Gut

Leading article

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.[1] 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.[2] 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,[3] 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.[4 5] Mutations in peptide transporting molecules (TAP) may also affect presentation of T cell epitopes.[6] 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[7 8] cell surface associated mucin[9] and an annexin-like molecule.[10] 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.[11] A precedent for this is seen in melanoma where a number of self antigens are associated with protective immunity to tumours.[12]

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, certain phenotypes of MHC expression can render the tumour cells non-susceptible to direct cellular cytotoxicity by CTL or NK cells. Loss of polarisation at the luminal membrane of epithelial tumour cells gives rise to aberrant expression of mucin molecules (such as MUC-1).[13] Expression of mucin all over the cell membrane can mask surface immunoregulatory molecules and inhibit interaction between tumour and immune cells.[14] However, changes in mucin glycosylation may make the mucin a target for CTL activity and Th cell recognition without MHC restriction due to its repeating and possible TcR cross-linking properties.[15] 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,[16] which is another way in which the tumour may evade the immune response. A number of tumour infiltrating lymphocyte (TIL) populations have been identified in colorectal tumours, which may have an association with increased patient survival.[17 18] Indeed, TIL cultured in vitro and adoptively returned to patients have resulted in varying degrees of protection in other cancers.[12] NK cells are particularly abundant in colorectal TIL[19] as are CD4+ T cells which outnumber CD8+.[20 21] Colorectal tumour infiltrating T cells with a limited repertoire of T cell receptor variable regions suggests tumour specific clonal expansion[22 23] as does the possession of activation markers.[24] T cells with the γδ T cell receptor, which have been identified in neoplastic as well as in healthy intestinal mucosa,[25] have specific killing activity towards epithelial-derived tumours in a non-MHC restricted manner[26] and may provide another antitumour mechanism. Furthermore, both γδ T cells,[27] and NK cells[28] seem to recognise heat shock proteins (hsp), molecules which are constitutively expressed by colorectal neoplasia[29] (S Todryk, unpublished data). The upregulation of hsp by heating[30] or gene transfer[30a] 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) β[31 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 leukaemia inhibitory factor and prostaglandins may also have immunosuppressive effects.[34 35] 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.[36] 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) α 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,[38] 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,[39 40] but these may be reversible by cytokines such as IL-2.[41] 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 43]

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,[44–48] albeit at low levels, suggests that the immune defect could be reversed in vivo by immunotherapy.

Gene transfer mediated immunotherapy of colorectal cancer

IMMUNOSTIMULATORY GENES

Immunostimulatory gene transfer is a potentially powerful therapeutic approach for treating colorectal cancer that aims to mobilise the immune response to recognise and destroy tumour cells (box). Gene transfer therapy usually involves the resection of tumour and then infection in vitro of tumour cells with retro-, adeno- or herpes viruses[49] containing genes for cytokines and/or costimulatory molecules. This is followed by reinjection of the irradiated or unirradiated cells as a "cancer vaccine". When cytokine genes are transferred, tumour cells will secrete the cytokine and stimulate immune responses and inflammation by a local paracrine effect. When genes for costimulatory molecules (e.g. B7) are transferred the molecule will be expressed on the tumour cell surface and stimulate lymphocytes by direct contact. Local production of cytokines also avoids toxic effects of systemic cytokine administration. Together with the elimination of the inoculating tumour cells, this approach aims to elicit systemic immune memory and protection against secondary contact with parental tumour (subcutaneous or in the liver in the mouse model), which represents tumour regrowth and metastasis in humans. A number of transferred cytokines have shown varying degrees of protection in tumour models,[50] with IL-2,[51] GM-CSF[52–54] and IL-12[55 56] being most effective and consistent at inducing protective immunity in murine colorectal tumour models. We have found IL-12 and B7.1 to be a combination that elicits the greatest degree of protection[54] and IL-12 can also give rise to CTL that successfully treat colorectal tumour "metastases" in the lung.[57] The local release of these cytokines induces a cell mediated Th type 1 response (IL-12) or the stimulation of dendritic cell precursors (GM-CSF), which take up tumour antigens, migrate to the lymph nodes and prime T cells giving rise to effector and memory T cells.[58] CTL and/or NK cells mediate the tumour rejection in these models, with[51] or without[55] T cell help. The inflammatory environment created by the transferred cytokines also enhances the expression (together with MHC molecules) and recognition of less dominant self-antigen T cell epitopes which can then become targets for CTL.[11] In comparison to such murine models, humans may have carried their tumours for long periods prior to gene therapy, and both priming of naive cells and reversal of anergy will be required. In addition, selective pressure over many years will have caused the tumour to adapt to, and evade, the immune response. In human studies, the transfer of the B7 costimulatory molecule to colorectal tumour cell lines did not cause activation of T cells in vitro,[59] whereas the transfer of IL-2

