. Finally, mutational frameshifts such as those associated with APC gene expression can result in a stretch of unique protein sequence containing potential T cell epitopes.

Recently, a potentially effective route of DNA vaccination has emerged in which dendritic cells are pulsed or transduced with tumour antigen encoding DNA and can efficiently prime T cells. In light of the identification of a number of tumour-regression antigens in melanoma and...
other tumours, a requirement exists for the identification of further tumour antigens in colorectal cancer.

Conclusion and future prospects

A number of cells of the immune system may be manipulated in a gene therapy of colorectal cancer in order to tip the balance from protumour to antitumour mechanisms (fig 1). One of the challenges is to stimulate an effective immune response towards the various tumour phenotypes and locations by transferring genes encoding the appropriate immunostimulatory or cytotoxic molecules, or by immunising with the appropriate tumour antigen encoding DNA. Effective immune responses to colorectal neoplasia that express or fail to express MHC class I molecules—for example, may require different immunostimulatory molecules to activate different effector cells. Tumour burden and, in particular the size of the tumour, may very much determine the success of such therapies as an immunosuppressive environment may be created and the tumour may simply be proliferating too rapidly for the immune system to contain. Previous studies immunising patients with colorectal cancer with autologous tumour cells and bacillus Calmette-Guerin (BCG) have shown some improvements in survival rates86 and current gene transfer trials involve transfer of allogeneic MHC molecules (HLA-B7)87 and cytotoxic deaminase to colorectal tumours. Genes encoding IL-12, GM-CSF, B7, cytotoxic deaminase, and thymidine kinase have shown therapeutic efficacy in murine models of colorectal cancer. DNA vaccination studies in murine models that are currently translating to human studies include CEA.

Colorectal cancer is amenable to gene therapy as patients can be returned to a state of minimal residual disease following resection of the primary tumour. Latent micrometastases will be a more controllable target. To put these principles into practice we are currently working towards clinical trials comprising in vitro transfection of colorectal tumour cells with adenovirus encoding genes for tk and GM-CSF, followed by reimplantation of the cells as a "vaccine". These gene therapy approaches have the potential to be useful adjuvants to conventional treatments with potential advantages of being physiologically less toxic and providing systemic vigilance against tumour regrowth and metastasis.
Treatment of colorectal cancer by gene transfer


