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TRANSCRIPTIONAL TARGETING OF SUICIDE GENES IN CANCER GENE THERAPY

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment / of the requirements for the degree of Doctor of Philosophy

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À mes très chers Parents,

pour leur Amour, leurs Encouragements

et Sacrifices Infinis

Abstract

The use of tissue or tumor selective promoters in targeted gene therapy for cancer depends on strong and selective activity. Hexokinase type II (HK II) catalyzes the first committed step of glycolysis and is overexpressed in tumors, where it is no longer responsive to normal physiological inhibitors, e.g. glucagon. I show in a reporter gene assay activation of HK II in non-small cell lung carcinomas NCI-H661 and NCI-H460 at 61% and 40% of the activation observed with a constitutive promoter respectively, while it is only 0.9% in a number of different primary normal human bronchial epithelial cell lines (NHBEC). Similar results were observed in a variety of normal and tumor cells. Moreover, treatment of the transfectants with glucagon did not inhibit promoter/ activation in the transformed H661 cells, while endogenous HK II in NHBEC is suppressed by glucagon. H460 and H661 cells infected with a recombinant adenovirus carrying a HK II/LacZ expression cassette, Ad HexLacZ, demonstrated β-galactosidase activity, which correlated with the level of HK II promoter activation in these cells. Under similar conditions, no enzyme activity was observed in NHBEC. Cells were then infected with AdHexTk and treated with GCV. Our results demonstrate selectivity in toxicity with a 10-100 fold increase in IC_{50} between lung cancer cells and NHBEC. There was also a 100-fold increase in IC_{50} in normal human mammary epithelial cells (NHMEC) relative to breast carcinoma cells MCF-7. This represents a novel use of the hexokinase type II as a selective promoter in cancer gene therapy. Other factors important in suicide gene therapy were explored. Pharmacological modulation of the bystander effect using 8-bromo-cAMP, observed in HSVTk/GCV suicide killing, was demonstrated to enhance killing efficacy by 50% when a small proportion of a target population was

gene modified. The phenomenon of cellular resistance to HSVTk/GCV was examined and in a novel finding, we demonstrated dramatic interference from adenoviral proteins in cytotoxicity of this combination. Finally, based on indirect evidence suggesting ifosfamide activation by CYP3A4, I evaluated this combination as a potential new prodrug/suicide gene system. It was found to be inefficient at prodrug activation. Furthermore, co-expression of a reductase is not required, as has been suggested for CYP2B1, since reductase expression is induced by CYP3A4.

Résumé

L'utilisation des promoteurs spécifiques tissulaires ou tumoraux en thérapie génique ciblée pour le cancer dépend fortement de l'identification de promoteurs à la fois puissants et spécifiques. L'hexokinase de type II (HK II) catalyse la première réaction de la glycolyse. Cette enzyme est surexprimée dans les tumeurs et ne répond plus aux inhibiteurs physiologiques de la glycolyse, tel que le glucagon. Nous avons démontré, par un test d'activation de la transcription basé sur l'usage de gènes reporters, l'activation de HK II dans des cellules de cancer pulmonaire de type NSCLC, les NCI-H661 et NCI-H460. Cette activité était de 61% et 40%, respectivement de celle démontrée par un promoteur viral constitutif. Par contre, dans des cellules pulmonaires épithéliales/ humaines normales, les NHBEC, l'activation de HK II était de 0.9% seulement. Des résultats semblables ont été obtenus dans des cellules normales ou tumorales d'origines diverses. De plus, des cellules NCI-H661 transfectées avec le vecteur contenant le promoteur HK II couplé à un gène reporter et traitées avec du glucagon ne démontrent pas d'inhibition dans l'expression du gène reporter, alors que l'expression de l'hexokinase endogène dans les cellules NHBEC est réduite en présence de glucagon. L'infection des cellules H661 et H460 par un adénovirus recombinant, le AdHexLacZ, codant le gène de la β-galactosidase sous le contrôle du promoteur de HK II, démontre une activité de la β-galactosidase correspondant au profil d'activation du promoteur HK II tel que déterminé auparavant dans les tests de gène reporter. Dans les mêmes conditions d'infections, aucune activité de l'enzyme n'a été détectée dans les cellules NHBEC. Suite à une infection par un adénovirus recombinant, le AdHexTk, codant la thymidine kinase sous le contrôle du promoteur HK II, les cellules cancéreuses traitées au

gancyclovir étaient de 10 à 100 fois plus sensibles que des cellules normales. Ce résultat a été validé dans des cellules tumorales d'origines diverses. Cette stratégie constitue un usage innovateur du promoteur de HK II comme un outil de sélectivité en thérapie génique du cancer.

D'autres aspects importants de cette stratégie d'activation de gènes suicides ont été étudiés. Notamment, la manipulation pharmacologique de l'effet 'bystander' observé dans les populations de cellules traitées avec une combinaison de thymidine kinase et de gancyclovir a été testée avec le 8-bromo-cAMP. Cette intervention a augmenté de 50% le taux de toxicité observé chez des populations de cellules dont une très petite partie a été modifiée génétiquement. De plus, le phénomène de résistance au traitement de thymidine/ kinase et gancyclovir a été observé. Nos résultats suggèrent qu'une interférence des protéines adénovirales, présentes lors de l'usage des vecteurs adénoviraux pour le transfert de matériel génétique, pourrait en être la cause. Finalement, nous avons caractérisé la combinaison d'une enzyme du foie, la CYP3A4 et d'un pro-médicament, l'ifosfamide, pour un usage éventuel dans la thérapie génique du cancer. Nos résultats montrent que le transfert de la CYP3A4 dans des cellules cancéreuses n'est pas suffisant pour une activation significative de l'ifosfamide. D'autre part, la co-expression de la NADPH reductase n'est pas nécessaire pour le fonctionnement de la CYP3A4, comme a été le cas pour la CYP2B1, vu que l'expression de la réductase endogène est induite dans les cellules exprimant la CYP3A4.

Acknowledgments

I would like to express my deepest gratitude to my supervisor Dr. Gerald Batist. He has tremendous vision and enthusiasm that proved to be invaluable during the course of my Ph.D. training. His guidance and support, along with the time he invested in my development made this a truly positive experience for me.

I would also like to thank Drs. Jacques Galipeau and Moulay Alaoui-Jamali for constructive discussions that contributed to the development of the project.

Developing adenoviral vectors turned out to be a real challenge in our hands. Working with Helen Chan on this was a real pleasure. I would like to thank her for her outstanding help in this project, as well as for her friendship and support throughout. Several people from my lab also contributed friendly advice and help in various ways and I am thankful to them. These are Lily Yen, Shirley You, Xiang Chen, Joe Caruso, Jesse Paterson, George Carystinos, Josee Bergeron, and Marie Norbert. Many thanks to John Cho for a very special friendship throughout and hopefully beyond our days as bench neighbors.

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I would also like to thank researchers who provided very useful research materials. The hexokinase II promoter was a gift from Dr. Pedersen (Johns Hopkins University), the P450 reductase plasmids are a gift from Dr. David Waxman (Boston University), the CYP3A4 plasmid from Dr. Frank Gonzales (NIH), the Muc-1 promoter from Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund), the AdMucTk virus from Dr. Christina Addison (Dr. Frank Graham's lab, McMaster University), the AdRSVTk virus from Dr. Savio Woo (Baylor College of Medicine), the AdCMVLacZ form Dr. Bernard

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Last, but not least, I am immensely grateful for the Lady Davis Institute, for providing a great research environment and for being the set to the development of a life long relationship with a very special person to me, Pierre Beauparlant, whose support is far more extensive than words can ever say.

Preface

In accordance with the Guidelines for Thesis Preparation, the candidate has chosen to present results of her research in the traditional format. A general introduction is presented in chapter I. The materials and methods used in this thesis are presented in chapter II. The results are described in chapters III to V. They appear in part in the following articles, in that order:

1- Katabi,M.M., Chan,H.L.B., Karp,S.E., and Batist,G. 1999. Hexokinase Type II: A Novel Tumor-Specific Promoter for Gene-Targeted Therapy Differentially Expressed and Regulated in Human Cancer Cells. Hum Gene Ther 10:155-164.

The work presented in section 2 of chapter IV was based on an active collaboration/ project shared by another student in the lab. Therefore the results presented in figures 12, 13 and 15 are those of George Carystinos, and the results presented in figure 14 were generated by both.

2-Carystinos,G.D., Katabi,M.M., Laird,D.W., Galipeau,J., Chan,H.L.B., Alaoui-Jamali,M.A., and Batist,G. 1999. Cyclic-AMP Induction of Gap Junctional Intercellular Communication Increases Bystander Effect in Suicide Gene Therapy. Clin Can Res 5:61-68.

As an extension of this collaboration, results presented in figure 19 were also those of George Carystinos.

3- Katabi,M.M., Carystinos,G.D., Chan,H.L.B., Alaoui-Jamali,M., and Batist,G. 1999. Resistance to HSV/Tk toxicity is dependant on delivery vector context in different cell lines. Human Gene Therapy (submitted)

Results presented in section 5 of chapter V were obtained in the lab of Dr.David Waxman (Boston University) who is a collaborator on this project. This work will be submitted for publication in the near future. Finally, results are discussed in chapter VI and references listed in chapter VII.

The candidate also wishes to acknowledge excellent technical assistance from Helen Chan with adenoviral work, Annie Woo with tumor samples preparation, Dr.Taiqi Wang with in vivo work, and Dr. Naciba Benlimame with immunofluorescence studies.

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CHAPTER I: GENERAL INTRODUCTION

1. Gene Therapy: Promises and Results

Gene therapy was first publicly discussed in the early seventies, when its original goal was to correct hereditary human genetic disease, by introducing a normal copy of the deficient gene into the cell's genome (1). From seemingly simple beginnings, it evolved into a discipline encompassing strategies to treat a wide variety of diseases. Different targets have been explored, bringing the field very far away from corrective gene replacement. In fact, genes are used in many instances to augment the function of a cell, as a way to reverse a pathological state.

1.1 Ethical considerations

Somatic cell gene therapy for the purpose of treating a serious disease is an ethical therapeutic option. However, considerable controversy exits on germline gene therapy. From a scientific point of view, there hasn't been enough data collected on the long-term effects of modifying cells with a normal copy of a gene to fully assess the safety of such an approach. Until gene transfer becomes an exact science where homologous recombination between the defective copy of a gene and a normal one occurs during gene transfer, one cannot say that random integration in the genome of a normal copy of a gene is harmless in the long run. From a philosophical point of view and the perspective of informed consent, it isn't obvious that we have the right to administer a permanent genetic change to somebody that isn't even born or conceived yet. Other social implications of gene transfer that cause concern relate to the theoretically possible use of this procedure to enhance desired characteristics, such as intelligence or cosmetic features and not for therapeutic purposes.

