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Invited Review

Gene therapy strategies for intracranial tumours: glioma and pituitary adenomas

M.G. Castro, R. Cowen, J. Smith-Arica, J. Williams, S. Ali, S. Windeatt, V. Gonzalez-Nicolini, T. Maleniak and P.R. Lowenstein

Molecular Medicine and Gene Therapy Unit, School of Medicine, University of Manchester, Stopford Building, Manchester, UK

Summary. Intracranial tumours such as brain gliomas and pituitary adenomas pose a challenging area of research for the development of gene therapy strategies, both from the point of view of the severity of the diseases, to the physiological implication of gene delivery into the central nervous system and pituitary gland. On the one hand, brain gliomas are very malignant tumours, with a life expectancy of six months to a year at the most after the time of diagnosis, in spite of advances in treatment modalities which involve chemotherapy, surgery and radiotherapy. Gene therapy for these tumours is therefore a very attractive therapeutic modality which due to the severity of the disease is already in clinical trials. On the other hand, pituitary tumours are usually benign, and in most cases, treatment is successful. Nevertheless, there are some instances, especially with the macroadenomas and some invasive tumours in which treatment fails. Gene therapy strategies for these adenomas therefore needs to progress substantially in terms of safety, adverse side effects and physiological impact on the normal pituitary gland before clinical implementation.

In this paper, we will review gene delivery systems both viral and non-viral and several therapeutic strategies which could be implemented for the treatment of these diseases. These include cytotoxic approaches both conditional and direct, immune-stimulatory strategies, anti-angiogenic strategies and approaches which harness pro-apoptotic and tumour suppressor gene targets. We will also review the models which are currently available in which these gene therapy strategies can be tested experimentally.

This new therapeutic modality holds enormous promise, but we still need substantial improvements both from the delivery, efficacy and safety stand points before it can become a clinical reality.

Key words: Viral vectors, HSV1-TK, Prolactinomas, Glioblastomas, Apoptosis, Cytokines, Angiogenesis

Offprint requests to: Dr. Maria G. Castro, Molecular Medicine and Gene Therapy Unit, School of Medicine, University of Manchester, Stopford Building, Rm: 1.302, Oxford Road, Manchester M13 9PT, UK. Fax: (44)-(0)161-275-5669. e-mail: mcastro@fs1.scg.man.ac.uk

Introduction

Although gene therapy was first envisaged for the treatment of genetically inherited disorders such as hemophilia, cystic fibrosis and severe combined immunodeficiency syndrome, the majority of gene therapy studies currently underway are for the treatment of acquired diseases such as AIDS, cancer and cardiovascular disorders. Currently, 72% of gene therapy clinical trials are for the treatment of cancer (Lowenstein, 1997). The main reasons for this are that the therapeutic endpoints for the treatment and the population of cells which needs to be targeted are very well defined. In general, for the successful treatment of cancer, the required duration of transgene expression could be relatively short (i.e. sufficient to kill the tumour cells) compared to that required to treat inherited genetic disorders. Also because of the relatively rapid progression of most tumours, it is possible to monitor the clinical outcome of the treatment over a relatively short period. The availability of a large cohort of patients with severe and life threatening acquired diseases, such as cancer and AIDS, provides a clinical scenario in which to develop and test new strategies for gene delivery and therapy that might be applied later for inherited diseases (Martin Duque et al., 1998)

For cancer treatment the goal of gene therapy would be to induce tumour cell killing and/or induce anti-tumour immune responses (Lowenstein, 1997; Castro, 1999; Castro et al., 1999; Lowenstein et al., 1999). Below we will review several gene transfer and anti-tumour strategies, as well as disease models, in which to assess the efficiency of these novel therapeutic modalities, with special emphasis on intracranial tumours, i.e. gliomas and pituitary adenomas.

In vivo gene transfer vehicles

The ideal gene delivery system should be able to encode for large DNA inserts, transgene expression should be targeted to pre-determined cell-types and should provide regulatable long term expression. The vector should be easily produced on a large scale, be non-toxic and ideally systemic delivery should be

feasible. None of the gene delivery systems currently used for *in vivo* gene transfer are perfect with respect to all of the previously mentioned characteristics. The main gene transfer systems which have recently been used for gene therapy clinical studies involve retrovirus vectors vectors, herpes simplex type 1 virus vectors, adenovirus vectors, adeno-associated virus vectors and liposomes. The following section will discuss the salient characteristics of each of these systems.

Viral vectors for gene transfer

Retroviruses are composed of a diploid RNA genome that is packaged into a virion, surrounded by a lipid envelope containing both host cell membrane and viral proteins. For gene expression, the RNA genome must be reverse transcribed into double-stranded DNA using the viral enzyme, reverse transcriptase. This complementary DNA sequence is then integrated into the host cell DNA using the viral integrase protein contained within the retrovirus particle. The integrated provirus is then able to use the host cell machinery for viral gene expression. Retroviruses used for gene transfer are rendered replication defective by the deletion of the viral genes *gag*, *pol* and *env*, and retroviral vector production can be achieved in viral packaging cell lines which provide these deleted genes *in trans*. The packaging cell line is optimally constructed by stably inserting the deleted viral genes *gag*, *pol* and *env*, into the packaging cells' genome, such that each gene will reside on different chromosomes. This approach ensures that recombination of these genes is highly unlikely minimising the possibility of the generation of replication-competent retroviruses. Furthermore, the removal of the *gag*, *pol* and *env* genes eliminates these virally encoded mRNAs and therefore reduces the potential immune response to these antigenic peptides within transduced cells.

With stable integration of this vector into the host DNA and low immunogenicity, the therapeutic transgene should be expressed for the entire life of the infected cell. This is not the case, since transgene expression mediated by retroviruses decreases with time, due to promoter "shut off" or depending on the site of integration within the host genome. Also integration of retroviral vectors is limited to dividing cells and at present only moderate retrovirus titers of 10^5-10^7 plaque forming units (pfu)/ml can be produced.

The recently developed lentiviral vectors based on human immunodeficiency virus (HIV) and other lentiviruses can be used to express transgenes in both dividing and quiescent cells as these viruses can cross the nuclear membrane. *In vivo* experiments with lentiviral vectors have achieved transgene expression in brain, retina, muscle and liver (Naldini, 1998). Further studies in terms of the safety of these vectors will need to be undertaken and higher titers produced before their clinical use can be approved.

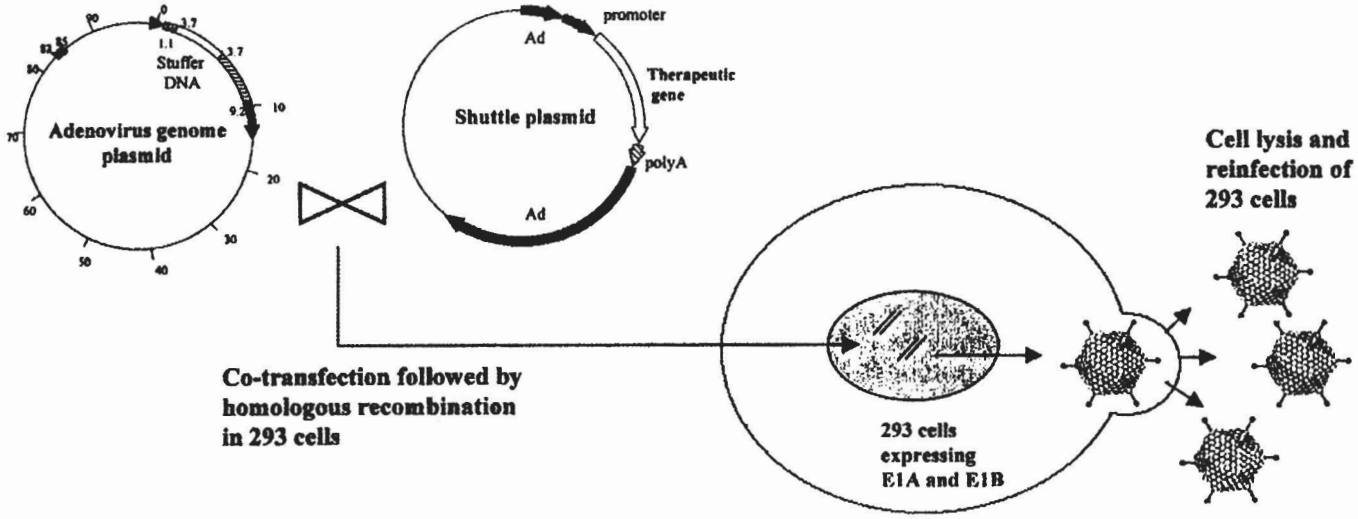
Adenovirus vectors possess a double-stranded DNA genome of 36Kb, and replicate independently of the host cell genome. These vectors have many attractive properties, being capable of transducing a broad spectrum of human tissues. High levels of gene expression can be obtained in both dividing and non dividing cells. Several routes of administration can be used including intravenous, intrabiliary, intraperitoneal, intravesicular, intracranial and intrathecal injection, or direct injection into the target organ. The adenoviral genome is composed of early (E) and late (L) regions that contain different complex transcriptional units divided according to the onset of viral DNA replication. Infection involves the fiber protein which binds to the coxsackievirus and adenovirus cell surface receptor (CAR). The penton base of the adenoviral capsid engages integrin receptor domains ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) on the cell surface resulting in internalization of the virus into an endosome. Once within the endosome, changes in the internal pH cause conformational changes of the viral coat proteins resulting in viral escape into the cytoplasm. The capsid then translocates to the nuclear membrane, triggering dissociation of viral coat proteins to expose the viral DNA. The viral DNA enters the nucleus and is transcribed episomally without cell division. The E1 regions, E1A and E1B encode proteins responsible for transcriptional regulation of the viral genes. Removal of these genes renders the virus replication-defective. The 293 human embryonic kidney cell line has been engineered to express E1A and E1B proteins, in order to transcomplement the E1-deficient viral genome, and allow for the production of recombinant adenoviruses (rAds) of high titer (10^{13} pfu/ml) (Fig. 1) (Lowenstein et al., 1996; Southgate et al., 2000).

Most adults have had prior exposure to wild-type adenovirus and therefore possess anti-adenovirus antibodies. These viruses elicit a strong immune response when administered into the periphery which limits the duration of transgene expression (Elkon et al., 1997). This immune response also precludes the treatment of diseases requiring repeated administration (Yang et al., 1996; Thomas et al., 2000a). Some groups report relatively short transgene expression with a maximum duration of expression of 2 months (Le gal la salle et al., 1993), whilst other groups including our own show much longer transgene expression, i.e. 6-12 months in the brain (Geddes et al., 1997; Dewey et al., 1999; Thomas et al., 2000b). Within the periphery the immune response against rAds is well characterised but the immune responses to recombinant adenoviruses administration into the CNS are less well understood. Importantly, adenoviral vectors can elicit rapid inflammatory responses within a few hours post administration into the brain (Cartmell et al., 1999).