Table 1 Candidate antigens for DNA vaccination against colorectal cancer

Antigen	*Class of antigen*	*T cell responses*	*Antitumour effect/association*
p21 *ras*	Mutated oncogene product	Human CTL, Th1	No
p53	Mutated tumour suppressor	Murine CTL	No
CEA	Embryonic gene product	Human CTL	In mice
MUC-1	Epithelial mucin	Non-MHC CTL	In mice
MAGE	Melanoma associated antigen	Human CTL in melanoma	In melanoma
GA733	Surface molecule	Murine CTL, Th	In mice[88]
Annexin-like molecules	Placental/structural protein	Th	No

stimulated NK cells in vitro but not tumour specific CTL.[60] In human melanoma, exogenous IL-12 and IL-2 in combination, but not B7, yielded the best in vitro CTL responses.[61]

In vivo infection of tumour cells with tumour targeting viruses (systemic or local administration) may also be feasible in gene therapy[62] 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 intratumoural 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 promotor.[63] This in vivo gene delivery approach should affect the growth of the targeted tumour and elicit protective immunity 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.[64] In addition to tumour cell modification, transfer of the gene for TNF-α to TIL from melanomas has been achieved,[65] 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).[62 66] This approach has been successful at reducing growth of a tumour challenge in a mouse colorectal tumour model[67] (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.[68] The use of suicide genes may also reduce the need for tumour cell irradiation, which could adversely affect the vaccine's efficiency.[69] 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,[70] or in vivo delivery (adenovirus).[71] 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.[72] 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 heterogenous human population may necessitate the use of larger antigenic fragments that encompass many epitopes.

Mutations in oncogenes 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.[73] Peptides from this region of *ras* also bind MHC class II molecules with high promiscuity,[74] 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.[75] 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[76 77] 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.[78] Polymorphic epithelial 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.[79 80] Although most frequently associated with melanoma, the MAGE family of genes has also been found in colorectal neoplasia[81] 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.[82]

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.[83 84] In light of the identification of a number of tumour-regression antigens in melanoma and

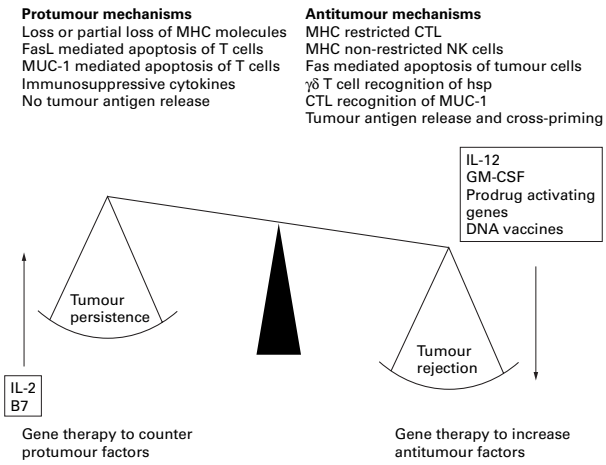

Figure 1 Tipping the balance in colorectal cancer. A number of protumour mechanisms outweigh antitumour mechanisms and allow colorectal tumours to survive and proliferate. Gene therapy with cytokine, immunostimulatory or suicide (prodrug activating) gene transfer to tumour cells, or DNA vaccination, aims to tip the balance towards antitumour mechanisms and tumour rejection by enhancing antitumour cellular immune responses. Correction of immune deficiencies associated with the tumour could work in synergy with enhancement of antitumour immunity and many of the genes shown could apply to both sides of the balance.

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 the 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.[85] 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 rates[86] and current gene transfer trials involve transfer of allo-MHC molecules (HLA-B7)[87] and cytosine deaminase to colorectal tumours.[3] Genes encoding IL-12, GM-CSF, B7, cytosine 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 micrometastses 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 reinjection of the cells as a "vaccine".[89] 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.

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