1.2 Target diseases and progress to date

The first experiment in human gene therapy began in 1990 with the aim of treating adenosine deaminase (ADA) deficiency. Initial trial results from two patients were published in 1995 (2) with the conclusion that the therapy was safe and beneficial to the recipients. Another study also conducted in a limited number of patients showed long term expression of ADA in blood (3). A lot of progress has been made since then. At the beginning of 1997 (4), more than 2100 patients had received gene therapy, compared to 392 in December 1994. In terms of number of patients with various diseases, malignancies rank first (68%), followed by AIDS (18%), and cystic fibrosis (8%). Among patients being treated for malignancies, melanomas rank first, followed by metastatic cancers and glioblastomas. Among the five most used genes, there are markers such as the neomycin resistance gene, β-gal, or therapeutic genes such as Env/Rev, HLA-B7, IL-2, and TK. There has been a marked decline in the use of ex-vivo gene transfer strategies from 1994 to 1996 where 39% of all patients are now on such protocols as opposed to 52% in 1994. This decline will help make gene therapy more accessible to a wider number of patients and at a lesser cost. The sections below describe some of the approaches used in gene therapy for various disorders. This description provides a global perspective of the scope of this discipline. Cancer gene therapy strategies are covered in more details in section 2.2.

1.2.1 Acquired immunodeficiency syndrome

Strategies for the treatment of AIDS include the use of genetically modified T cells to improve the immune response in HIV positive patients or to block HIV replication. Anti-HIV ribozymes have been targeted to highly conserved sequences of the HIV genome (5)

and transferred to autologous CD8 depleted mononuclear cells. Expression of specific antisense RNAs targeting the 5' leader region of HIV-1 mRNA transcripts has been used to block HIV-1 replication (6). Expression of RevM10 or Gag antisense RNA in cells of the myeloid lineage caused a marked decrease in HIV-1 replication. Ex-vivo modification of CD4+ T lymphocytes with vectors encoding transdominant Rev (7) and/or antisense TAR and re-infusion in patients was successful. One clinical trial with this approach has resulted in increases in CD4 counts that last up to 28 weeks (8). Moreover, combination approaches where powerful delivery vectors combine several of these approaches such as ribozymes, RNA decoys or mutant transdominant protein have also proven successful at inhibiting infection by different classes of HIV-1 (9).

1.2.2 Inherited single gene defects

Several inherited single gene defects that cause debilitating diseases, including several inborn errors of metabolism, have been the targets of gene therapy approaches. Some examples are cystic fibrosis, muscular dystrophies, Gaucher's disease, hypercholesterolemia, phenylketonuria, Hurler and Hunter syndromes or mucopolysaccharidosis type I and II (10), respectively, sickle cell anemia (11), hemophilia type B (12; 13) and adenosine deaminase deficiency, which results in severe combined immunodeficiency (2; 3). Cystic fibrosis treatments are the most documented among these approaches, where the cystic fibrosis transmembrane regulator (CFTR) gene is delivered by adenovirus infection to the airway epithelium (14). This strategy hasn't been successful yet to treat patients (15), due to the inefficacy of adenoviruses to attach to and internalize at the apical plasma membrane of differentiated airway epithelial cells (16), and to the strong immune response first generation vectors have generated upon

administration to patients. Correction of chloride ion transport defect as reflected by transepithelial electrical potential difference was not measurable. Other methods of delivery using lipid-based vectors are now being explored (17). In Duchenne muscular dystrophy (DMD) patients, healthy myoblast transfer was proposed to replace defective dystrophin. This cell-based therapy wasn't successful due to the low survival observed in the transplanted myoblasts and the ensuing low levels of normal dystrophin levels (18). Gene transfer for the treatment of DMD was made possible with the observation that a dystrophin minigene was therapeutically active in Duchenne patients. The reduced gene is compatible with the size requirements of viral delivery vectors. The strategy was tested using adenovirus vectors and retroviruses but was more successful with the latter. Adenoviruses caused a strong immune response, when injected directly in muscles or when used in an ex-vivo approach with genetically modified myoblasts. As a result, only short-term gene expression was achieved and hence no therapeutic effect (19; 20). Retroviruses were used more efficiently through the implantation of producer cell lines in an mdx mouse model. Healthy muscle fiber formation and active skeletal muscle remodeling (21) were documented. Gaucher's disease treatment strategies have been focused mainly on the ex-vivo transduction of hematopoietic stem cells with the glucocerebrosidase gene (22; 23). Treatments for phenylketonuria, where liver phenylalanine hydroxylase is absent have also been attempted using gene therapy. This deficiency causes the accumulation of phenylalanine in different organs and if left untreated, can lead to mental retardation. Delivery of the enzyme has been successful to the liver and skeletal muscle in animal models. Skeletal muscle gene delivery has been demonstrated to be a viable alternative to liver directed therapies since access is easier

and secretion of the required protein is very efficient from this tissue (24). Although gene transfer was demonstrated in all of these contexts, therapeutic benefit did not follow when these strategies were tested in humans. Problems maintaining gene expression have made it difficult to achieve a therapeutic level of recombinant protein and restore function in the organ of interest. Also, immune responses to the delivery vehicles have prevented the success of repeated injections of recombinant viral vectors.

1.2.3 Occlusive coronary artery disease

Vascular diseases are the number one killer in Western countries. Recent insights into the molecular mechanisms of their pathogenesis have allowed the identification of novel targets to treat a set of related conditions (25). Restenosis is a common cause of relapse in patients undergoing angioplasty treatment. It is characterized by an excessive vascular cell proliferation in response to injury. Atherosclerosis is another condition where excessive vascular cell proliferation can cause disease. Transcription factors involved in upregulating cell-cycle regulatory genes such as E2F were used as therapeutic targets. E2F decoys were efficacious at inhibiting cell proliferation both in vivo and in vitro (26). Other strategies were aimed at directly blocking cell cycling by expressing p21, a cyclin-dependent kinase inhibitor to block G1/S progression (27). Patients with familial homozygous hypercholesterolemia don't express the low density lipoprotein receptor. They suffer from atherosclerosis and die early from myocardial infarction. Ex-vivo treatment strategies were designed to correct the defect with autologous transplant of retrovirally modified hepatocytes expressing the human LDL receptor (28; 29). While the treatment was assessed to be both safe and feasible, it didn't demonstrate a homogenous gene transfer efficiency among patients in the study. Metabolic correction also varied

considerably. Other delivery vectors are being explored for similar strategies. Anti-atherogenic proteins, such as plasma apolipoprotein AI and lecithin-cholesterol acyltransferase were transferred to muscle cells for correction of atherosclerosis. Stable expression was achieved with adeno-associated virus (30). Angiogenesis is desired in certain disease states such as myocardial ischemia and diabetes. Delivery of vascular endothelial growth factor VEGF using adenovirus resulted in normal levels of neovascularization in a diabetic mouse model (31). In a pilot study with 5 patients, direct naked DNA injections of VEGF in 5 patients with ischemic angina resulted in improved myocardial perfusion as shown by coronary angiography (32).

1.2.4 CNS diseases

CNS diseases associated with neurodegeneration such as Alzheimer's or Parkinson's disease have been chosen as targets for gene therapy strategies. Neurotrophic factors protect neurons and prolong their survival in vivo. However, direct delivery of neurotrophic factors to the brain causes side effects related to the wide dissemination of these factors in the brain and not to a localized site of injury. Genetic delivery of these molecules was a good alternative to improve targeting. In pre-clinical models, intraparenchymal delivery of ex vivo genetically modified autologous cells expressing nerve growth factor (NGF) to the basal forebrain region showed a 92% protection from cholinergic neuronal degeneration after injury (33). Direct in vivo administration of these factors with adenovirus or adeno-associated virus vectors also conferred protection and promoted axonal regeneration (34; 35) in animal models.

1.3 Limitations

From the results presented in the previous section, one can conclude that gene therapy hasn't lived up to the promise of the miraculous treatment it was initially portrayed to be. Although some success at gene transfer was demonstrated, therapeutic outcomes did not always follow. The current limitations of gene therapy are mostly in the delivery vehicles. A lot of progress has been made in the design of better viral vectors, as described in section 3. Lipid-based delivery systems are also being explored, but their description is beyond the scope of this introduction. All gene therapy trial results published to date were obtained with early generations of these vectors and therefore don't reflect this progress. The development of appropriate preclinical models of gene therapy that parallel the context of natural diseases will allow for a better prediction of toxicities and efficacy and contribute to progress in this field. Safety issues concerning the use of viral vectors and scale-up problems to manufacture clinical-grade safe stocks are also being addressed. Finally, a more focused effort on in-vivo delivery strategies as opposed to ex-vivo ones will also contribute to making gene therapy a clinical reality, where genes could be administered like traditional drugs.

2. Cancer Gene Therapy

Trials for cancer gene therapy that have been approved in the USA have involved malignancies that are considered incurable. This clinical situation, which is unlike the one for many genetic diseases for which life expectancy is measured in years rather than weeks or months, has been considered more appropriate ethically for untested technologies. For this reason, applications of gene therapy to cancer are expected to be the fastest growing area of human gene therapy.

2.1 Cancer

Major advances in our understanding of cancer causation have demonstrated that cancer is a genetic disease. It is the accumulation of several genetic lesions culminating in uncontrolled proliferation that leads to the clonal expansion of certain cell types (36). These genetic lesions are found in genes involved in pathways regulating cellular proliferation, differentiation, or death by apoptosis. As a consequence, progress has been made in methods of cancer diagnosis, treatment and prevention.

2.1.1 Epidemiology data

An estimated 129,200 new cases of cancer and 62,700 deaths from cancer occurred in Canada in 1998 (37). Three types of cancer account for at least 50% of new cases in each sex: prostate, lung and colorectal cancers in males, and breast, lung and colorectal cancers in women. Lung cancer remains the leading cause of cancer death among Canadian women in 1998, accounting for an estimated 6,500 deaths compared to 5,300 deaths from breast cancer. However, lung cancer incidence is still less than one-half of breast cancer, which at 19,300 new cases remains the leading cancer in incidence among Canadian women. Among Canadian men in 1998, prostate cancer continues to be the leading form of cancer in incidence with an estimated 16,100 new cases diagnosed compared to 12,200 for lung cancer. Lung cancer is still the leading cause of cancer deaths among Canadian men in 1998; the estimated 10,600 lung cancer deaths far exceeds the 4,300 deaths due to prostate, the second leading cause of male cancer deaths. These differences in incidence and mortality are related to severity of the disease which is measured by deaths to cases ratio. Lung cancer has the poorest prognosis of the leading cancer deaths. International comparisons with Canadian cancer statistics of the fifteen

most common cancers show very high similarities of incidence rates between Canada and the United States, as well as Western European countries. The three top cancers for men and women are the same. Asian countries show a lower rate of prostate cancer and a higher rate of stomach and liver cancers. For women, breast cancer rates are considerably lower in Japan, China, Ecuador and Zimbabwe than in Canada. Cervical cancer, on the other hand is higher in these other countries. In incidence, breast cancer is either the first or second most common cancer among women in all of the countries reported.