The latest generation of adenoviral vectors contain only the viral inverted terminal repeat (ITR) sequences, and the packaging signals. All of the remaining adenoviral genes have been removed and replaced by stuffer DNA therefore the presence of a helper virus is required, to provide essential viral genes *in trans*, for

their production. These vectors are termed gutless, high capacity or helper dependent vectors. To avoid contamination of preparations with helper virus, lox P sites have been inserted to flank the packaging signal of the helper. Virus production is then carried out in 293 cells expressing Cre recombinase to excise the helper packaging signal (reviewed by Morsy and Caskey, 1999). These vectors can sustain long term transgene expression, even in the presence of a peripheral priming with a first generation adenovirus, making them very attractive for long term gene therapy applications (Thomas et al., 2000a). Further advances in adenoviral vectors for cancer gene therapy have used conditional (or restricted) replicative recombinant adenoviral systems such as the ONYX system (Bischoff et al., 1996), with restricted viral replication limited to p53 deficient tumour cells. This vector is now being assessed in clinical trials (Kirn et al., 1998).

Adeno-associated viruses (AAV) are single-stranded DNA, non autonomous parvoviruses capable of integration into the genome of non dividing cells. AAV's are not thought to be associated with human disease and do not elicit a strong T-cell immune response, although they do elicit a neutralizing B-cell antibody response. AAV's have two distinct life cycle phases. Wild-type virus will infect a host cell, integrate and remain latent, if the cell is infected in the absence of adenovirus, or another helper virus. In the presence of helper adenovirus, the lytic phase of the virus is induced, which is dependent on the expression of early adenoviral genes, and leads to active virus replication. The AAV genome is composed of two open reading frames (called *rep* and *cap*) flanked by inverted terminal repeat (ITR) sequences. The *rep* region encodes four proteins which mediate AAV replication, viral DNA transcription, and endonuclease functions used in host genome integration. The *rep* genes are the only AAV sequences required for viral replication. The *cap* sequence encodes structural



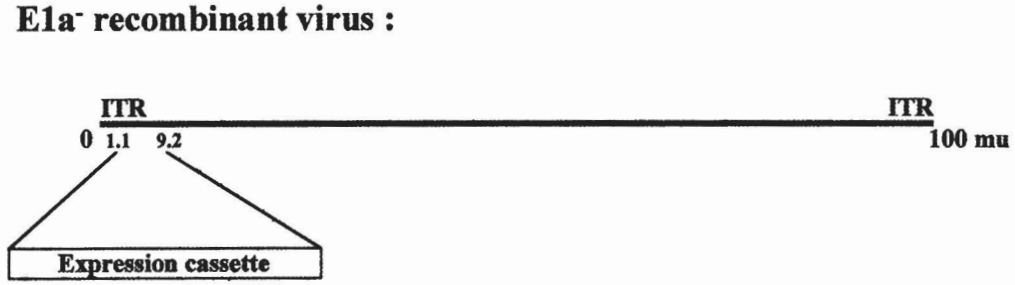


Fig. 1. Generation of recombinant E1-substituted adenovirus vectors (rAds) expressing either a cytotoxic or immune stimulatory molecule for cancer gene therapy. The shuttle plasmid provides the expression cassette (encoding the promoter, therapeutic gene and polyadenylation signal) with flanking homology from either side of the Ad E1a gene region. The adenovirus genome plasmid contains the entire Ad5 genome with prokaryotic, "stuffer" DNA inserted into the E1a gene at 3.7 map units, making this plasmid too large to be packaged into a capsid. The shuttle plasmid and adenovirus genome plasmid are co-transfected into 293 cells. Homologous recombination between the plasmids results in the expression cassette replacing both the E1a region and intervening stuffer DNA. The recombinant plasmid size is now sufficiently reduced to be packaged resulting in the generation of E1a- recombinant virus. Propagation of the rAd vector is achieved in 293 cells which have been stably transfected to provide E1 complementation. A linear representation of the rAd genome is shown at the bottom of the figure, showing the position of the expression cassette. The numbers on the adenovirus genome plasmid and the linear viral genome refer to the Ad5 genomic sequence expressed as map units (mu).

proteins that form the viral capsid. The ITRs contain the viral origins of replication, provide encapsidation signals, and participate in viral DNA integration. Recombinant, replication-defective viruses that have been developed for gene therapy lack *rep* and *cap* sequences. Replication-defective AAVs can be produced by cotransfecting the elements necessary for AAV replication into permissive 293 cells and titers as high as 10^{14} pfu/ml can be produced. Recombinant AAV vectors can only carry a transgene of 5Kb which has limited their usefulness for gene therapy (reviewed by Grimm and Kleinschmidt, 1999).

The Herpes simplex virus (HSV) is a large ~150Kb, double-stranded, DNA virus that replicates in the nucleus of infected cells. It has a broad host range and can infect dividing and non dividing cells (Lowenstein et al., 1994, 1995; Goya et al., 1998, Tomasec et al., 1999), where it can persist for a long time in a non integrated state. Much of the large genome of HSV is redundant enabling large deletions to be made which allow the insertion of large sequences of foreign DNA into the viral genome by homologous recombination. However, before HSV vectors can be used *in vivo* the viral preparations must be rendered free of replication-competent virus, which is not without complications. These vectors induce a potent immune response against the immediate early (IE) genes of the virus but deletion of many of these genes has resulted in vectors with reduced toxicity. The generation of plasmid "amplicons" is achieved through the cotransfection of one plasmid which contains the HSV origin of replication and packaging signal, with a cosmid encoding the HSV genome containing a defective packaging signal, to provide all factors necessary for amplicon replication and packaging. This yields a viral vector containing only plasmid sequences, eliminating any HSV associated immunogenicity, and providing a large cloning capacity. First generation amplicon systems have been dogged by helper virus contamination but newer methods of production are in development (Geller, 1997).

Alternatively two viral vectors can be combined to harness the favourable attributes of each individual vector. With this aim adenovirus/retrovirus vectors are being developed where an adenoviral vector induces target cells to function as transient retroviral producer cells *in vivo* (Bilbao et al., 1997). Another well-characterised example of a viral hybrid is the Sendai virus (hemagglutinating virus of Japan [HVJ]) liposome complex. Here the F-protein from the HVJ is fused with liposomes containing DNA expressing the gene of interest. *In-vitro* experiments suggest that the HVJ-liposome complex is more efficient than cationic liposomes alone (Nakanishi et al., 1985). Due to its apparent efficiency numerous transgenes have been expressed from the HVJ-liposome complex *in vivo*. Expression of HSV-1 thymidine kinase from HVJ-liposome complexes resulted in 80% of nude mice submeningeal gliomatosis to be cured when treated with ganciclovir, suggesting that this delivery system may be applicable for the *in-vivo* gene therapy of human malignant glioma (Mabuchi et al., 1997). Interestingly, studies investigating the persistence and repetitive injection of HVJ-liposomes expressing the reporter gene ß-galactosidase in the liver showed that second and third re-administration resulted in the same levels of expression as seen in the initial injection (Hirano et al., 1998).

To improve the specificity of gene delivery and expression of therapeutic genes the inclusion of targeting elements into these delivery vehicles and the use of regulatory expression systems are actively being pursued. A bispecific molecule can be complexed to a viral vector which blocks native receptor binding and retargets the virus to a tissue-specific receptor. Alternatively genetic modification of the virus coat protein to redirect binding to a novel ligand is also being attempted (reviewed in Curiel, 1999; Stone et al., 2000; Wickham, 2000).

Non-viral vectors

Although in the field of gene therapy viral vectors have taken centre stage, when considering delivery vehicles for therapeutic transgenes, many non-viral vectors have shown great promise. The generation of a host immune response by adenoviruses, low integration efficiency of certain retroviruses, toxicity of Herpes simplex viruses and general concern over possible generation of wild-type viruses expressing human transgenes has led some researchers to begin developing non-viral vectors.

Mouse skeletal muscle and myocardial tissue have been shown to be highly receptive to plasmid DNA (pDNA) transfection with expression of the transgene persisting over 2 months *in vivo*. The strength and longevity of expression in muscle has been suggested to be due to the unique architecture of muscle fibres, which facilitate the uptake of pDNA. Numerous other tissues *in vivo* have been shown to be susceptible to pDNA mediated transfection, including brain and gastro-intestinal tissue but the level of transgene expression is limited.

Plasmid DNA complexed with cationic lipids, to form liposomes has been shown to be highly successful in transfecting cell lines. Numerous cationic lipid compounds have been developed (reviewed by Gao and Huang, 1995). Various tumour models have been shown to be responsive to cationic liposome mediated gene therapy. Human interleukin-2 (IL-2), diptheria toxin A-chain and Herpes simplex virus 1 thymidine kinase (HSV1-TK) have been successfully delivered into tumours using cationic liposomes with favourable results. Such success has lead to the development of clinical trials using cationic liposome mediated gene therapy for cancer. A phase I immunotherapy study using cationic liposomes to deliver the human HLA-A2, HLA-B13 and murine H-2K genes into cutaneous metastasis resulted in complete regression of two

tumours, and the other four tumours showed a partial local response (Hui et al., 1997). An alternative trial expressing HLA-B7 suggested that gene therapy using cationic liposome vectors for tumours was both feasible and non-toxic (Rubin et al., 1997).

One limitation of liposomes is that once endocytosed by the target cell they fail to escape from the endosome, eventually becoming degraded. It is thought that the presence of protonatable groups on the liposome may facilitate escape from the endosomes. Indeed the use of the cationic polymer polyethylenemine, which contains numerous protonatable groups, improves the vector properties of liposomes (Abdallah et al., 1996). The same limitations apply to cationic liposome complexes as for pDNA, in that the large amounts of DNA required to transduce target cells *in vivo* do not generate high levels of transgene expression. The most successful studies using cationic liposomes express soluble factors in experimental paradigms that require a low percentage of transfection. However, in the absence of an immune response to such non-viral vectors and the ability to systemically re-administer cationic liposomes such technology might prove useful in certain gene therapy protocols.

Targeted polyamine conjugates have attempted to utilise the natural endocytic pathway inherent to cells to gain efficient access into the cytoplasm, thus obviating the need for such large quantities of DNA. Targeted polyamine conjugates involve the production of polyamine-DNA aggregates, covalently linked to specific proteins on their exposed surfaces, which recognise specific epitopes on the surfaces of target cells. The polyamine condenses the DNA into tightly packed structures, which can then be coupled to any desired protein. The versatility of such a system is that any protein can be linked to the polyamine conjugate and can potentially be targeted to any cell type within the body. Numerous receptors have been targeted using this system, including epidermal growth factor receptor, erythrocyte growth factor receptor and ErbB2 receptor (Cristiano and Roth, 1996).

Anti-tumour strategies

Cancer gene therapies have used several strategies to achieve tumour cell killing. Due to the fact that most cancers exert their morbidity and mortality because of the spread of metastasis, the challenge is not only to target every tumour cell in the primary lesion, but also target small groups of metastatic cancer cells in locations distant from the main tumour mass. Current gene transfer vectors do not allow us to achieve high levels of gene transfer in an *in vivo* setting. Therefore alternative strategies which do not rely on highly efficient gene transfer are being developed which include, (1) conditional cytotoxic cell killing and (2) immune stimulation by ectopic cytokine expression and/or engineering immune recognition of tumour cells.

Conditional cytotoxicity approaches for cancer gene therapy

Conditional cytotoxicity utilises the conversion of an inert prodrug to a pharmacologically active cytotoxic drug at the tumour site. The most commonly used approach is Gene Directed Enzyme Prodrug Therapy (GDEPT). A "suicide" gene encoding the enzyme is selectively delivered to tumour cells. This enzyme must be capable of metabolising a non or weakly toxic prodrug to an active, highly toxic drug. The toxic drug is produced either within the tumour cells or the tumour mass, allowing its local diffusion to non transduced tumour cells producing a "bystander effect".

Thymidine kinase (TK) from Herpes simplex virus 1 (HSV1) is the most intensely studied conditional cytotoxic gene. Its substrates include the anti-herpes drug ganciclovir (GCV) which HSV1-TK monophosphorylates to an intermediate, which is subsequently phosphorylated by cellular kinases to a di- or triphosphate form, that can be incorporated into DNA as a nucleoside analogue. Phosphorylated GCV can only kill dividing cells, as tumour cells are usually the most actively dividing cells in tissues, it can be argued that this gives the therapy a level of selectivity. HSV1-TK/GCV exhibits a strong bystander effect both *in vitro* and *in vivo*. Mouse glioma tumour studies showed that only 10% of tumour cells needed to be transduced to achieve total tumour regression (Chen et al., 1994). GCV has FDA approval, therefore on the basis of good pre-clinical data (Ezzeddine et al., 1991; Dewey et al., 1999) this HSV1-TK/GCV pairing has been used in human gene therapy trials. *In vivo* gene transfer using both retroviral and adenoviral delivery of HSV1-TK in clinical trials have been well tolerated by patients with recurrent brain tumours, with extension of patient survival reported (Egilmez et al., 1996; Klatzmann et al., 1998).

Cytosine deaminase in combination with 5-fluorocytosine (CD/5-FC) has also moved into human gene therapy clinical trials. Cytosine deaminase is ubiquitous within bacteria and fungi but absent from mammalian cells. This enzyme deaminates the antifungal drug 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) which induces cell death by inhibiting both DNA and RNA synthesis, therefore it only kills dividing cells. Huber et al. (1993) infected a colorectal tumour cell line *ex vivo* then implanted the transduced cells into mice demonstrating anti-tumour effects as a result of local 5-FU production. A bystander effect was also observed when mixtures of transduced and non transduced cells were implanted. These experiments suggest that as few as 2% of the tumour cells need to be transduced to eliminate the tumour.

Nitroreductase (NRD) is an enzyme from the *E. coli* strain K12 and is used in combination with the prodrug CB1954, a weak alkylating agent. NRD converts its prodrug into the 4-hydroxylamino derivative, which after acetylation via thioesters becomes a powerful

alkylating agent capable of cross linking DNA. The active drug therefore kills both quiescent and dividing cells and shows promise for *in vivo* tumour cell killing (Searle et al., 1998).

Carboxypeptidase G2 (CPG2) is an enzyme isolated from the bacteria *Pseudomonas* strain RS-16, which cleaves the C-terminal glutamate moiety from benzoic acid mustard prodrugs. This cleavage results in the release of cytotoxic nitrogen mustards which are toxic to both quiescent and dividing cells by DNA alkylation. This enzyme was first used in antibody directed enzyme prodrug therapies (ADEPT), targeting CPG2 to a colorectal tumour xenograft in athymic mice, by conjugation of the enzyme to an antibody recognising the carcinoembryonic (CEA) antigen (Blakey et al., 1995), thus achieving significant anti-tumour activity upon administration of prodrug. Clinical trials using this antibody targeting approach are ongoing in patients with advanced, drug resistant colorectal cancer. GDEPT approaches are now being investigated (Marais et al., 1996, 1997).

Dual enzyme/prodrug combinations are currently being investigated with promising results. HSV1-TK and cytosine deaminase have been simultaneously expressed from stably transfected gliosarcoma cells implanted into nude mice and after prodrug administration, toxicity of the double suicide system was shown to be 2-3 times higher than if the cytotoxic effects of each prodrug was purely additive (Rogulski et al., 1997). These enzyme prodrug combinations are also being assessed for their effects on radiosensitisation, for their use in conjunction with radiotherapy (Kim et al., 1998).

Pro-apoptotic and tumour suppressor genes

Promising targets for cancer gene therapy are pro-apoptotic and tumour suppressor genes (Favrot et al., 1998; Watson and Lowenstein, 1998). Apoptosis is regulated by a complex cascade of proteases of the interleukin-1ß converting enzyme (ICE) family culminating in cell death. Over expression of these ICE proteases, also known as caspases, is sufficient to induce apoptosis. Abnormalities in this apoptotic cascade, such as gene deletions, mutations or aberrant gene expression, are almost always present in tumour cells, including gliomas (Ueki et al., 1996). Apoptosis can also be mediated via the Fas/FasL (Fas ligand) pathway. An increase in cell surface expression of Fas in glioma cells, increases their susceptibility to Fas antibody mediated apoptosis *in vitro* (Weller et al., 1995). We have recently generated recombinant adenovirus vectors expressing FasL, under the control of cell type specific promoters to reduce systemic toxicity (Larregina et al., 1998; Morelli et al., 1999). When used in an *in vivo* model of intracranial glioma, over expression of FasL increased the survival of tumour implanted animals (Ambar et al., 1999).

Mutation of the tumour suppressor gene, p53 is the most common genetic alteration in cancers. Inactivation of p53 occurs early in glial tumourigenesis and mutations are commonly found in low grade astrocytomas. Replacement of a defective p53 gene has been described for many tumour models. Gomez-Manzano et al. (1996) used adenoviral delivery of a wild-type p53 gene into two glioblastoma cell lines expressing either a wild-type or mutant p53 gene. In these experiments the wild type p53 cells showed an inhibition in proliferation and mutant p53 cells underwent apoptotic cell death. In anaplastic astrocytomas mutation of the retinoblastoma (Rb) gene and/or p16 gene is most common. The mutation, methylation or deletion of p16 is also found in a variety of human tumour cell lines, including 80% of glioma cell lines. Restoration of p16 expression, again using an adenovirus vector for delivery into p16 deficient human glioma cell lines i.e., D-54M, U-251MG and U-87MG produced growth arrest (Arap et al., 1995). Within glioblastoma multiforme alterations to chromosome 10 have also been identified, indicating that a glioma-associated tumour-suppressor gene may reside within this region. Microcell-mediated transfer has been used to introduce chromosome 10 into tumourigenic U-251MG glioma cells, which when injected into nude-mice did not form tumours. Two tumour suppressive regions were mapped to chromosome 10 and recently a tumour suppresser gene MMAC1/PTEN was identified within this chromosome which is commonly mutated in human glioblastoma multiforme (Rasheed et al., 1997). *In vitro* proliferation of U87MG cells was inhibited by MMAC1 expression. Cheney et al. (1998) have generated a replication-defective adenovirus encoding MMAC1/PTEN, and infection of MMAC1 mutated cells with this virus rendered them almost completely non-tumourigenic, as compared to untreated and control cells, suggesting that the *in vivo* gene transfer of MMAC1/PTEN could potentially be useful in cancer gene therapy for aggressive gliomas.

The pathogenesis of pituitary tumours has also been extensively studied to identify pro-apoptotic and tumour suppressor genes which could be used as effective transgenes for gene therapy (Clayton et al., 1997). Mutations have been described in the G protein, $G_s\alpha$, which occur in approximately 40% of growth hormone (GH) secreting tumours. Rarely occurring *ras* mutations in invasive tumours, loss of heterozygosity on chromosome 11, and near the Rb locus on chromosome 13 have also been implicated, but not reproducibly identified. The pituitary tumour-transforming gene (PTTG) was isolated from rat GH4 pituitary tumours and recently the human homologue was cloned. Human PTTG is abundantly expressed in pituitary tumours and potently transforms cells both *in vitro* and *in vivo*. Furthermore, PTTG can be used as a marker for the invasiveness of hormone secreting tumours, with more invasive tumours expressing the highest amounts of PTTG (Zhang et al., 1999).

Inhibition of angiogenesis

The inhibition of angiogenesis is thought to be a

promising strategy that could lead to the development of novel anti-neoplastic therapies. It is well established that tumour growth is angiogenesis dependent. The primary target for an anti-angiogenic intervention is the inhibition of tumour growth by targeting the tumour neovasculature. The potency of blocking the tumour blood supply has been demonstrated directly in a mouse model of tumour vascular targeting (Huang et al., 1997). With the molecular characterisation of receptors and ligands that stimulate and modulate the ingrowth of blood vessels in tumours, strategies can now be employed to disrupt this process.

Therapeutic targeting of the tumour vasculature is extremely appealing as angiogenesis is down-regulated in healthy adults, so targeting should lead to minimal side-effects even after prolonged treatment. Also, the fact that tumour associated angiogenesis is a physiological host mechanism, targeting the tumour vasculature should not lead to the development of host resistance (Boehm et al., 1997). Tumour capillaries supply hundreds of tumour cells indicating that targeting angiogenesis should lead to a potentiation of the antitumour effect.

The inhibition of angiogenesis has already been proven as a powerful tool in a number of experimental animal tumour models and some of the first substances identified have now entered clinical trials (Boehm et al., 1997; O'Reilly et al., 1997). Amongst the targets for these agents are vascular endothelial growth factor (VEGF)/VEGF-receptor complex, alpha (v) integrins and tie1 and 2 receptors, all members of the tyrosine kinase receptor family. VEGF has a narrow target cell specificity and acts primarily on endothelial cells. VEGF's mediate their endotheliotropic activities through receptor tyrosine kinases [VEGF-R1 (*flt-1*), VEGF-R2 (*flk-1*/KDR) and VEGF-R3 (*flt-4*)] whose expression is upregulated during angiogenesis.