2.1.2 Current treatments

Three approaches are mainly used for cancer treatment: surgery, irradiation, and chemotherapy. Chemotherapy is the main method of treatment for leukemias, and is mostly used in an adjuvant setting in other cancers, following surgery or irradiation therapy or sometimes in combination with another modality. The general principle of cancer chemotherapy is to target dividing cells. However, heterogeneity in the types of cells that constitute a tumor renders this approach difficult, since cells could be either dividing or in a non-cycling resting state, referred to as G0. Resting populations include cells that have the potential to divide or cells that are no longer able to divide, but they are generally thought to be more resistant to cytotoxic drugs.

There are three general classes of traditional anti-cancer drugs: alkylating agents, antimetabolites and natural products. Alkylating agents include the nitrogen mustards cyclophosphamide, ifosfamide, melphalan, and chlorambucil. Their mechanism of action is through nucleophilic attacks on nucleotide bases and the formation of mismatches and DNA cross-links. Nitrosoureas are also part of alkylating agents. Their lipid solubility allows them to cross the brain barrier for treatment of intracranial tumors.

Antimetabolites target cells in S-phase, since their mechanisms of action are based on altering metabolic pathways involved in providing precursors for DNA and RNA synthesis. Examples of antimetabolites are methotrexate, 5-fluorouracil (5-FU), cytosine arabinoside (Ara-C), 6-mercaptopurine and 6-thioguanine. Natural products include adriamycin, actinomycin D, bleomycin, the vinca alkaloids vincristine and vinblastine, etoposide (VP-16), and mitomycin C. Other commonly used drugs are cisplatin and carboplatin They have a variety of mechanisms of action. Hormone-based treatments, such as tamoxifen, have emerged into routine clinical practice for certain types of cancer. These treatments are generally used in combination, and a typical treatment would include drugs from different categories. Although such regimens have been effective in many cases, there is a need for alternatives. Drug resistance is very common in tumor populations and the high toxicity of these drugs limits potential increases in administered doses. Toxicities include myelosupression, damage to mucosal cells, nausea, vomiting, hair loss, damage to gonadal cells, and potential carcinogenicity. Other toxicities might include neurological, cardiac or renal damage. The extent of each toxicity varies with the drugs described previously, depending on their mechanism of action. Developments in newer therapies have included antimetastatic, differentiation, and anti-angiogenic agents. Gene therapy approaches have attempted to address the problems of toxicity with the design of tumor selective treatments or drug resistance with more efficient intra-tumoral drug activation. These two aspects are addressed by the strategies described in this thesis.

2.2 Strategies of cancer gene therapy

Gene therapy treatments for cancer can be divided into two broad categories where the aim of the treatment is either genetic correction of the abnormal tumor cell to reverse its phenotype or its destruction through a genetic modification.

2.2.1 Immunogene therapy

In its early days, immunogene therapy was mostly based on ex-vivo approaches, due to the lack of adequate gene delivery vectors. It can be divided into two groups: tumor cell vaccines or vaccines based on recombinant cytokines or tumor antigens directed to various cell types to boost the host's immune response.

Tumor cell vaccines are divided in autologous tumor cells, genetically modified/ fibroblasts and tumor infiltrating lymphocytes (TILs). TILs were thought to be a useful tool for the treatment of cancer cells because of their propensity to home in tumors (38; 39) and were extensively used in humans with modest results (40). Autologous tumor cells offer the advantage of being the most relevant to the patient since they display the patient's own antigens. This approach was successful in animal models where irradiated tumor cells were modified with GM-CSF, and injected into animals (41). However, due to the difficulty of growing certain tumor types in vitro, genetically modified fibroblasts were used with a mixture of irradiated tumor cells instead. Allogeneic partially matched (HLA) tumor cell vaccines have also been used. This was possible due to shared tumor associated antigens (TAAs), such as the MUC-1 protein on tumors of epithelial origin or MART on melanoma cells.

The development of in vivo immunotherapy seems to hold more promise with the direct injection of vectors carrying various cytokines in tumors. The most widely used

cytokines in these various vehicles for cancer vaccines are IL-2 (42), IL-12 (43), IFN-γ, GM-CSF (44), IL-10 (45), TNF-α(46), and RANTES (47). Initial clinical trials demonstrated that direct intratumoral IL-2 injections (48) in adenoviral vectors are safe, but efficacy in a controlled study has yet to be demonstrated.

Injections into tumors or muscle cells of vectors carrying TAAs were also tested (49). However, tumor associated antigens also vary widely and the injection of a certain TAA only generates a clonal immune response (50). In patients, approaches using adenoviral vectors were not very successful due to the short lived presence of the vaccine. Immunologic assays did not demonstrate consistent immunization in patients receiving these vaccines (51). Alternative approaches using synthetic peptide vaccines to deliver/ tumor antigens have been more successful (52).

More than just antigen recognition is needed to activate T cells into effector functions. Tumor cells are also deficient in antigen presentation skills. Therefore even a heightened immune response may not allow tumor epitopes to be recognized. Co-stimulatory molecules such as B7-1 and B7-2 are needed (53-55), since the lack of expression of these molecules by tumors contributes to their poor immunogenicity. Enhancement of tumor immunogenicity by injections of MHC I or II as well as co-stimulatory molecules has been observed. One limitation to this approach is the heterogeneity and unpredictability of loss of co-stimulatory molecules in human cancers. Replacement of multiple molecules may be required.

Other strategies of immunomodulation involve the use of antisense technology to inhibit the expression of immunosuppressive and differentiation factors expressed by tumor cells. One example is inhibition of transforming growth factor β, (TGF-β) which,

amongst other functions, inhibits cytotoxic T lymphocytes by down-regulating IL-2 receptors.

2.2.2 Tumor suppressor genes and oncogenes

On one hand, the inhibition of several oncogenes has been investigated as a potential target for cancer gene therapy (56). Antisense treatments to K-Ras have been effective in slowing down the growth rate of lung tumors in vitro and in vivo in nude mice (57; 58). Alternative methods to inhibiting the expression of oncogenes include the use of ribozymes and intracellular single-chain antibodies. On the other, introducing a normal copy of a tumor suppressor gene is a paradigm based on the idea that a homozygous loss of function is required for cells to show the mutant phenotype. Therefore, the introduction of a single copy of the wild type gene should be enough to restore function. One thoroughly explored example is the p53 gene. This tumor suppressor normally induces cells to undergo apoptosis by controlling cell cycle progression and interacting with several proteins involved in this pathway. It is mutated in a large percentage of cancers (59-62). The introduction of wild type p53 in retroviral or adenoviral vectors has provoked apoptosis in tumor cells in an animal model of human non-small cell lung cancer (63; 64) and has shown some benefits in two different phase I trials (65; 66). In another context, it restored sensitivity to radiation (67). Another strategy taking advantage of the p53-mutated status in tumor cells is based on the use of adenoviruses (68; 69). The E1B 55-kDa protein of the virus targets p53 for destruction. It allows the wild type virus to force cells into cell cycle progression and DNA synthesis to ensure replication of its genome and propagation through lysis of the host cell. An adenovirus harboring a mutation in E1B 55kDa, ONYX-015, can replicate selectively in p53 mutant

cells (70) and cause cell death through lytic infection (71). Preliminary results of clinical trials in 90 head and neck patients showed 40-100 % destruction of p53 mutant tumors after direct injection. Virus injection in neighboring normal tissue did not lead to cell damage (72). Other strategies aimed at restoring apoptosis in cancer cells are based on the introduction of E2F-1 (73) or p21 Waf-1 proteins (74).

2.2.3 Chemoprotection

While it is not a therapeutic modality in itself, this strategy aims at augmenting existing cancer treatments, namely chemotherapy. The main approach is to enhance the resistance of bone marrow or blood derived stem cells by introducing drug resistance genes, such as MDR-1 (75). The gene product, P-glycoprotein, functions as a cellular efflux pump for a variety of hydrophobic drugs and is increased in cancer cells. It was successful in improving patients' recovery from myelosupression after intensive systemic chemotherapy (76). Another transporter, the multidrug-resistance associated protein (MRP) was identified and used to confer resistance to natural product drugs (77). Other genes involved in DNA repair pathways or cellular metabolism that causes drug resistance have been explored. The GST-Yc enzyme is involved in protecting cells from the effects of alkylating drugs. Its expression has been shown to protect cells from the hematopoietic system from the effects of alkylating drugs such as chlorambucil, mechlorethamine and melphalan (78; 79). Cytidine deaminase was used to confer resistance to the antimetabolite ara-C (80; 81). This strategy relies on the assumption that a higher dose chemotherapy is indeed going to translate into clinical benefit and that other toxicities, unrelated to myelosuppression, wouldn't be limiting. Animal studies do

show some promise in the treatment of ovarian carcinoma but clinical results have yet to demonstrate therapeutic efficacy.

2.2.4 Tumor killing by inserting toxin or suicide genes

The concept of using prodrugs in treatment of cancer was explored many years ago, by designing prodrugs compatible with enzymes that were endogenously expressed or overexpressed in tumor cells (82). However, it wasn't very successful, due to the lack of a strong and consistently expressed activating enzyme. The idea became of interest again when delivery of activating enzymes was possible. Two methods were explored: antibody-directed enzyme prodrug therapy (ADEPT) or gene-directed enzyme prodrug therapy (GDEPT). ADEPT was successful experimentally in a breast cancer xenograft/ model where it was directed against c-erbB2 p185 (83). It was also useful in a renal cell carcinoma model (84) and melanoma model (85; 86) where the antibody was directed to the p97 melanotransferrin antigen. These antibodies were conjugated to carboxypeptidase-G2 and β-lactamase, respectively. Carboxypeptidase G2 has been used to activate a variety of prodrugs including benzamide, which is cleaved to a benzoic acid mustard drug (87), and CMDA (a mustard prodrug) (83). Carboxypeptidase A was used to cleave methotrexate-α-phenylalanine and other prodrugs based in this compound (88). In a human lung adenocarcinoma an antibody linked to cytosine deaminase was used (89) to activate 5-fluorocytosine into 5-fluorouracil. However, clinical studies of these strategies showed clear disadvantages of ADEPT (90). The antibody/enzyme conjugate is not very stable in circulation and was eliminated by an immune response, essentially limiting the approach to a single round of therapy. Moreover the antibody conjugate could be internalized and inactivated right at the tumor site, or the activated drug could be

too bulky to enter tumor cells. The activated drug also caused myelosupression. This unexpected side effect was due to activation of the prodrug in the circulation by unbound conjugate. The only successful clinical trial report with this strategy relies on the use of an immunotoxin made of diphteria toxin linked to human transferrin. Human transferrin receptors are highly expressed on rapidly dividing cells. Therefore the approach was ideal for direct brain injection for the treatment of gliomas. Delivery was achieved using a high flow microinfusion technique and success, determined as 50% tumor reduction was measured in 60% of patients (91). However, local brain injury occurred at the higher doses, probably due to killing of proliferating vascular cells.