The introduction of a dominant-negative *flk* (VEGF-R2) receptor into C6 glioma cells using a retroviral vector caused tumour involution in a nude mouse model (Milauer et al., 1994). A different approach was adopted by Saleh et al. (1996) who used stable cells expressing an antisense VEGF, which when expressed reduced quantities of VEGF compared to the parental cell line. In a nude mouse model, the tumour cells expressing antisense VEGF showed reduced ability to form neoplasms and this correlated with a significant decrease in blood vessel formation. The expression of antisense bFGF cDNA has also been associated with reduced proliferation of C6 cells. However, Schmidt et al. (1999) suggest that bFGF is only an essential co-factor for angiogenesis in glioma cells, since it is present in sparsely vascularised low-grade tumours.

Angiostatin and endostatin are two recently discovered anti-angiogenic factors which may hold potential for the treatment of disseminated cancers. Blezinger et al. (1999) used intramuscular injection of an expression plasmid encoding the endostatin gene to inhibit the growth of a subcutaneous primary tumour and lung metastases in an *in vivo* renal cell carcinoma mouse model.

Activation of the immune response

The attraction of the immune system from a therapeutic viewpoint is that once appropriately activated against tumour specific determinants, a small activation signal can produce a long lasting, body wide protection. These properties make the immune system an attractive target for current attempts at gene therapy of cancer, since no gene delivery system or chemotherapeutic drug available has the required specificity to target all tumour cells throughout the body of cancer patients. Studies of mechanisms involved in recognition and elimination of tumour cells have shown a key role for T lymphocytes in conferring the specificity of tumour rejection. In particular CD8+ cytotoxic T lymphocytes (CTLs) were identified as the important effector cell population in the elimination of tumour cells. Whilst responses are mainly mediated by CD8+ CTLs, the induction of these responses are dependent on the presence of CD4+ helper T cells. In addition to these antigen specific effectors, roles have also been identified for natural killer cells and non-specific effector cells such as macrophages and eosinophils. It is now believed that both the innate and adaptive immune responses act in concert, through specific signalling pathways, to generate anti-tumour immune responses (Fig. 2). We will review the two most commonly used gene therapy approaches to enhance the host's immune response against tumours, i.e. cytokine based gene therapy and immune enhancement of tumour cells.

The underlying rationale for using cytokines in patients with cancer is to increase the patient's inhibited immune response to the autologous tumour. Cytokines may act to enhance local antigen presentation by the tumour by means of inducing expression of MHC-I antigen on tumour cells, MHC-II antigen on antigen presenting cells (APCs) or by increasing the expression of co-stimulatory molecules, or the tumour antigens themselves.

Local expression of cytokines may also act to enhance antigen uptake and processing by tissue APCs, with subsequent migration of these cells to secondary lymphoid tissue and presentation to resident T and B cells. Cytokines induce Th1 and Th2 sub-populations of helper T cells, direct activation of natural killer and cytotoxic T cells, differentiation of granulocyte and macrophage progenitors and expansion of dendritic cells.

Ultimately it is expected that these cytokine effects will result in either tumour cells which are better APCs themselves, or the body's normal APCs become better at presenting tumour antigens and that tumour cells will become targets of immune-activated anti-tumour cytotoxic T cells. The potential role of cytokines as adjuvants of the immune response has been demonstrated

by various studies, which illustrate the regression of human tumours following their systemic administration. However the dose required to produce optimal results showed unacceptable levels of toxicty. Subcutaneous implantation of genetically modified cells secreting cytokines, including IL-2, IL-4, IL-6, INF-γ, IL-12, GM-CSF and TNF-α outside of the CNS has been successful in generating cellular mediated immune responses, whilst at the same time minimising the toxic effects associated with high dose systemic administration (Rosenberg, 1991).

Interleukin 2 (IL-2) is a critical and potent cytokine, the pleiotropic effects of which include stimulation of cell-mediated killing activity of CTLs, enhancement of natural killer activity, induction of lymphokine activated killer cells and activation of tumour infiltrating lymphocytes. IL-2 therapy of CNS malignancies has limited application as demonstrated by various studies. Direct administration of recombinant IL-2 in clinical trials produced even more marked neurological deterioration in patients, with increasing peritumoural oedema. Studies using IL-2 transfected cells in mouse glioma models have produced interesting results showing an increase in tumour latency and increased survival of animals receiving IL-2 secreting cells (Glick et al., 1995). Less promising results have been obtained with *in-situ* transfection of tumour cells using retroviral vectors encoding the IL-2 gene. Other attempts at using IL-2 to augment the immune response for the treatment of glioblastoma have combined the use of cytokines with suicide gene therapy systems in which the "bystander" effect is believed to have an immune component (Palu et al., 1999).

INF-γ is another T cell derived cytokine that is central to the interaction of T cells with APCs and activation of macrophages and astrocytes. INF-γ has been shown to induce the expression of MHC I and II expression on both professional APCs and on malignant cells *in vitro*. Although clinical trials of systemic and intratumoural INF-γ administration in malignant glioma were unsuccessful, several lines of evidence suggest that this cytokine could augment the efficacy of cellular immunotherapy.

TNF-α is a pleiotropic cytokine that does not only kill tumour cells directly but induces haemorrhagic necrosis of some tumours *in vivo* by virtue of its effects on endothelial cells and the coagulation system. Additionally it affects the stimulation of T cell proliferation, activation of CTLs and the induction of adhesion molecule expression on endothelial cells and on tumour targets. Despite the success of TNF-α as an

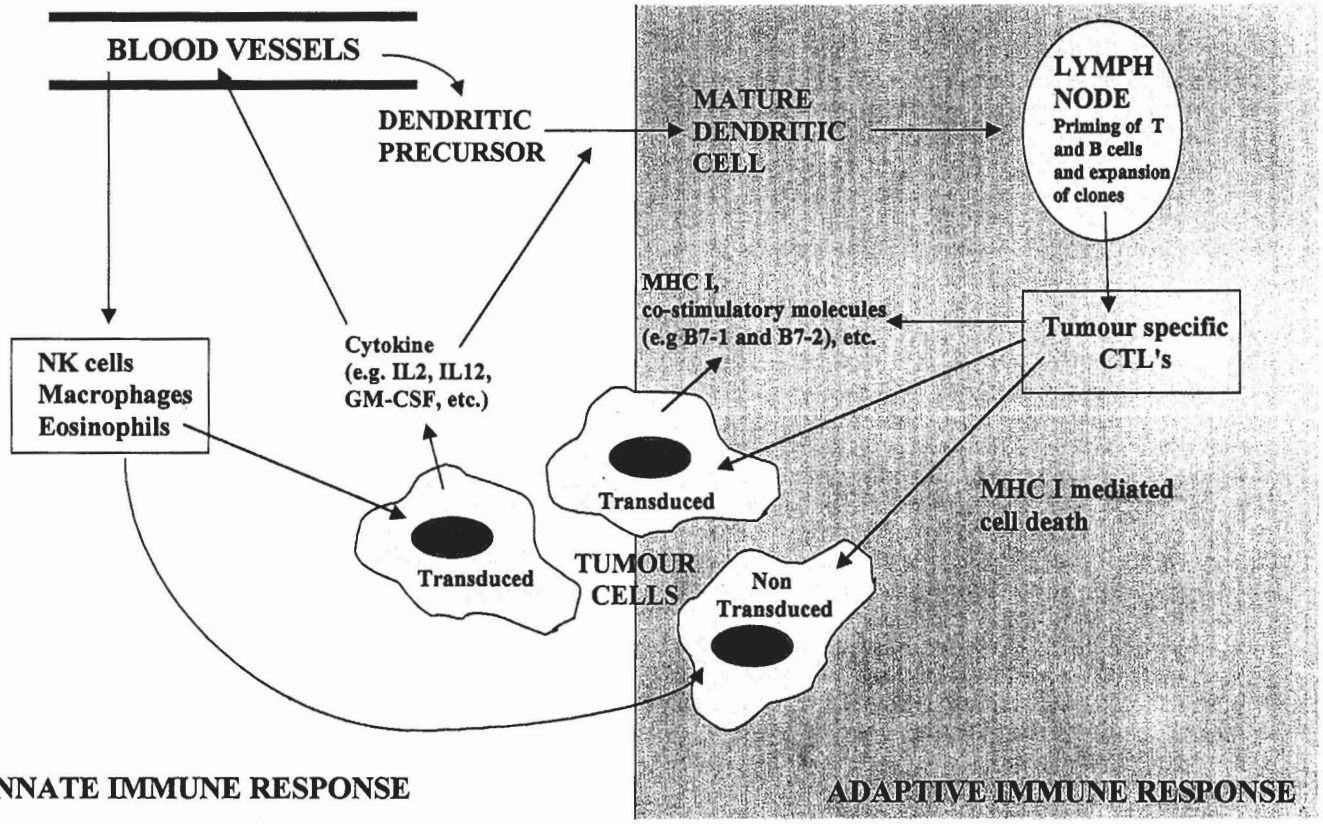


Fig. 2. Immune enhancement of tumour cells, following transduction with a cytokine and/or a co-stimulatory molecule, to evoke both specific and non-specific tumour cell killing. Cytokine expression from tumour cells can enhance both the innate and the adaptive immune response against transduced and non-transduced tumour cells. Cytokines may act to enhance MHC mediated antigen presentation, uptake and processing by professional antigen presenting cells (APCs) such as dendritic cells or promote a cytotoxic natural killer (NK)/macrophage/eosinophil response.

antitumour agent in different experimental animal models, clinical trials with TNF-α for malignant gliomas, as well as for other tumours, have been disappointing (Vaquero et al., 1995).

Unlike other cytokines interleukin 4 (IL-4) promotes an anti-inflammatory response when expressed at the site of tumour growth, and also has anti-angiogenic properties. IL-4 induces macrophage and eosinophil mediated immune responses and retroviral tumour delivery of IL-4 in a rat brain model has caused significant tumour regression (Benedetti et al., 1999).

GM-CSF stimulates growth, differentiation and activation of granulocytes, monocytes/macrophages, microglia and dendritic antigen presenting cells. The importance of this cytokine was demonstrated by Dranoff et al., (1993) who showed that vaccination with irradiated GM-CSF transduced tumour cells produced specific and marked inhibition of tumour growth in mouse models of adenocarcinoma and melanoma. Of 10 cytokines investigated in this study, GM-CSF was shown to promote the greatest growth inhibition. This cytokine has been evaluated for the treatment of brain glioma and brain metastasis (Yu et al., 1997) with encouraging results.

To increase the immune response against cancer cells several approaches are being developed. The tumour cells can be allogenized by the expression of a highly immunogenic molecule on the surface of the tumour cell. Non-self class I MHC antigens can act as strong alloantigens and be recognized as distinct targets by cytotoxic T lymphocytes. Expression of non-self antigens on the surface of tumour cells has been achieved by coupling purified protein derivatives to the surface of cells. Antibody responses to the purified protein derivatives of tuberculin (PPD) and *Mycobacterium bovis* BCG (bacille Calmette Guerin immunotherapy), as well as tetanus toxoid have been tested.

Alternatively, recognition of pre-existing tumour antigens can be increased by enhancing T cell activation. The mechanisms involved in anti-tumour immunity are extremely complex but it is known that in order to activate resting T-cells the antigen must be seen by the T-cell in association with a major histocompatibility complex molecule (either MHC class I or II). Despite the fact that many tumours express MHC molecules to present antigen, an adequate tumour destroying immune response is rarely raised.

Further interaction with a co-stimulatory molecule and/or certain cytokines is still required to push the T cell into active proliferation and differentiation. Once the T-cell is pushed from resting G0 this activated cell no longer requires co-stimulation to react with the target antigen, with binding strength now dependent upon up-regulation of accessory binding molecules such as CD2 and LFA-1, resulting in cytokine production. Several co-stimulatory molecules have been discovered and their use in enhancing tumour immunogenicity investigated. The co-stimulatory molecules B7-1(CD80) and B7-2(CD-86) are involved in a pathway to both positively and negatively regulate the T cell response. Both bind to the T-cell surface receptors CTLA-4(CD152) and CD28 with differing affinities. CD28/B7 binding results in T-cell proliferation and recent studies suggest CTLA-4 has an essential inhibitory function in maintaining the homeostasis of the pathway. This antagonistic relationship has implications in allograft rejection where inhibition of B7-2 binding to CTLA-4 achieved long term graft survival as well as tumour immunotherapy, where providing B7-CD28 costimulation results in the activation of T-cells to otherwise non immunogenic epitopes (Judge et al., 1999).

Despite genetic modification of tumour cells to express these co-stimulatory molecules, many tumours are still not rejected and recent studies focus upon the co-administration of both these co-stimulatory molecules with immunostimulatory cytokines. Hurwitz et al. (1998), used a combination of B7, interferon-gamma (IFN-γ) and granulocyte-macrophage colony stimulating factor (GM-CSF) expression *in vivo*, in a mouse mammary carcinoma (SM10) model. GM-CSF gave no induction but B7/IFN-gamma co-expression resulted in rejection of the tumour by CD8+ T-cell activation and protection from rechallenge with SM10 tumours as well as from a second syngeneic mammary tumour.

The necessity to express combinations of immuno-modulators, including interleukins such as IL-12, within cancer cells to achieve an additive and adequate immune response has been tested by viral delivery. Vaccinia virus expressing B7-1/murine IL-12 (subunits p35 and p40) were intratumourally injected into a colon carcinoma mouse model. Metastasis was measured by the development of lung tumour nodules which was reduced by 95% with treatment. Survival duration was further extended by the exogenous administration of IL-12 (Carroll et al., 1998). More recently other co-stimulatory molecules, CD40 and CD70 have been compared to CD80 in their ability to induce T-cell activation. CD40-transfected MHC class II-negative P815 tumour cells (murine mastocytoma) became highly immunogenic and induced long-lasting, tumour-specific memory CTLs *in vivo*.

Immune enhancement of intracranial tumours is still in the early stages of research. Nakaichi et al. (1995) directly injected DNA from *Mycobacterium bovis* BCG into the lesion within a rat brain tumour model, with subsequent histology showing increased leukocyte infiltration. Komata et al. (1997) stably transfected U251 human glioma cells with B7-1 and used exogenous addition of IL12 to stimulate a mixed lymphocyte culture. The co-stimulatory molecule or cytokine alone was not effective, but in combination a CTL proliferation was achieved. Visse et al. (1999) investigated the effect of subcutaneous immunizations with glioma cell clones expressing either rat IFN-γ, rat IL-7, or rat B7-1, on the growth of pre-existing intracerebral brain tumour isografts in the rat. IFN-γ and IL7 achieved significant increase in survival but B7-1 expressing cells required immunisation with high

numbers of cells.

Tumor models in experimental neuro-oncology

Before the reviewed gene therapeutic strategies can be implemented for the treatment of human disease it is imperative to assess their efficacy and potential side effects in experimental animal models. In this section we will outline the most relevant animal models which can be used for testing gene therapy paradigms for both brain gliomas and pituitary adenomas.

Glioma models

Animal models of brain tumours have proved invaluable for evaluating the efficacy of potential new anticancer therapies and for the study of experimental neuro-oncology providing fundamental toxicity and statistical survival data. A valid model for glioma must fulfil several criteria if the therapeutic response is to closely mimic that of human brain tumours. The tumour should be composed of glial-derived neoplastic cells which are amenable to *in vitro* culture. Tumour growth rates should be predictable and reproducible maintaining glial like growth characteristics *in vivo*, such as neovascularisation, invasion, alteration of the blood brain barrier and lack of encapsulation. The tumour should not invade the epidural space or beyond the brain and within a syngeneic animal the tumour should be non or weakly immunogenic to allow adequate host survival time for therapy administration, and determination of therapeutic efficacy. Canine and feline models have been described but rodent models are most widely used and will therefore be the focus of this review.

Cell or tissue transplantation

The tumours induced by either carcinogen or virus have been maintained by cell culture or subcutaneous passage. Stereotactic delivery of the cells or tissue can be performed with accuracy into a precise region of the brain. Transplantation can be performed into a syngeneic host but as the brain shows partial immune privilege many models were generated by heterotransplantation into a non-syngeneic animal. Heterotransplantation of the cells or tissue can also be achieved into an immune compromised animal to produce a xenograft model.

The C6 glioma was induced in randomly bred Wistar rat progeny by the transplacental effects of N-nitrosourea (NNU). This tumour was cloned and is now available as a continuous cell line (ATCC No. CCL107). This cell line exhibits astrocytic morphology and the glial cell markers, glial fibrillary acidic protein (GFAP) and S100 protein. It has been used extensively *in vitro* and *in vivo* where the tumour has shown invasion into surrounding parenchymal tissue, neovascularity and necrosis. The major weakness of the C6 cell line is its immunogenicity, because of its generation in an outbred rat it has no syngeneic host therefore is highly immunogenic (Trojan et al., 1993).

The extensively used 9L gliosarcoma was induced in inbred CD Fischer rats using repeat injections of N-methylnitrosourea (NMNU). The tumour was cloned and initially exhibited glial characteristics, but with serial passage these characteristics changed to give this 9L gliosarcoma cell line the features of a sarcoma, which when transplanted lost the ability to invade the parenchyma. Its inability to invade the parenchyma made it ideal to study the effects of genes that modulate tumour angiogenesis. 9L gliosarcoma tumours expressing retrovirally transfected oncogenic factors such as *ras* were shown to be vascularised and capable of parenchymal invasion (Schwartz et al., 1991). Like the C6 cell line, there is not a syngeneic rat strain for the 9L gliosarcoma, therefore it is highly immunogenic and unsuitable for survival studies to assess the long term therapeutic effects of gene therapies. The T9 glioma model is an identical model to 9L as the clone was derived from the same tumour.

The BT_4A glioma was induced in BD IX rat progeny by exposing the parent to N-ethylnitrosourea (ENU). The BT_4A glioma cell line has low immunogenicity in a syngeneic host, in which 100% tumour take can be achieved to produce an invasive tumour expressing the glial marker S-100 protein (Mella et al., 1990).

The transplacental exposure of ENU to CD Fischer fetal rats resulted in the generation of the RG2/D74 glioma. Two identical clones were cultured from this tumour in two different laboratories, hence the clone was designated two names, RG2 and D74. This cell line produces a tumour in syngeneic Fischer rats which is invasive, giving uniform lethality and has proved impossible to immunize against. This lack of immunogenicity makes it an attractive choice to model the effects of anticancer drugs, tumour suppressor and immune activation genes (Tjuvajev et al., 1995). Using the same methodology the F98 glioma was also induced in CD Fischer rats. The F98 cell line derived from this tumour is only weakly immunogenic in the syngeneic host if at all, and based on its *in vivo* morphology has been designated as an anaplastic glioma with characteristics that closely resemble human glioblastoma and like RG2 is a good model for brain tumour gene therapy.

Another syngeneic rat model was produced by repeated injection of NMNU into an inbred Lewis rat giving rise to the CNS-1 glioma (Kruse et al., 1994). The CNS-1 cell line expresses both GFAP and S-100 and *in vivo* inoculation gives rise to an invasive tumour (Fig. 3). More studies are required to test the immunogenicity of this relatively new model. However, survival times within the syngeneic Lewis rat have been shown in our laboratory of up to 1 month with a tumour load of 5-10,000 cells implanted into the striatum (Dewey et al., 1999). Recently our laboratory has shown the CNS-1 cells to be a useful model for evaluating the efficacy of gene therapy treatment using the HSV1-TK/GCV