Another method of activating a prodrug in situ consists of introducing the gene into cancer cells. The paradigm of suicide genes or GDEPT was first introduced by Moolten (92) using herpes simplex virus thymidine kinase (HSVTk) and gancyclovir (GCV). HSVTk catalyzes the initial phosphorylation of GCV, a guanine analogue. Cellular enzymes can then metabolize GCV-monophosphate to active GCV tri-phosphate, which acts as a chain terminator in DNA synthesis (93). Several other combinations of enzymes/prodrugs have since been explored (94; 95) and the most promising to date are cytosine deaminase and 5-fluorocytosine (96; 97), E.coli nitroreductase and CB1954, a bifunctional alkylating drug (98), and E.coli purine nucleoside phosphorylase, which activates a deoxyadenosine analog to 6-methylpurine, which is toxic to the cells (99).

Optimization of an existing pair, HSVTk and GCV, has led to the creation of HSVTk-i mutants by random sequence mutagenesis of the substrate binding site. The mutants were made more specific for GCV or acyclovir (ACV) binding than to thymidine. Stable transfectants of the mutant Tk were 10 fold more sensitive (smaller IC_{50}) to GCV than

stables made with the wild-type HSVTk (100). The generation of lipophilic GCV derivatives led to the identification of E-GCV, an elaidic acid derivative of the parent nucleoside analog, that is 10-fold more inhibitory to HSVTk-1 than the parent compound. The mechanism of this increased sensitivity in Tk transfectants exposed to E-GCV is the longer retention time of E-GCV anabolites in the cell (101).

The ideal enzyme for prodrug activation should not be present in normal human cells to restrict toxicity of prodrugs to target tissues. However, another interesting approach based on GDEPT consists of increasing activating enzymes in tumor cells, even if such enzymes are found elsewhere in the organism. Transfer of activating genes directly into tumor cells may conceivably increase in situ drug availability thereby providing for a more effective anticancer effect and an improved therapeutic index. In human tumors, levels of cytochromes P450 are very low, therefore metabolic activation of administered drug is achieved through liver metabolism followed by release into the blood and tumor uptake. Cytochrome P450s (CYP) are a super family of enzymes, involved in metabolism of drugs and other xenobiotics. They are widely expressed in the liver and localized in the endoplasmic reticulum (102). Over fifteen isoforms of the human family of these oxidases have been characterized and implicated in drug metabolism. The conventional cancer chemotherapeutic agents cyclophosphamide (CPA) and ifosfamide (IFA) are alkylating agent prodrugs that undergo bioactivation catalyzed by human liver P450 enzymes with electron input from the flavoenzyme NADPH P450 reductase (103; 104). The primary 4-hydroxy metabolite is formed in the liver and spontaneously decomposes, both in circulation and within the target tumor cells, to yield acrolein and an electrophilic mustard, which exhibits the DNA cross linking and cytotoxic effects associated with the

parent drug. P450 isoforms involved in CPA and IFA metabolism have been identified as CYP2B1 (rat isoform) or its human equivalent CYP2B6 (105; 106), for cyclophosphamide and CYP3A4 for ifosfamide (107-109). Transducing the activating gene into tumor cells would be advantageous since more of the drug that is already available in the blood and taken up by the tumor cells would be activated in situ, thereby increasing tumor cell killing. Stable transfections of MCF7 human breast cancer and 9L rat glioma with CYP2B1 demonstrated increased sensitivity to cyclophosphamide (110; 111). Direct in vivo(112) delivery approaches using adenoviruses and retroviruses were also used to test this strategy and showed significant growth inhibition in treated tumors in a model of 9L glioma. A bystander effect was also reported with the use of this suicide/ gene system and was demonstrated to be mediated by diffusible metabolites (113).

To avoid unwanted toxicities, targeting is as critical in GDEPT as finding the ideal enzyme/prodrug combination (114; 115), and methods to achieve selectivity are discussed in section 4 and further explored in this thesis.

3. Choice of Viral Vectors for Gene Delivery

3.1 Overview

A variety of viral vectors with different characteristics have been used for gene delivery. Since all current gene delivery systems suffer from some limitation, there is no one ideal vector system. The choice of vector depends strongly on the strategy in use, and on desired attributes for achieving a positive outcome. This includes characteristics such as integrating versus non-integrating vectors, size of the transgene and capacity of the vector, ability of the vector to target certain cell types, and finally feasibility of having the required number of viral particle synthesized for *in vivo* delivery. For example, the

introduction of normal copies of tumor suppressor genes or the use of antisense molecules to block the expression of oncogenes requires the use of a delivery method that is very efficient since the gene product needs to be expressed in a majority of cells to control tumor growth. Moreover expression should preferably be persistent, through integration in the cellular genome. On the other hand, strategies that introduce a suicide gene in tumor cells followed by systemic prodrug treatment have been reported to have a bystander effect. In this case, even if 10-20% of tumor cells are expressing the gene product, complete tumor regression can be expected. Similarly, strategies relying on boosting the immune system will not require a very high gene transfer rate since the nature of the immune response, once it is re-activated, is systemic. Certain distinctions can be made, in the case of immunogene therapies aimed at correcting deficiencies in antigen presentation in tumor cells by expression of co-stimulatory molecules, where a high proportion of tumor cells would have to acquire a copy of the gene.

Another concern with delivery vectors is safety. For viral vectors, main points to be considered are pathogenicity, and clearance following the initiation of an immune response. Great progress has been made with non-viral delivery methods as a safer alternative, with lipid based technology (116) or with naked DNA injections. Different formulations exist, where plasmid DNA is encapsulated within a lipid sphere or simply emulsified with various cationic lipids. Naked DNA injections have also been used with a good success rate for intramuscular gene delivery. A detailed description of these delivery vehicles is outside the scope of this thesis. The main setback in this non-viral approach is reduced transduction efficiency compared to viral vectors. Specific viral

delivery systems will be discussed below and progress with each of these tools will be presented.

3.2 Retroviral vectors

Retroviral vectors in the form of the murine leukemia virus (MLV) and its derivatives were the first vectors to be used in gene therapy (117-119). This is because the retroviral life cycle was well studied and the technology to generate recombinant retroviral vectors free of replicating helper virus was well developed (120). The genome comprises three genes termed gag, pol, and env. They are flanked by two elements required for integration called long terminal repeats (LTR). Retroviruses are enveloped virions of about 100 nm in diameter. Like all viruses, retroviruses have a specific requirement for interaction with a cell surface receptor molecule for infection. Amphotropic retroviral vectors bind to a receptor that is present on most human cells. It resembles proteins of membrane transport systems, and is known to be involved in phosphate transport. The natural function of these proteins seems unrelated to their roles in virus infection. Retroviral vectors can accept up to 8 kb of non-viral DNA, which replaces all viral genes, and retain LTRs and a packaging sequence. Integration of proviral DNA proceeds only if the target cells undergo cell division shortly after infection. This characteristic has made retroviruses the vehicle of choice for targeting of brain tumors, where surrounding normal tissue is quiescent. Integration of the provirus in the host's genome poses a finite insertional mutagenicity risk but no such side effect has been reported. The viral promoter in the long terminal repeat (LTR) is constitutively active in many different cell types. Targeting of retroviral vectors using tissue-specific control elements has been difficult because of interference from internal viral promoters and the influence proviral

integration site can have on transgene expression. However, recent developments in self-inactivating retroviral vectors (121) have made retroviruses potential candidates for the incorporation of tissue specific promoters. An attractive feature of retroviruses is that they essentially produce the desired protein products only. Hence there is no induction of host immunity from the production of additional proteins. The problem with *in vivo* use of these vectors is their low transduction efficiency. Moreover, cells transduced with retroviruses appear to lose expression of transgene through loss of proviral sequence or methylation of the integrated DNA. Finally, they can only be synthesized in titers up to 10^7 particles per ml.

The generation of replication competent retroviruses from early producer cell lines caused a safety concern for the use of retroviruses as delivery vehicle in humans (122; 123). Several technical advances have made possible the production of retroviral vector stocks free of wild type virus in the final stock preparation. Some of these strategies include having the structural proteins expressed separately and from heterologous promoters instead of the LTR (124). More recently, the development of human packaging cell lines also decreased the chance of recombination since they don't have naturally occurring homologous provirus sequences in their genome (125; 126). There is no pre-existing immunity to the murine derived vectors, but in the case of retroviral vectors packaged in mouse cell lines, reaction with the human complement markedly reduces their half-life in vivo. Retroviruses currently in use are based on the Moloney MLV with variations in the envelope protein that account for modifications of tropism. A notable advance is the use of the vesicular stomatitis virus G pseudotype system, in which the VSV-G protein replaces the normal retroviral envelope protein. In this case, virus titers

attainable are up to 10^9 and tropism is very broad (127). Since the VSV-G protein is very toxic when constitutively expressed, different packaging systems for production of these pseudotyped viruses express it either after transient transfection (128), in an inducible fashion using a tet-repression strategy (129; 130) or a LoxP-Cre recombinase system (131). The new VSV-G generation of retroviruses has been efficiently used *in vivo* in two syngeneic mouse models: a colorectal carcinoma and a melanoma. Transduction efficiency of 20% was achieved as a result of the higher titer in the retroviral preparation used (132).

3.3 Adenoviral vectors

Early interest in adenoviruses as gene delivery vectors stems from their capability of infecting non-dividing cells *in vivo* (133-135). Advances in production systems and the development of easy to manipulate plasmids for the generation of recombinant adenoviruses enhanced their utility. In addition, high titers are easily achievable (10^{11} to 10^{12} per ml).

Adenoviridae (136) are double stranded DNA viruses of 36,000 bp. Over 100 serotypes have been identified, and the one mostly used in gene transfer studies is the human type 5 adenovirus (Ad 5). They do not usually integrate in the host DNA. Rather, they replicate as episomal elements in the nucleus of host cells. The virion is an icosahedron of about 70 nm in diameter, consisting exclusively of proteins and DNA. The protein shell is composed of 252 subunits (capsomeres), of which 240 are hexons and 12 are pentons. Five and six neighbors surround pentons and hexons subunits, respectively. Each of the twelve pentons contains a base, which forms part of the capsid and a projecting fiber whose length varies among different serotypes. The fiber interacts with a cellular receptor

protein, the Coxsackie and Adenovirus Receptor (CAR). It has a 222-amino acid extracellular domain, a membrane-spanning helical domain, and a 107-amino acid intracellular domain (137). The presence of this receptor has been associated with a more efficient gene transfer (138). Adenovirus entry is also facilitated by the secondary interaction between the penton base protein and integrins on the cell surface (139).

The viral genome is stable and does not undergo rearrangements. Insertions of foreign genes are maintained intact through successive rounds of replication. However, there is a correlation between genetic stability of adenoviral vectors and net insert size. The vectors with larger inserts rearrange extremely rapidly after serial passage in packaging cells (140; 141). The virus replicates efficiently in permissive cells and remains concentrated within the cell long after yields have reached maximum levels, making collection and concentration of virus extremely easy. Adequate packaging cell lines based on 293 cells, with the relevant complementation proteins for helper independent vectors were generated to package these vectors. 293 are human embryonic kidney cells, containing and expressing the left 17% of the Ad5 genome (142). More recently the viral-cellular junctions of Ad5 sequences were characterized and integration of Ad5 DNA in 293 cells was mapped to chromosome 19 (143). These cells complement E1 deleted vectors and allow for their replication. Other packaging cell lines which have better defined adenoviral sequence content were developed to reduce the possibility of homologous recombination occurring and the generation of wild type virus (144-146). Other proteins were introduced to complement the corresponding deleted vectors in the E2 (147-149) or E4 (150) regions (151; 152). The E3 region, which is deleted in all of these constructs, is not required for packaging in 293 cells. In addition to mammalian based systems for

generation of recombinant adenovirus by homologous recombination, other systems based on *E.coli* have been generated and they offer the advantage of speed and ease of manipulation over mammalian cultures. A recombinant adenoviral plasmid is generated with a minimum of enzymatic manipulations, using homologous recombination in bacteria rather than in eukaryotic cells (153-155). This simplifies the generation and production of such viruses since homogeneous viruses can be obtained from this procedure without plaque purification.

The adenoviral virion can package up to 105% of the size of its wild type genome, allowing for inserts of 1.8-2 kb. Several generations of adenoviral vectors have been developed for use in gene therapy based on deletions in several regions of the viral/ genome. They can accept foreign inserts to generate helper independent virus with a greater capacity then 2 kb. First generation adenoviruses have deletions in their E1 and E3 regions, and can accept up to 8 kb inserts (156-158). The E1 deletion is an important factor in rendering these vectors replication defective. However, considerable viral protein expression was still observed even though these vectors were replication deficient. Other generations were developed to reduce immunogenicity of the first generation vectors and to reduce leaky viral protein expression in infected cells and improve duration of expression *in vivo*. Vectors were made with E1 and E2 deletions (147; 159), where the DNA binding protein, composed of a DNA polymerase and a terminal protein (pTP) essential for initiation of replication at the ITR, is absent. These viruses show complete abolishment of early and late protein expression. Vectors with E1 and E4 deletions have also been generated (151; 160), and have also shown reduced viral protein expression. The newest generation of adenoviruses is called the "gutless vector"

(161; 162) since all the viral proteins between the ITRs have been deleted. The packaging of these vectors is helper dependent, and requires more elaborate preparation and purification procedures.

Inflammatory responses have developed in hosts infected with recombinant Ad5. This has stopped or delayed the use of these vectors in a clinical setting because of short term expression of the encoded transgene. Two types of inflammatory response have been observed. On the one hand, there was a cellular response based on T-cell activation and rapid elimination of cells expressing the transgene. On the other hand, a humoral B-cell response characterized by neutralizing antibodies prevented repeat injections of the virus. It is not clear whether it is the leaky expression of viral proteins or the expression of foreign transgenes that is responsible for immune responses (163). Some reports have demonstrated activation of NFκB upon virus injection, followed by ICAM-1 upregulation, even when the vector was devoid of transgene or was UV irradiated. This was seen only at very high multiplicities of infection (MOIs) (>1000) (164). At lower MOIs, stimulation of the Raf/MAPK pathway and IL-8 secretion were reported (165). Several reports have shown that newer generations of adenoviruses where E2 or E4 regions have been deleted gave more promising *in vivo* results (159; 160; 166; 167), namely reduced hepatotoxicity and improved transgene persistence. Others concluded there was no difference between first and second generation adenoviruses (168; 169). Experiments conducted with the newest generation of adenoviral vectors, the gutless or gutted vectors, demonstrate a dramatically improved profile of safety and persistence of gene expression (170; 171). However these studies are hard to interpret since they used a variety of transgenes and different strains of mice (172). A study done by Lusky et al

(173) was aimed at characterizing the immune response to different generations of adenovirus by comparing isogenic vectors devoid of transgenes that differed only in regions of adenoviral genome that have been deleted. They reported no difference in cellular or humoral immune response activation when experiments were done in immunodeficient or immunocompetent animals. The conclusion of this work is that reactivity is in fact due to the transgene and not to the virus itself. A report that might shed some light on this controversy demonstrates that adenovirus infects antigen presenting cells (APC), more specifically dendritic cells, and enhances T-cell recognition to cells expressing transgenes in the context of adenovirus (174). This immune response was not observed in the case of other viruses like AAV that didn't infect APCs, and/ transgene expression in that case lasted longer than was ever observed with adenovirus. This was further demonstrated in a recent study where APCs were infected with an adenovirus encoding Fas-ligand (175). These modified APCs led to the elimination of adenovirus reactive T-cells. T-cell tolerance that is specific for adenovirus was induced. Some groups claim this immune response is an advantage to the use of adenovirus in certain cases, such as cancer for instance, where short-term expression is desirable. Even more, the enhancement of immune response to tumor cells infected with adenovirus has made it a vector of choice in vaccination strategies (176). While it may be true when gene delivery is via direct intratumoral injections, this view is debatable when injections are systemic. In the case of the latter, toxicities have been reported due to viral dissemination to major organs such as lung or liver and strong inflammatory responses in these organs. Another concern with gene transfer is germ line transmission of the transgene. Since the approaches described previously are all meant for somatic cells, the risk of gene transfer

to germ line had to be assessed. Two studies addressing this concern were reported by Wilson's group. They were done on baboons (177) and C3H mice (178) and they have shown that the use of E1/E4 deleted vectors is safe since no dissemination of the virus to the gonads was seen at low doses. Further studies demonstrated gene transfer to the brain and gonads after systemic adenovirus injection. DNA analyses on the offspring of these animals, when one or both parents had received adenoviral injections showed no evidence of germ line transmission.

3.4 Adeno-associated vectors

Adeno-associated virus (AAV) is a defective member of the parvovirus family. It is called defective because it requires the presence of other helper viruses such as adenoviruses or herpes simplex viruses to produce lytic infections. Otherwise it simply integrates in the host genome and produces a latent infection. There are no known human pathologies associated with AAV, but it is widespread in the human population. About 80% of people have antibodies against AAV. The AAV genome is encapsidated as a single stranded DNA molecule and is composed of three structural proteins termed Cap proteins, and at least four non-structural proteins termed Rep. It also contains two inverted terminal repeats and a packaging signal (179). It integrates at a specific site on chromosome 19 (180), and this feature makes it a safe tool for gene therapy. However AAV vectors haven't demonstrated that specificity of integration (181; 182), and require more optimization to conserve this feature. AAV type 2 has been widely used for gene therapy. The AAV vector is made by replacing the Cap and Rep genes with a transgene of interest. The vector capacity is 4 to 5 kb. Production techniques were laborious and required the purification of AAV particles from the wild type Ad particles. More recent

progress (183; 184) has allowed the production of AAV without using a wild type adenovirus, and an increase in viral titers. Another advantageous feature for the use of this vector is the wide range of cell types infected in various organisms. It enters the cell via a heparan sulfate proteoglycan (185), which may explain its broad tropism. Complete lack of immune response upon injection of this virus in various organs and persistence of gene expression make it a very promising gene therapy tool. Recent success in the field of gene therapy came from two studies aiming to correct hemophilia B in canine and murine models using AAV for delivery of factor IX in different target organs (12; 13). Serum levels of factor IX remained constant for 8 and 16 months, respectively, and protein levels were sufficient for partial phenotypic correction throughout the period of the study. Another strategy aiming at the use of exogenously regulated gene delivery *in vivo* was highly successful using AAV (186). Regulated gene expression is desirable to maintain concentration of the protein within a therapeutic window and avoid toxicity. Delivery of components of this system, namely the hybrid transcription factors construct and the promoter/transgene construct, was done using two different AAV vectors. Simultaneous expression of both in the same cell was required for successful control of gene expression. This was achieved *in vivo*, confirming the high efficiency of AAV for *in vivo* gene delivery.

3.5 Others

Other vector systems are in earlier developmental stages but are showing great promise. Herpes simplex virus-based vectors are being developed for use in the central nervous system (187). Lentiviral vectors, based on HIV virus, have also been used (188). They are part of the retrovirus gene family, but they can integrate in the genome of non-dividing

cells. Efficient and sustained gene transfer (up to 6 months) in muscle and liver (189) of mice, as well as neurons of the striatum and hippocampus of adult rats (190) has been shown with these vectors *in vivo*. This gives them a broader range of application than traditional retroviral vectors. Newly developed packaging cell lines (191) should also improve their tropism by integration of the VSV-G protein in a tetracycline inducible system.

4. Targeting

4.1 Transcriptional targeting

4.1.1 Tissue-specific promoters

A successful approach in cancer gene therapy using transcriptional targeting depends upon high expression levels and specificity of gene expression. Several tissue-specific promoters that target neoplasms of a single origin were analyzed and used for this purpose (114). Examples include the tyrosinase promoter to target melanomas (192; 193), the CEA promoter to target gastric carcinomas (194) and lung adenocarcinoma (195), and the alpha fetoprotein promoter (AFP) for hepatomas (196). The secretory leukoprotease inhibitor promoter has been used to target breast, ovarian, and lung cancer cells overexpressing this gene (197). The MUC1/Df3 promoter was used in targeting breast carcinoma in animal models. MUC1 encodes a mucin gene expressed in a variety of epithelial cells (198), and frequently over-expressed in breast cancer due to transcriptional activation (199-201). The MUC1 promoter has previously been used both *in vitro* and *in vivo*. In both conditions it was capable of driving thymidine kinase (HSVTk) gene expression in cells that express MUC1, but not in those that do not express MUC1 (202; 203). It has therefore been considered a useful breast

cancer-specific promoter for use in suicide gene therapy. The drawback of this targeting tool is expression of the suicide gene in normal tissues of epithelial origin.