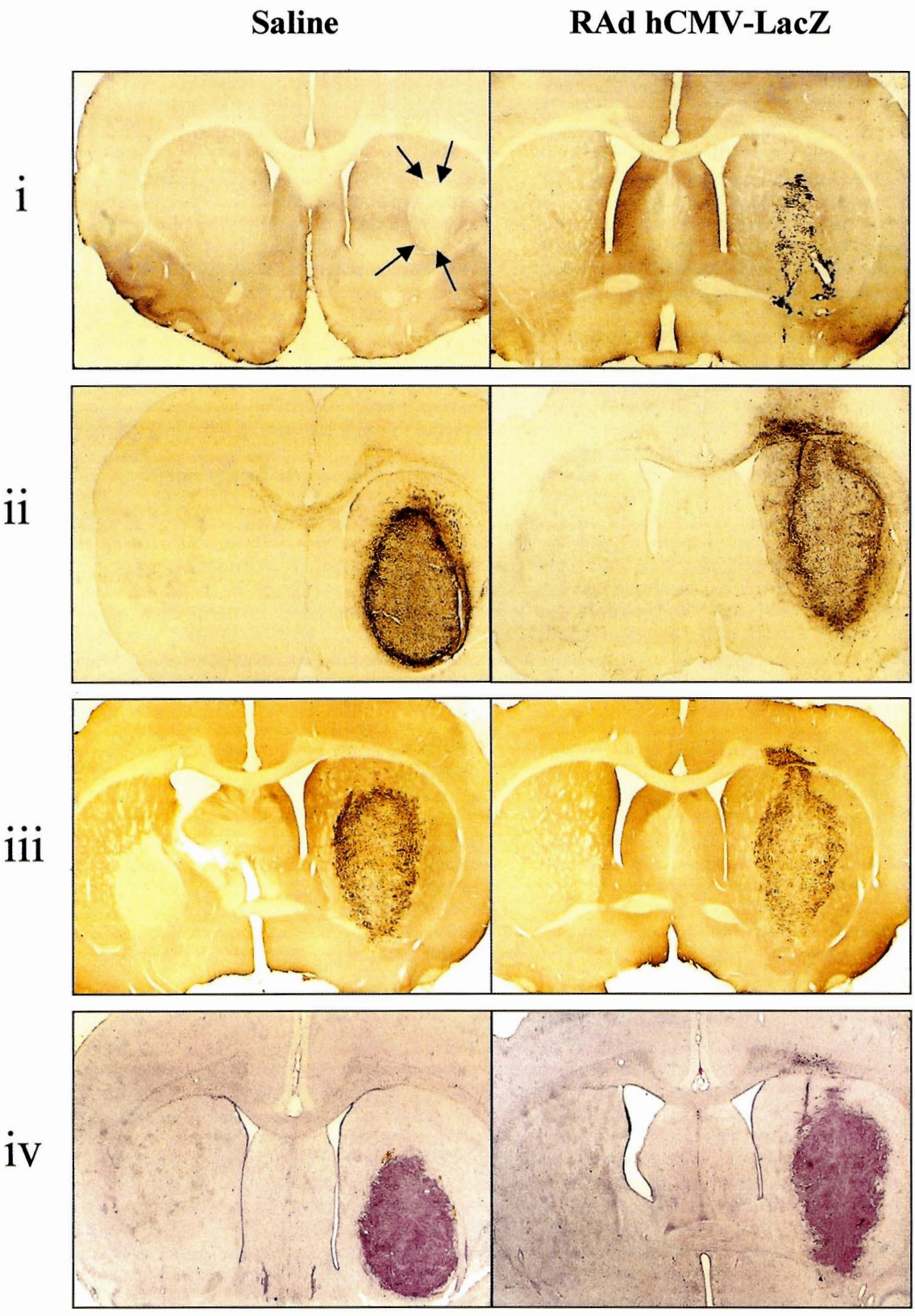


Fig. 3. An intracranial tumour was produced by implanting 5000 CNS-1 cells into the right striatum of male Lewis rats (Dewey et al., 1999). Either saline or an adenovirus encoding the LacZ gene under the control of the hCMV promoter (10^7 pfu) was injected 3 days later. Ten days later the rat was anaesthetised and fixed by cardiac perfusion. Serial vibratome sections (50 μm thick) were cut and processed for immuno-histochemistry to assess (i) ß-galactosidase expression, (ii) ED1 to identify monocytes, macrophages and microglia (iii) CD8 positive lymphocytes and (iv) Nissl, which stains all nuclei identifying the tissue damage. (Cowsill et al., 2000). Black arrows highlight the intracranial CNS1 tumour.

strategy. Long term survivors after HSV1-TK/GCV treatment showed widely distributed transgene expression of HSV1-TK throughout the brain parenchyma and chronic inflammation at the site of tumour implantation/viral delivery (Dewey et al., 1999; Cowsill et al., 2000).

The VM murine astrocytoma model was derived from a spontaneous murine astrocytoma from which the cell line 497-P(1) was isolated and characterised. This cell line is GFAP positive and highly tumourigenic. The resulting tumour is locally invasive and necrotising making this syngeneic model useful for gene therapy studies. However the 497-P(1) cells have been shown to be inherently resistant to the efficacious human anticancer agent procarbazine.

Heterologous transplantation of human brain tumour tissue or derived cell lines into an animal was greatly facilitated by the generation of the nude mouse, although previous implantation of human tissue into immune privileged sites had achieved some limited success. This mutant mouse is athymic therefore cannot mount an adaptive immune response. Characterisation of human tumour xenografts grown in this mouse has demonstrated that the tumours retain many of the morphological traits of the original biopsy. Subcutaneous human glioblastoma tissue has been successfully grown in this artificial environment but analysis has shown tumour heterogeneity, possibly due to differing selection of certain cell sub-populations (Jones et al., 1981).

Transgenic glioma models

The ability to insert foreign DNA, to introduce specific genes into the germ line of mammals has paved the way for the development of a new generation of transgenic glioma model. Serendipitously, it was found that if the large T-antigen gene of the simian monkey virus (SV40), under the regulation of its own promoter and enhancer sequences was placed in transgenic mice, choroid plexus tumours were reproducibly generated as this T antigen is capable of transforming cells (Brinster et al., 1984). Moreover, if a tissue specific transcriptional control element was included cell transformation and hence tumour formation arose in a tissue specific manner. Using the SV40 promoter and the moloney murine sarcoma virus enhancer to control large T-antigen expression, transgenic mice were created which developed midline brain neoplasms at 18 weeks comparable to primitive human medulloblastoma. These tumours displayed photoreceptor like features presenting S-100 and rhodopsin. Transgenic model generation is still in its infancy and is not a trivial undertaking. Even in the most well planned experiments the success rate is poor with random integration into silent genomic areas or resulting in unexpected tissue expression, however some exciting glioma models are appearing which use glial directed expression of oncogenes. Expression of the *neu* oncogene, originally isolated from a rat glioblastoma, under the control of the myelin basic protein (MBP) transcription regulatory element produced high grade, necrotic glioma, presenting oligodendrocytic antigens (Hayes et al., 1992). The transforming gene of the Rous sarcoma virus (RSV), v-*src*, under the control of the glial fibrillary acidic protein (GFAP) gene regulatory elements found to be expressed in astrocytes, generated a transgenic model of astrocytoma. This model allows astrocytoma development to be studied as the tumours progressed from small foci expressing vascular endothelial growth factor (VEGF) to a tumour with the morphology and growth characteristics of glioblastoma multiforme (Weissenberger et al., 1997). The transgenic glioma models to date do not allow screening of new therapeutic agents. Levels of transgene expression are variable even between siblings, resulting in tumour progression at differing sites and unreproducible survival times. To achieve greater control of oncogenesis, efforts are being directed towards harnessing inducible promoters.

Human glioma cell lines in culture

In vitro studies using cultured glioma cell lines derived from surgical biopsies are an effective tool for assessing gene therapy treatment strategies, allowing the mechanisms involved in determining treatment susceptibility and those involved in acquiring resistance to treatment to be studied. Further benefits also include assaying cultured cells from patients in order to provide individualized treatment. When considering a gene therapy treatment strategy, and testing it in cultured cell lines, it is important to take into account the amount of cells to be used in the experiment, the duration and regime of treatment, and how to determine the successful end point (Darling et al., 1997). It is also necessary to keep in mind the morphology of the tumour. Treatment effectiveness could also be altered due to the heterogeneity of the cells within the tumour (Bradford et al., 1997). The passage number of the cultured cell lines is another factor to consider. Using cells directly from the tumours ('primary culture') will provide cultures that are the most representative of the tumour, since they have not gone through any selection process. These primary cultures should be used for testing a strategy that has been through *in-vitro* testing on short term cultures. Approximately 90% of surgical biopsies of human glioblastomas can be established to short term cultures. However, cultures are sometimes unstable, slow growing and have a limited life span (Darling et al., 1997) and continual passaging has been shown to alter the DNA profiles in these cells and therefore could affect treatment susceptibility. The majority of gene therapy studies using cultured human glioma cell lines involve the use of the thymidine kinase/ganciclovir system but other approaches include the delivery of p53, the pro-apoptotic molecules Fas ligand and FADD gene therapy . The ability of adenoviruses to infect human glioma cells based on the coxsackie/adenovirus receptor (CAR) and

integrins expressed on their surface has also been studied. Levels of adenoviral infection varied according to the level of expression of CAR, but not the integrins. Our laboratory is studying adenovirus delivery of a variety of conditional cytotoxic or directly cytotoxic genes to short term cultures of human malignant gliomas, including HSV1-TK and FasL (Maleniak et al., 1999). Both HSV1-TK/GCV (Fig. 4) and over expression of FasL induced human glioma cell death *in vitro*.

Animal models for pituitary adenomas

Pituitary adenomas account for ~16% of intracranial tumours. If gene therapy is to be a useful tool for the treatment of these adenomas, good animal models of the disease states have to be developed to test the efficacy and safety of gene delivery into the pituitary gland (Castro, 1999; Castro et al., 1999). In this section we discuss some of the current models that may be of use to study potential gene therapy strategies.