4.1.2 Tumor-specific promoters

An optimal tumor specific promoter will selectively target neoplastic cells as opposed to normal ones. The advantage in this case is the increased safety and versatility for the type of targeted cancers. Some examples of such promoters that have been used in GDEPT are the erbB2 promoter (204) for targeting breast and pancreatic tumors, and the heat-inducible heat-shock protein 70 promoter sequence (205) for use in cancer hyperthermia. Taking advantage of the pRB mutation harbored by many tumors, an E2F-responsive promoter has been successfully used to target gliomas, with less toxicities than normally/ seen with standard non-specific promoters in the context of adenoviral vectors (206). Other features of cancer cells implicating specific genes can be exploited to target suicide genes to tumor cells. A striking feature of rapidly growing tumor cells is their high rate of glucose utilization compared to their normal counterparts (207). Glucose is channeled mainly through the glycolytic pathway, which is used not only for rapid energy production but also for the provision of biosynthetic precursors, such as pyruvate, necessary to sustain a high rate of cellular division. Hexokinases (HK), also called ATP:D-hexose-6-phosphotransferase (208), catalyze the first committed step of glycolysis. There are four mammalian hexokinases. They are composed of two highly homologous 50 kDa subunits and are product inhibited by glucose-6-phosphate to varying degrees. In rats, HK I is expressed in brain and kidney, HK II is found in insulin responsive tissues such as skeletal muscle, adipose tissue and heart. In normal liver, HK IV is most abundant. These enzymes are found in the cytosol or bound to the

mitochondrial membrane (209). Tumors at various stages of malignancies show an increase in HK II activity compared to normal tissues (210; 211). Two factors are involved in this increased activity: a tendency of the tumor enzyme to bind to the outer mitochondrial membrane where it has preferential access to ATP (212), and overproduction of HK II. The latter is the result of a gene amplification (213) and transcriptional upregulation. Indeed, the level of HK II mRNA is increased in rat AH130 and AS-30D hepatoma cells, human hepatoblastoma HepG2 cells and in renal cell carcinoma (214; 215). Protein analysis of HK II that is expressed in two rapidly growing tumor cell lines, Novikoff ascites (216) and AS30D hepatoma has shown, at least at the primary sequence level, that the overexpressed type II isoform is essentially identical to/ the type II hexokinase expressed in muscle cells. However, it is quite possible that tumor-specific post-translational modifications do exist due to altered signal transduction pathways. This in turn may alter the kinetic properties of the enzyme expressed in tumor cells. Mathupala et al. have cloned the HK II promoter and found a difference in its regulation in rat hepatoma cells compared to normal rat hepatocytes (217). More recently, they reported that two point-mutations in p53 overexpressed in the AS-30D hepatoma cell line resulted in HK II promoter activation. Two functional p53 motifs were identified in the proximal promoter at –4250 and –4195. Some of the transcription factors and promoter elements involved in HK II regulation by insulin, cAMP, and glucose have also been identified (218-220). The hexokinase II promoter seems to be a good candidate for an ideal promoter, and was selected for further study in this project.

4.1.3 Interference in viral vectors

Control elements of transgene expression have been used in viral vectors for *in vivo* gene delivery. While this strategy has been successful in many instances for retaining promoter specificity in the viral context and effectively limiting gene transfer, there have been several reports of interference from delivery vehicles. The most likely explanation is that strong viral promoters present in vectors used for gene delivery can override cellular promoters and compromise their utility. A reported example is the neuron-specific enolase gene promoter that showed great promise in transgenic specificity studies (221) and lost all tissue specificity when expressed in a replication defective herpes simplex virus vector (222). Another example that shows the influence of viral context is that of the erbB2 promoter. Whereas it was successfully used in a retroviral vector to target the expression of cytosine deaminase to erbB2 positive breast and pancreatic carcinoma cells (204), it lost specificity in the context of an adenovirus vector (223). One solution has been to introduce the expression cassette in the antisense orientation to the viral promoter. Targeting of HSVTk expression with the erbB2 promoter was not possible in an adenoviral vector in both sense and antisense orientation, whereas it was demonstrated again in a retroviral and plasmid construct. This was also reported by another group attempting to target gene expression to the lung under the control of the CFTR promoter (224). The reasons for this loss of specificity in adenoviral vectors have not been determined yet and point to the need for case specific testing of promoters in the desired viral vehicle. Other strategies that have been used consist of replacing viral enhancers with cellular ones. The U3 region of the retroviral LTR promoter has been replaced by the muscle creatine kinase promoter element (225) to effectively restrict its activation.

The MoMLV enhancer was replaced with the tyrosinase promoter to restrict expression to melanoma cells (121). Replacement of viral elements that are thought to be responsible for overriding cellular promoters with the promoters themselves is a viable alternative.

4.2 Transiently regulatable targeting

With the use of a specific promoter for transcriptional targeting, gene expression is constitutive in the target tissue. A desired feature in certain cases is the exogenous control of gene expression by administration of a pharmacological agent. There are some cellular promoters that possess this feature, due to cAMP responsive elements in the tyrosinase promoter, glucocorticoid responsive elements in the AFP promoter, or androgens in the PSA promoter. However, basal expression from these promoters in target tissues is already high and it would be only possible to enhance it. Ideally, a regulatable system would have no basal expression in the absence of the inducer and very high expression in its presence, or vice-versa if the regulator is a repressor. In addition, responsive elements for the inducer should not be present in mammalian tissue to avoid non-specific effects on endogenous genes. Finally the inducer should have no toxic side effect in the organism. Synthetic inducers of bacterial or mammalian origins have been developed. One of the first systems to be described is based on the bacterial tet operator and allows the repression of gene expression in the presence of tetracycline (226). It is based on the idea that a chimeric tet repressor, tTA, made of the herpes simplex virus VP16 activating domain and the tet repressor would activate gene expression from the tet operon. Binding of tet to this chimeric repressor inactivates it and transcription is shut down. Targeting of this system was achieved by directing expression of tTA from a specific promoter. The myosin promoter for cardiac muscle restricted expression of tTA, and expression of the

transgene of interest was directed with a tet operon promoter. Variations on this system were made to direct expression of both components from the same vector, and the discovery of a mutant tTA, rtTA, turned the repression-based system into an induction one. Several improvements have been made to the original system to address leaky expression and in vivo use compatibility issues (227). Other systems that are more compatible with *in vivo* use (228) and that take advantage of regulatable combinations have been developed, including ecdysone (insect hormone), rapamycin and RU486 responsive systems. Two of the most promising ones have recently been demonstrated to be functional *in vivo*. In an adenovirus mediated delivery system (229), a chimeric transactivator was expressed. It is made of a yeast GAL4 DNA binding domain, fused to/ the transactivation domain of p65, a component of the NF-κB complex, and to the ligand binding domain of a mutated progesterone receptor that responds to RU486. Downstream to the transactivator, a transgene was expressed that encodes human growth hormone (hGH) coupled to the 17-mer GAL4 binding site. To achieve tissue specific expression of growth hormone, the regulator was expressed from the liver-specific transthyretin promoter. Finally the expression cassette was inserted in a gutless adenovirus vector to avoid immune responses that were observed with the use of minimally deleted adenoviral vectors (see section 3.3). Ten days after systemic administration of $1x10^9$ pfus of recombinant adenovirus in C57B6 mice, a single intraperitoneal RU486 (mifepristone) administration induced a 10,000 fold increase in serum levels of hGH that peaked at 12 hours. Expression was repeatedly inducible over 60 days, and sustainable expression was obtained by subcutaneous implantation of biodegradable mifepristone pellets. Levels of mifepristone required for activation were well below the levels required to have anti-

progesterone activity, making the system safe to use. Another *in vivo* demonstration of the success of this approach came from adeno-associated virus delivery of rapamycin regulatable system (186). Due to the size restriction of AAV, two different viruses were used for delivery. The rapamycin inducible system consists of two chimeric proteins, based on FKBP12 and FRAP, whose interaction is mediated by rapamycin and required for gene activation. FKBP12 was fused to the DNA binding domain ZFHD1, and FRAP's rapamycin binding domain FRAP was fused to the transactivation domain of NF-κB p65 subunit. One vector expressed the two chimeric proteins from the same transcriptional unit and another expressed the transgene erythropoetin (Epo) driven by a promoter recognized by ZFHD1. Reconstitution of the system *in vivo*, which requires the two AAVs to transduce the same target cell, was achieved. Injection of virus in murine skeletal muscle of immunocompetent animals resulted in 100-fold increase in plasma Epo levels after a single rapamycin dose. The inducibility of Epo was sustained after 6 months of viral injection. The same approach in monkeys resulted in sustained expression up to 3 months. After 4 months levels of Epo were back to pre-therapy levels. The utility of an inducible system is obvious in the case of correction of a protein deficiency. Plasma level of the expressed protein can be closely controlled and turned on only when needed.

4.3 Transductional targeting

Restriction of transgene entry is another method of targeting, based on selective transduction of target cells. This is achieved by modification of virus tropism of retroviral and adenoviral vectors, or by taking advantage of the natural characteristics of certain viruses to infect the tissue of interest. For example, retroviruses have been the vectors of choice for CNS tumor-directed therapies since they can infect replicating cells only. The

use of regional delivery has also been explored as a targeting method by perfusing a specific organ but it is a limiting method of delivery.

Ecotropic retroviruses target a cell surface receptor which is an amino acid transporter expressed on murine cells. Transcriptional targeting of ecotropic viral receptors expression to tissues of interest in an adenoviral vector, combined with the use of an ecotropic virus for gene delivery has been explored with the AFP promoter in hepatoma cells in vitro (230). While this approach was successful in vitro, it is not probable to be efficient in vivo due to limited delivery to target cells with both vectors and the combined decrease in transduction efficiency. Tropism of retroviral vectors was also modified through the use of bispecific antibodies that can recognize cellular receptors and are specific to viral envelope proteins. Also, envelope proteins were modified to express ligands to known receptors on the tissue of interest. Limitations to this approach include the extremely low titers attained with the modified viruses, making it an unrealistic approach for in vivo use. Adenoviruses have also been modified to restrict their binding to certain cell types, by the incorporation of ligands in their fiber proteins. A FLAG octapeptide was incorporated in the HI loop of the viral fiber without affecting the three dimensional organization of the protein (231). Based on this demonstration, further developments incorporating ligands of interest in this fiber region are conceivable. Other modifications include the generation of chimeric fiber proteins with the knob region originating from Ad3 adenovirus and the shaft from Ad5 (232). This allows the exploitation of different tropism profiles exhibited by various adenovirus serotypes. Other approaches utilizing antibodies have been developed to generate bispecific

antibodies and complex them to adenoviruses to target them to receptors present on cells of interest (233).

5. Suicide Gene Therapy: HSVTk/GCV Paradigm

5.1 Characterization

The combination of herpes simplex virus thymidine kinase (HSVTk) and gancyclovir is the most thoroughly explored GDEPT combination. It is the paradigm we chose to use to characterize the use of specific promoters for suicide gene targeting. Since the system was originally devised (92), it has been examined in a number of experimental systems, as well as in clinical trials of melanomas (234), human gliomas (235-237) and mesotheliomas (238). Results from these trials have been equivocal in some cases, showing that certain aspects of this therapy, such as efficient delivery and restriction of gene expression to tumor cells can be further improved to optimize clinical efficacy.

Different models of cancers have been explored, with various viral vectors used for delivery and eukaryotic promoters driving the expression of HSVTk. Some of these models are an osteosarcoma model with the expression of HSVTk driven by an osteocalcin promoter (239), a hepatocellular carcinoma model (240) and a gastric carcinoma model (194) with the CEA promoter, all tested in human xenografts in nude mice with adenoviral-mediated gene delivery. It has also been investigated in a melanoma model using a tyrosinase promoter to drive HSVTk by direct injection of the DNA construct in tumors. In other models, HSVTk transcription was driven off strong constitutive viral promoters such as rous sarcoma virus (RSV) or cytomegalovirus (CMV) promoters. A study done in a rat glioma model demonstrated regression after in vivo adenoviral mediated gene transfer (241). A SCID mouse model of human ovarian

carcinoma was also successfully used to demonstrate effectiveness of the system (242). It was used in a malignant mesothelioma (tumor arising from the mesothelium lining of the thoracic cavity) model, demonstrating the efficacy of this strategy using an adenoviral vector (243; 244).

Nucleoside analogues have long been used as cytotoxic agents in cancer chemotherapy, and continue to play an important role. As members of the general class of antimetabolites, nucleoside analogues are cell cycle specific, and require activation to be metabolized by cellular enzymes. These characteristics, however, may also be important factors in the development of intrinsic or acquired cellular resistance to nucleoside analogues (245). Since the drug needs to be inside the cell in order to be activated, cellular uptake can play a role in metabolic resistance. There are at least five different transporters involved in nucleoside transport that differ in sensitivity to inhibitors and selectivity for substrates, as well as in their distribution on normal and neoplastic cells (246). The expression of these transporters in various tumor cell lines might play a role in sensitizing them to GCV.

5.2 Cellular resistance and toxicity

As noted, the combination of Tk/GCV has been studied in a number of tumor models using a variety of promoters and delivery systems. Although in highly selected circumstances it is effective at cell killing, a number of reports have directly or indirectly identified a spectrum of cellular sensitivity to activated GCV. In one case, in a transgenic mouse model of breast tumors, resistance was attributed to lack of Tk expression in residual tumors that were originally retrovirally transduced (247). The importance of Tk expression levels on sensitization to GCV was also confirmed by other groups (248; 249).

A correlation was also found between Tk levels and efficacy of bystander effect, in cells that were capable of establishing GJIC (250). One can conclude that Tk expression level is a limiting factor for success of this approach. However, in other reports, the dependence of the resistance phenotype on Tk levels was ruled out by establishing equivalent levels in cells under study that were stably transfected with Tk (251).

Toxicities observed in animals treated with HSVTk/GCV have been reported and they can be grossly divided in toxicities related to the delivery vehicle or to the combination itself. Toxicities were observed in a disseminated model of human breast cancer in nude animals (252) treated with GCV and an adenoviral construct of RSVTk (AdRSVTk), suggesting that it is the non-specific transduction of normal organs, such as the liver, that caused organ damage upon GCV administration. Doses used were $6\text{-}8\text{x}10^8$ pfus delivered intraperitoneally. Toxicity was related to the amount of virus injected. In the same animal model, *ex vivo* gene transfer led to a greater increase in survival in the treatment group, demonstrating the need to better target delivery *in vivo*. The possible involvement of an immune response in the observed liver toxicity cannot be ruled out since athymic (only T cell defective) animals were used. In this model, a humoral response to the treatment can still be mounted, while in other models of disseminated disease where SCID (B cell and T cell defective) animals were used, no toxicity was reported upon in vivo transduction with AdRSVTk. The same problem was reported by another group of researchers in a syngeneic rat model of colorectal liver metastases using CC531 cells (253). Similarly, when gene transfer was done *ex-vivo* with an adenoviral construct of CMVTk (AdCMVTk), animals treated with GCV after tumor implantation were tumor free compared to controls that developed large tumors 18 days after beginning of treatment. In

contrast, *in vivo* intraportal gene delivery of $5x10^9$ pfus resulted in severe hepatic toxicity as documented by serum levels of various liver enzymes. This effect appears to be specific to killing of hepatocytes by HSVTk/GCV as animals receiving the control AdCMVβ-gal virus did not exhibit any of these side-effects. Damage was also related to viral dose. The conclusion from these studies is that HSVTk/GCV toxicity is not restricted to dividing cells, as was originally thought, and targeting of this construct for *in vivo* use with adenoviral vectors is essential. Another study in a diethylnitrosamine-induced (DENA) model of hepatocellular carcinoma in rats also showed considerable liver toxicity in AdCMVTk/GCV treated animals compared to AdCMVβ-gal/GCV treated animals (254). However, in this model, cellular proliferation due to DENA was documented and toxicity in normal tissue was attributed to the proliferating status of these cells.

Another complication in interpreting these results is the humoral and immune response that has been documented to both Ad and Ad-encoded transgenes. However since controls treated with a different adenovirus or with Ad vector alone were not killed, one cannot solely rely on an immune response explanation for the non-selective killing seen with this strategy.

5.3 Importance of the bystander effect (BE)

Current delivery systems, such as adenoviruses or retroviruses, are unable to reach the total tumor population *in vivo*. However, a striking phenomenon was observed, the "bystander effect" (BE), where cytotoxicity was seen not only in genetically altered cells, but in non-transfected or transduced neighboring cells (255; 256) as well. This could represent an important therapeutic opportunity, since not all of the tumor cells need to be

directly targeted. The mechanism of the BE may include a number of possibilities, however there is compelling evidence that gap junction-mediated intercellular communication (GJIC) is important in several gene therapy pro-drug/suicide gene systems, using retroviral or adenoviral gene-delivery systems (257-261). Evidence supports the hypothesis that gap junctions allow passive diffusion of the activated metabolite to neighboring cells, thereby enabling the drug to target a greater number of the cell population. However, in some combinations of prodrug/suicide gene, the presence of functional gap junctions is not necessary, since the bystander effect is mediated by cytotoxic metabolites diffusing in the medium (262).

GJIC is considered fundamental in cell-cell interactions, since gap junctions (GJ) mediate the transfer of ions, nucleotides, small regulatory molecules as well as drugs or their metabolites between adjacent cells (263). They are made up of hemichannels called connexons in the membrane of one cell joined in mirror symmetry with the same number of hemichannels in the apposing cell membrane. Connexons are formed from members of a multi-gene family of distinct but functionally related proteins called connexins (Cx) (264; 265). In general, cancer cells have reduced or non-existent levels of connexins and greatly reduced GJIC as compared to normal cells (266; 267). Connexin transfection and GJIC induction leads to decreased rate of proliferation, increased differentiation, and reversal of cell transformation phenotype (268; 269).

Connexin 43 (Cx43) is one of the major connexins in breast tissue which is often lost or reduced in cancer (266; 267; 270). Studies have shown that transfection or transduction of Cx43, resulting in increased GJIC, leads to an increased bystander effect seen in vitro in suicide gene therapy (257; 258). Other studies examined the effect of increased Cx43

by transfection in tumor cells subsequently grown *in vivo*, and demonstrated that the BE is enhanced (271). Together these studies point to the potential utility of increasing GJIC as a part of suicide gene therapy. Adding transfer of the Cx43 cDNA to the suicide gene therapy approach would be useful for enhancing the BE. However, it is also counter productive to use a gene transfer approach to enhance the bystander effect since every cell would have to be targeted with the connexin gene. The utility of the bystander effect is precisely the fact that a whole tumor mass can be eliminated without having the gene expressed in every cell. Chemical induction of Cx43 might have a more potent effect, since a greater proportion of the cancer cell population can be reached as compared to gene delivery. Utilizing a chemical inducer of GJIC therefore has great potential to amplify the efficacy of suicide gene therapy.

There are a number of classes of chemicals that have been shown to increase Cx43 and subsequently GJIC including cyclic-AMP (cAMP) (272; 273), retinoids (274), and carotenoids (275). cAMP is an antiproliferative and differentiation agent that has a very wide range of action (276). The use of cAMP in the clinical setting is limited by its toxicity, yet its properties have lead groups to work on the cAMP-dependent pathway in cancer in search of analogues and downstream factors to target.

Another factor that hasn't been ruled out yet in an *in vivo* setting is the contribution of the immune system to the observed bystander effect. In this case, the inserted TK gene could be acting as a therapeutic tumor vaccine. Also, cytotoxicity could be leading to an inflammatory response and to the recruitment of macrophages to the tumor site, leading to a generalized re-activation of the immune system against tumor cells. This observation has been called by some the "*in vivo* bystander effect". Supporting evidence relies on the

demonstration of an infiltration of immune cells in the tumor site after HSVTk/GCV administration and necrosis of the targeted tissue (277; 278) and on the fact that this was observed in immunocompetent animals (193; 279). It was due to TNF, IL-1, IL-6, and IL-12 cytokine release by apoptosing tumor cells (280). This release upregulates the expression of immunostimulatory molecules in the tumor such as ICAM-1 and B7.1 and B7.2 (281). These immune changes in the tumor microenvironment happen over the course of several weeks, by which time tumor burden is too high to be handled by the re-activated immune system. This model has led to the combination of suicide gene and immunostimulatory approaches. Co-administration of the Tk and IL-2 gene in adenoviral vectors resulted not only in necrosis and regression of the tumor itself but rendered animals resistant to a subsequent challenge with tumor cells (282) in a liver metastasis model of colon carcinoma. It was also demonstrated that this antitumor immunity was due to the appearance of tumor specific $CD8^+$ T cells. A follow up on this study also showed that subsequent relapses occurred even in the combination treatment group. Disease-free survival was achieved by GM-CSF administration to enhance activity of APCs and further enhance the induction of an antitumor response (283). The combination of IL-4 and HSVTk in retroviral producer cells gene delivery also enhanced antitumor effects, however in this case it was also demonstrated that IL-4 treatment on its own also induced tumor regression in a glioma model (284). Other studies reported a distant bystander effect whereby non-modified tumors at a remote site from the genetically modified tumor mass regressed (234; 285; 286). This seems to be in accordance with the hypothesis of a reactivation of the immune system. But the puzzling fact is that this was observed in immunocompromised animals, in nude (287) and SCID (288) models. Nude

mice are defective in a transcription factor whose absence impairs thymus development rendering them deficient in T cells. Mice with the SCID mutation encode a deficient DNA dependant protein kinase which is involved in immunoreceptor gene recombination, causing a complete absence of functional T as well as B lymphocytes (289). Since lymphocytes are eliminated as effectors of the distant bystander observation, inflammatory cells and natural killer cells are thought to be responsible. However there isn't enough evidence at the moment to deduce a conclusive mechanism.