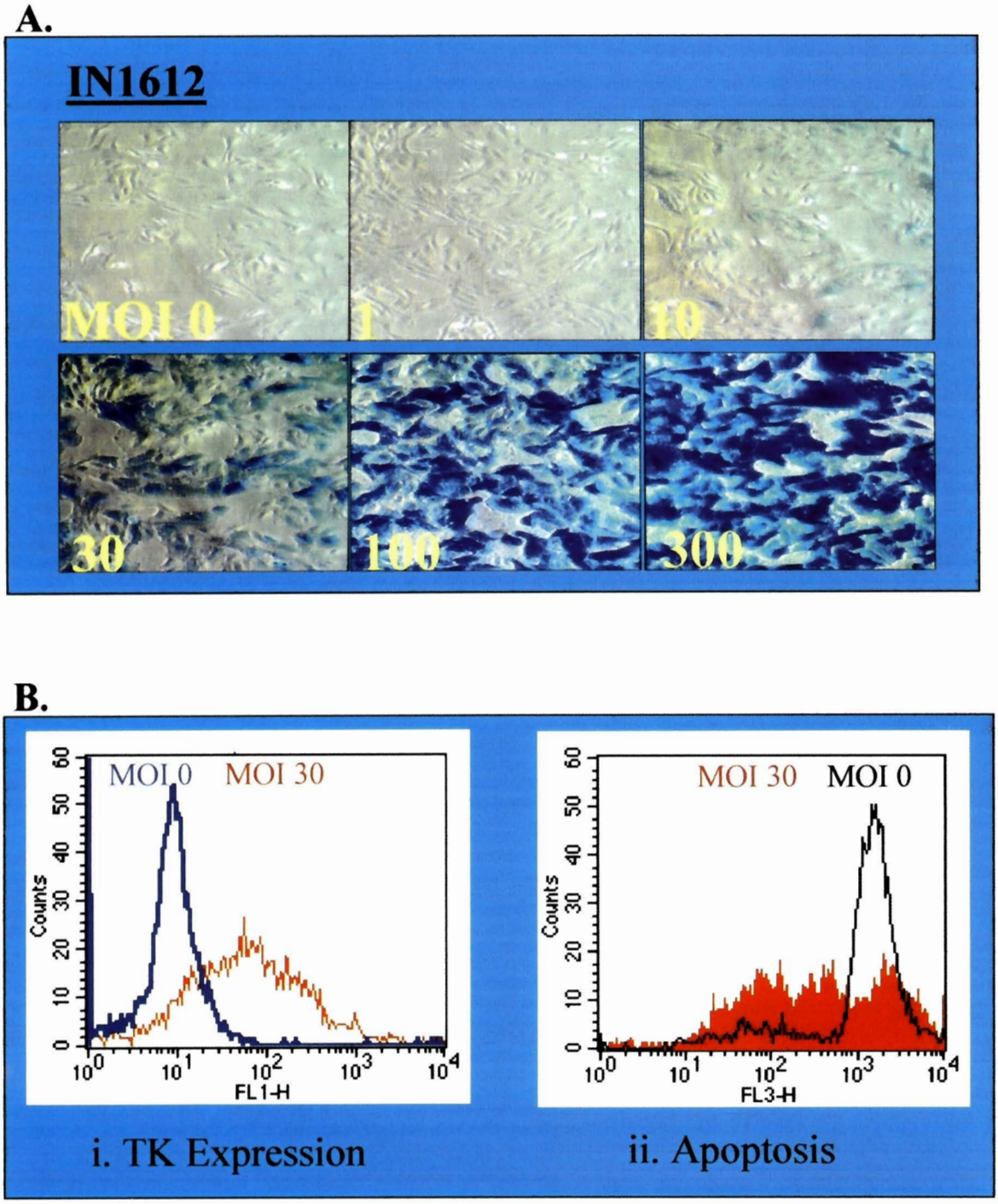


Fig. 4. A. Infection of the human glioma tumour cell line, IN1612, with an adenovirus encoding the LacZ gene under the control of the hCMV promoter. The cells were infected at different multiplicities of infection (MOI) with the virus and then analysed by histochemical staining for ß-galactosidase activity (Castro et al., 1997). **B.** Human glioma tumour cell line, IN1612, was infected at an MOI of 30 with an adenovirus encoding for HSV1-TK under the control of the hCMV promoter. (i) Analysis of the infected cells by flow cytometry (Morelli et al., 1999) using an HSV1-TK specific antibody showed that the cells were expressing HSV1-TK. (ii) Ganciclovir was administered 3 days post infection and the cytotoxic effect measured by analysis of apoptosis using propidium iodide and flow cytometry (Morelli et al., 1999).

Prolactinomas

There are three main types of animal models used for the investigation of prolactin-secreting adenomas, i.e., hormonally induced, implantation of tumour cells, and hereditary prolactinomas. It has been known for a long time that oestrogen stimulates lactotrophic growth in the pituitary and many groups have used this to create an animal model to study prolactinomas. The hyperplasia is induced using a variety of oestrogenic compounds including subcutaneous diethylstilbestrol (DES) implants, subcutaneous oestrogen (E1) implants and subcutaneous implants or injections of oestradiol-17ß. The problem with this treatment is that the removal of the stimulus results in the regression of the hyperplasia (Treip, 1983). Also if the efficacy of a treatment is being studied in this model, the continuous stimulus of the hyperplasia by the presence of oestrogen could result in impairment of the experimental therapy. In our laboratory, we are using an oestrogen/sulpiride induced lactotroph hyperplasia model. We used trans-auricular injection of an adenovirus vector expressing HSV1-TK followed by GCV treatment to test the efficacy of this modality for use in the treatment of prolactinomas (Windeatt et al., 2000). Most recently we have developed targeted recombinant adenovirus vectors with HSV1-TK transcription driven by the human prolactin promoter, thereby restricting expression of this conditionally cytotoxic gene to lactotrophic and mammosommato-trophic cell types in the anterior pituitary (Fig. 5) (Windeatt et al., 1999, 2000). Pituitary specific expression of both ß-galactosidase and HSV1-TK, driven by the human prolactin promoter has also been achieved both *in vitro* and *in vivo* (Castro et al., 1999; Windeatt et al., 1999, 2000). Cell-type specificity conferred by the human prolactin promoter was also combined with the tetracycline inducible system generating a cell-type specific and regulatable system for transgene expression within lactotrophic pituitary cells (Fig. 6) (Smith-Arica et al., 1999). This cell-type specific and regulatable system driving the expression of therapeutic transgenes will be valuable for the development of strategies which could be translated to the clinical situation (Castro, 1999; Castro et al., 1999).

Two of the most common transplantable tumour cell lines have been derived from the prolactin/ACTH secreting 7315a cell line. Implantation of the MMQ cell line into Buffalo rats results in increased prolactin levels, with increased spleen weight and visible white pulp hyperplasia. The second cell line, 235-1 cell has been shown to secrete prolactin alone and was able to form tumours when implanted into Buffalo rats and nude athymic mice. Trouillas et al. (1990) took propagated spontaneous prolactinoma tissue designated SMtT-W from Wistar/Furth rats and after serial passaging transplanted the cells under the skin or the kidney capsule. The kidney capsule gave 100% transplantation success whereas under the skin only 20% were successful. The serum prolactin levels increased with growth hormone and ACTH levels remained normal.

A model of prolactinomas has also been generated by crossing Okamoto spontaneously hypertensive rats (SHR) with the Koletsky rat. The new substrain (SHR/N:Mcc-cp) developed cardiomyopathy and congestive heart failure. However, in the examination of the pituitaries from 53 animals, 70% of them had developed prolactinomas between 14-18 months of age. 80% of the males and 56.5% of the females developed prolactinomas but due to congestive heart failure and cardiomyopathy the rats die at the same time as the formation of the pituitary tumours.

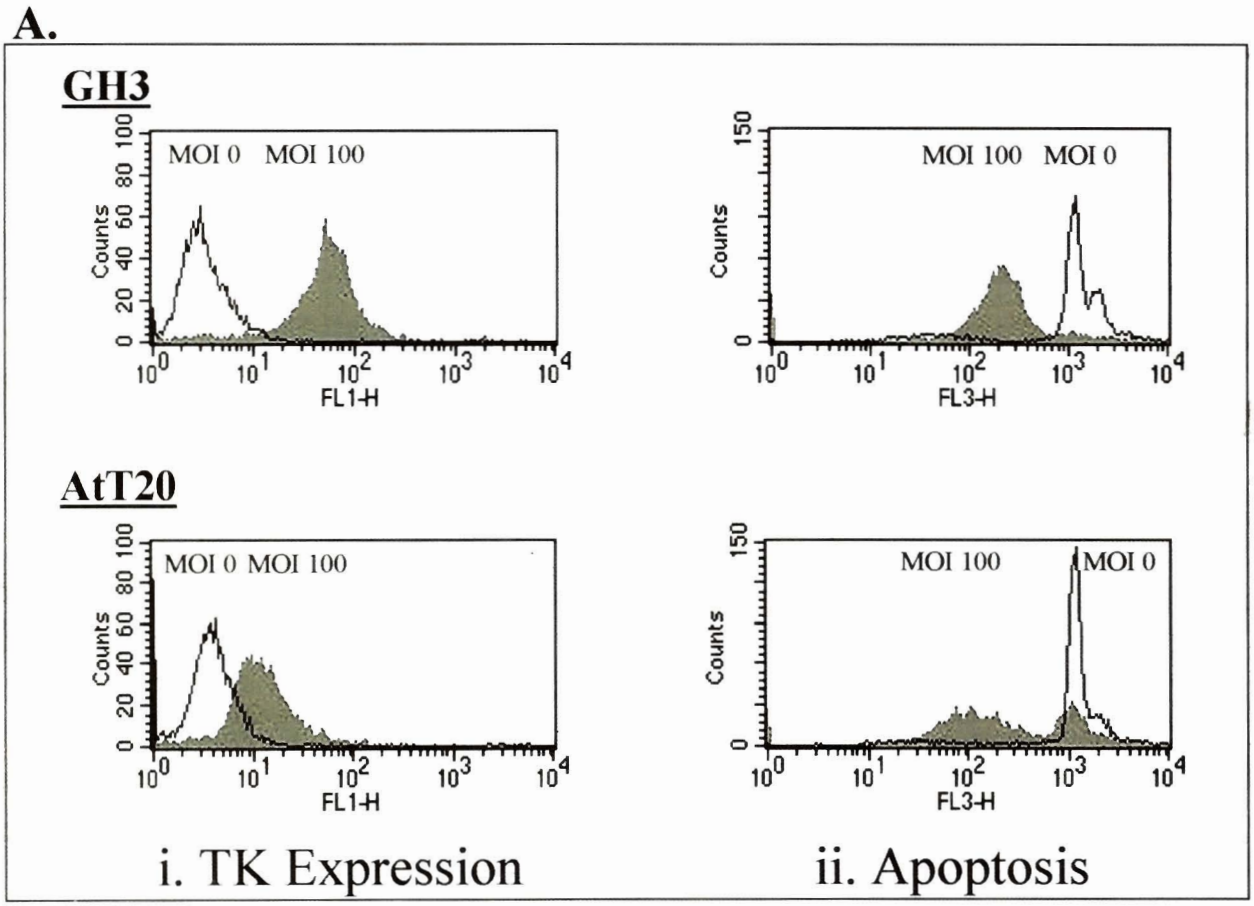
Prolactin-secreting pituitary tumours are also frequently observed in ageing female and male Sprague Dawley and Wistar rats with an incidence as high as 80% in Sprague-Dawley female rats. These spontaneous prolactinomas develop at 26-28 months of age and are recognised by an increase in prolactin levels, 13 times higher than the levels in young rats.

Growth hormone tumours

Transgenic somatotroph tumours have been developed in mice. The transgenic mice have the large SV40 T antigen under the control of the bovine arginine vasopressin promoter. There were also tumour occurrences in the intermediate lobe in a minority of cases that were immunoreactive for ACTH and POMC mRNA. Other models have used transplantable cell lines that secrete growth hormone including GC, MtT-W-15 and GH3 cells. Recently, an adenovirus expressing the HSV1-TK under the control of the GH promoter has also been successfully used in a transplantable tumour model using GH3 cells in nude mice (Lee et al., 1999). Tissue from human growth hormone secreting adenomas has also been subcutaneously transplanted into athymic mice and was found to be a reliable model.

Cushing's disease

For this syndrome, two models have been generated from transgenic mice. The first was generated using the polyoma early region promoter linked to a cDNA encoding polyoma large T antigen (PyLT). Normal pituitary morphology was observed at 4 months of age with microadenoma formation noted at 9 months. At 13-16 months of age the mice had adenomas that were 5mm in size. Subcutaneous transplants of these transgenic pituitaries to non-transgenic immunocompetent mice resulted in the development of tumours with morphology and ACTH immunoreactivity similar to the primary tumour. The second transgenic mouse model expresses leukemia inhibitory factor (LIF) driven by a pituitary glycoprotein hormone alpha-subunit (alpha GSU) promoter. Pituitary glands showed corticotroph, somatotroph, and gonadotroph hyperplasia, and multiple Rathke-like cysts lined by ciliated cells. Mice showed dwarfism, low IGF-1, hypogonadism, low FSH, truncal



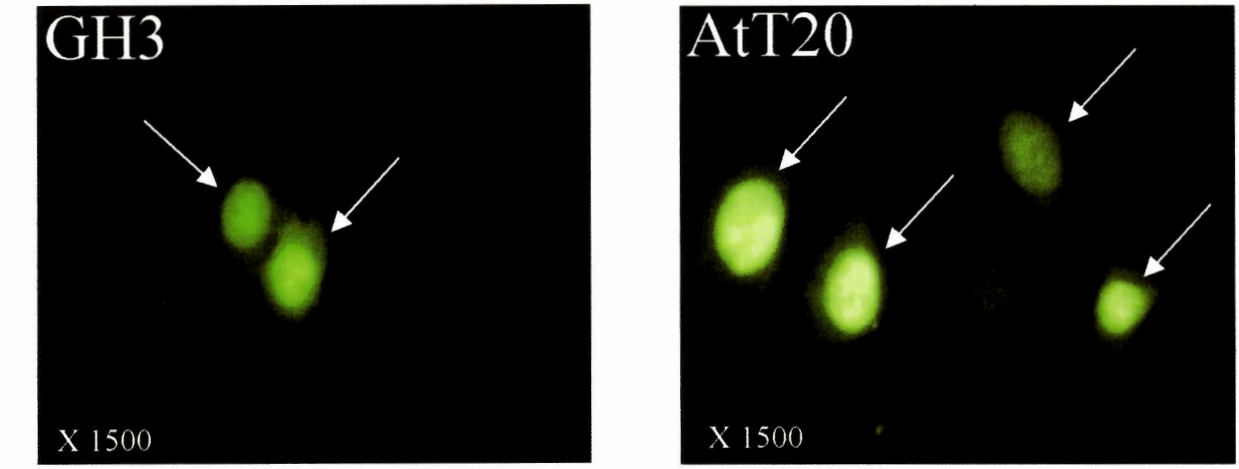
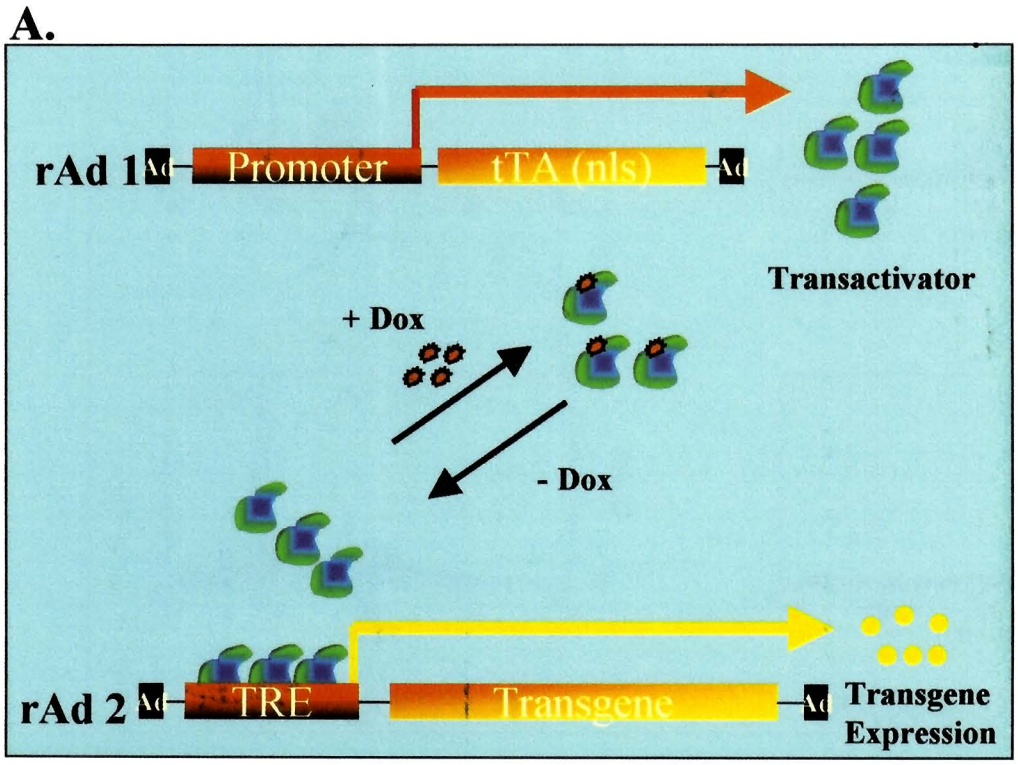


Fig. 5. A. Pituitary tumour cell lines, GH3 and AtT20, were infected with an adenovirus expressing HSV1-TK under the control of the hCMV promoter at an MOI of 100. (i) After 48hrs the percentage of cells expressing the HSV1-TK transgene was determined using flow cytometry. (ii) The cells were then exposed to the prodrug, ganciclovir and the cytotoxic effect measured by analysing for apoptosis using propidium iodide and flow cytometry (Windeatt et al., 2000). **B.** Expression of HSV1-TK from GH3 and AtT20 cells infected with an adenovirus encoding for HSV1-TK, under the control of the hCMV promoter, at an MOI of 30 (Windeatt et al., 2000). Forty eight hours post infection the cells were fixed and immunostained using an antibody specific for HSV1-TK expression. The white arrows indicate HSV1-TK expressing cells.

obesity, and Cushingoid features like thin skin and high cortisol levels. Recently, Westphal et al. (1999) have generated transgenic knockout mice that contain a null mutation in the neuroendocrine protein 7B2. These mice showed elevated ACTH and corticosterone levels, with adrenocortical expansion and proceeded to die of severe Cushing's disease before they were 9 weeks of age. For the study of additional intermediate lobe tumours a



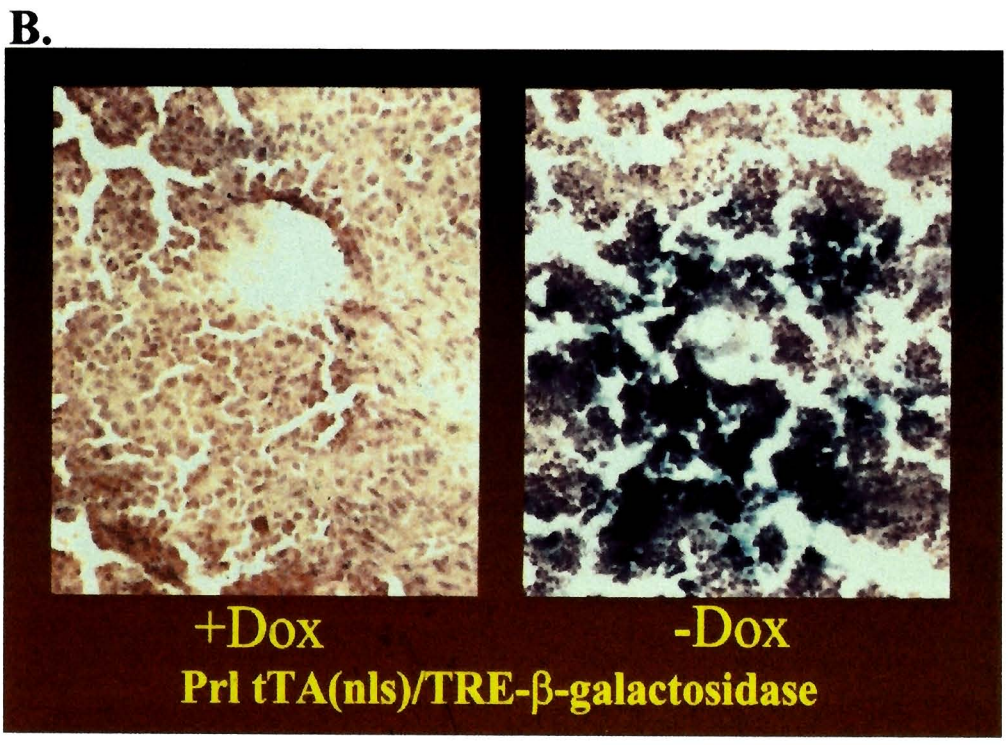


Fig. 6. A. Diagram of the tetracycline system encoded within a dual adenovirus vector system. In virus 1 the insert within the E1 region contains a promoter element (constitutive or cell-type specific) driving the expression of the transactivator with a nuclear localisation signal (tTA[nls]). In virus 2 the insert contains the tetracycline response element, (7 operator sequences and TATA box [TRE]), driving the expression of the transgene. In the absence of tetracycline (Tc), or its analogues doxycycline (Dox), the tTA(nls) binds to the TRE and initiates transcription of the transgene. However in the presence of Dox, the tTA(nls) does not bind to the TRE preventing transgene expression (Gossen and Bujard, 1992). **B.** Combined lactotroph cell-type specific and tetracycline regulatable expression of the reporter gene ß-galactosidase, in a transplantable tumour model. MMQ cells were implanted in Buffalo rats and infected with the two rAd vector system. The human prolactin promoter (Prl) drives expression of ß-galactosidase only in the absence of doxycycline (Smith-Arica et al., 1999).

heterozygous immunocompetent Rb+/- mouse has been used to study spontaneously arising melanotroph tumours. These tumours were treated with an adenovirus containing the Rb cDNA that inhibited tumour growth, decreased tumour cell proliferation and prolonged the animals life span (Riley et al., 1996).

Null cell adenoma

The model for these tumours was developed using transgenic mice which were bred carrying the temperature sensitive mutant of SV40 T antigen under the control of the human FSHβ regulatory elements (Kumar et al., 1998). They developed diffuse gonadotroph hyperplasia that progressed to nodular adenomas with decreasing immunoreactivity for FSHβ and LHβ. The ultrastructural characteristics of these tumours were similar to that of human null cell adenomas.

Conclusions

While significant hurdles need to be overcome before successful clinical implementation of gene therapy strategies for intracranial tumours, the results of the pre-clinical and clinical trials now in progress will yield information on areas in need of future development. Specific tissue targeting of the gene therapy delivery systems remains an area of intense efforts. Also, once the gene has been transferred, the duration of transgene expression and the potential immune responses elicited against the vector and/or transgene need to be further studied and this will lead to improvements in both the vector systems and therapeutic strategies used. Finally, the delivery vectors also have to be improved to minimise their potential to cause adverse side effects (Dewey et al., 1999; Lowenstein et al., 1999; Cowsill et al., 2000).

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