6. Rationale and objectives

Gene directed enzyme-prodrug therapy is a novel method of improving on traditional chemotherapy by increasing the therapeutic index of cancer treatments. Our aim is to develop new tools to improve tumor-cell targeting in this approach. We chose to study two potential candidates for a successful transcriptional targeting of cancer gene therapy. Muc-1, a tissue specific promoter and HKII, a tumor specific promoter. Evaluation of these promoters will be based on the HSVTk/GCV, a prototype of suicide gene/prodrug combination. Specific aims are:

1- Evaluate promoters in reporter gene based assays.

2- Validate the activation profile in adenoviral vectors, used as delivery vehicles for the promoter/suicide-gene expression cassette.

3- Demonstrate tumor cell specific killing in vitro

In addition, a new combination of suicide gene/prodrug will be studied, namely the CYP3A4 isoform of cytochrome P450, in combination with two enantiomers of ifosfamide, R-IFA and S-IFA (290). Specific aims are:

1- Generate cell lines stably expressing CYP3A4 and/or P450 reductase.

2- Evaluate efficacy and requirements for prodrug activation by a cytochrome P450 enzyme.

3- Evaluate the bystander effect with this combination.

CHAPTER II: MATERIALS AND METHODS

1. Cell Culture

All media and serum were bought from Mediatech (Herndon, VA). The human mammary carcinoma cell lines, MDA-231, MCF-7, MDA-MB-468, T-47-D, ZR-75-1, and non-small cell lung carcinomas (NSCLC) NCI-H460 and NCI-H661, as well as murine mammary carcinomas, DA3 and SP1 (ATCC, Rockville, MD), were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (RPMI 10%). HepG2, (ATCC, Rockville, MD), a human hepatocellular carcinoma, 888MEL, a murine melanoma, SW1222 and LS180, human colon adenocarcinomas, were all grown in α-MEM 10%. Normal human mammary epithelial cells (NHMEC) and normal human bronchial epithelial cells (NHBEC) are primary cultures grown in MEGM and BEGM, respectively (Clonetics, San Diego, CA). Several sets of NHBEC and NHMEC from different donors were tested. MGH-7, a poorly differentiated squamous cell carcinoma cell line established from human primary NSCLC tumors, was maintained in ACL-4 serum free media (Liu & Tsao, 1993). Ad5 E1 transformed 293 human embryonic kidney cells (Microbix Biosystems Inc., Toronto, ON), were used for recombinant adenovirus production and maintained in DMEM 10%. 293 GPG cells (129) were grown in DMEM 10% supplemented with 2 ug/ml puromycin (Sigma), 0.3 mg/ml G418 (Canadian Life Technologies, Burlington, ON) and 1 ug/ml tetracycline (Sigma) and were used for production of VSV-G pseudotyped recombinant retroviruses. This packaging cell line is a generous gift from Dr. Richard Mulligan (Howard Hughes Medical Institute, Boston, MA). All culture media were supplemented with 50 units/ml of penicillin and streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

2. Recombinant plasmids and viruses

2.1 Construction of recombinant plasmids

All plasmid DNA used in the study and in the production of recombinant adenoviral and retroviral particles was purified with the maxiprep kit from Qiagen (Santa Clarita, CA). All reporter gene assays were performed using the promoterless chloramphenicol acetyl transferase plasmid vector pCAT basic (Promega, Madison, WI). pHexII5150 CAT was constructed by inserting a *XbaI* cassette derived from the 29-1/Xba(pUC18) plasmid (Mathupala *et al.* 1995), a generous gift from Dr. P. Pedersen, (Johns Hopkins University, Baltimore, MA) in pCAT. The coding region and the first intron present in the *XbaI* cassette were removed from pHexII5150 CAT with a *BamHI* digest and re-ligated to generate the pHexII4557 CAT vector (fig.1A). pMUC1-1583 CAT contains the mucin-1 promoter and is a gift from Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, UK).

2.2 Construction of recombinant adenoviral vectors

All adenoviral vectors were made with the transfer plasmid pAdBN (Quantum Biotechnologies, Montreal, QC) or a modified version of this plasmid, pAdBN-AscI, created in this laboratory to accommodate the linearization requirements of various expression cassettes we used. The three "unique sites" available for linearization in pAdBN are *AseI*, *ClaI*, and *EcoRI*. The HK II promoter sequence included the first two sites. Therefore all expression cassettes that included the *EcoRI* site as well were shuttled in pAdBN-AscI. To make pAdBN-AscI, the "unique sites" fragment upstream of the left ITR was removed with an *EcoRI* digest, followed by a blunting reaction and ligation with

AscI linkers, to create an *AscI* site instead. The *AscI* site was removed from the multiple cloning site to make it "unique" in the pAdBN-AscI plasmid, as shown in fig.1B.

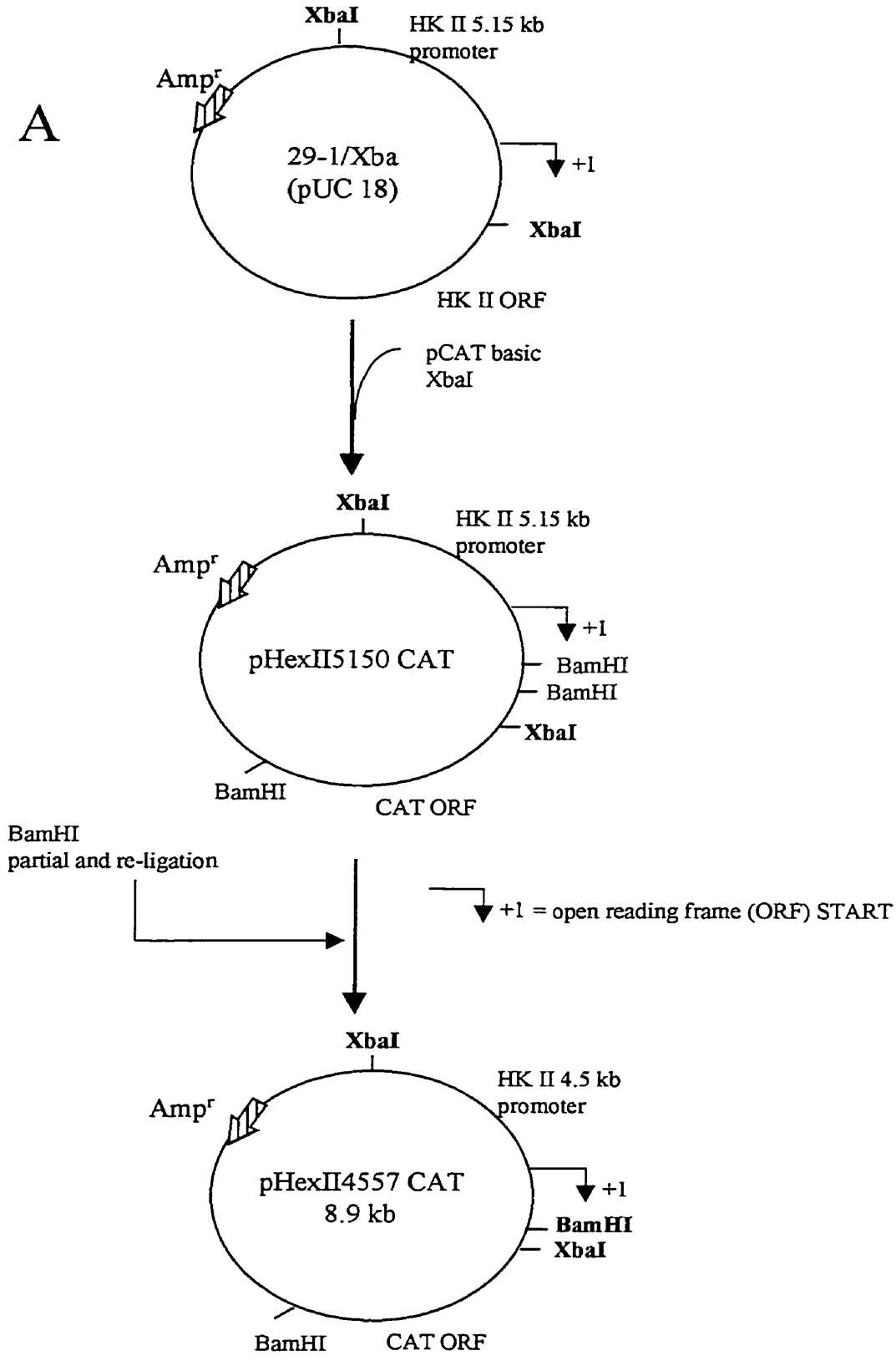
The pAdBN derived linearized constructs described below and shown in fig.1C, encoding various promoter-cDNA cassettes, were co-transfected in 293 cells with *ClaI* linearized Ad5 DNA that is E1/E3 deleted and missing the left ITR. All adenoviruses were generated by homologous recombination in 293 cells and plaque purified twice before amplification. The amplified viral stock was band purified twice on a cesium chloride gradient, purified on PD-10 columns (Pharmacia), eluted in viral storage buffer (10mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$, 10% glycerol) and titered in duplicate by limiting dilution on 293 cells. Viruses were stored at –80°C. The standard methods (291) for transfection, plaquing, amplification, and titration of adenoviruses were followed.

AdHexLacZ: the *XbaI-BamHI* cassette of pHexII4557 CAT was inserted in the corresponding sites of pUC18 (Pharmacia Biotech, Baie d'Urfe, QC) to generate pUCHexII4557. The *BamHI* cassette of the β-galactosidase coding region released from pSNS518 (Genomics One, Montreal, QC) was then inserted into the *BamHI* site of pUCHexII4557 to generate pUCHexIILacZ. The *HindIII-KpnI* cassette from pUCHexIILacZ was ligated into the corresponding sites of the transfer vector pAdBN, in antisense orientation to the left ITR. The construct was linearized with *EcoRI* and co-transfected with Ad5 ΔE1/E3 linear viral DNA in 293 cells. AdHexLacZ positive clones were selected by carrying out a β-galactosidase assay (as described below) on cellular extracts of 293 cells infected with different adenovirus clones.

AdHexTk: the viral thymidine kinase (vTk) cDNA from the pMC1Tk plasmid (Mario Capecchi, NCI, Bethesda, MD) was released using a *BsaI* digest, followed by a blunt-

Fig.1 Cloning strategy for plasmids and recombinant viruses. **A** : reporter gene construct for HK II. **B** : Modification of the transfer vector pAdBN to accommodate the transfer of various expression cassettes in adenoviral vectors. **C** : Different transfer vectors used to generate recombinant adenoviruses with different combinations of promoter and suicide or marker genes. **D** : Retroviral construct used to derive stable cell lines expressing CYP3A4.

A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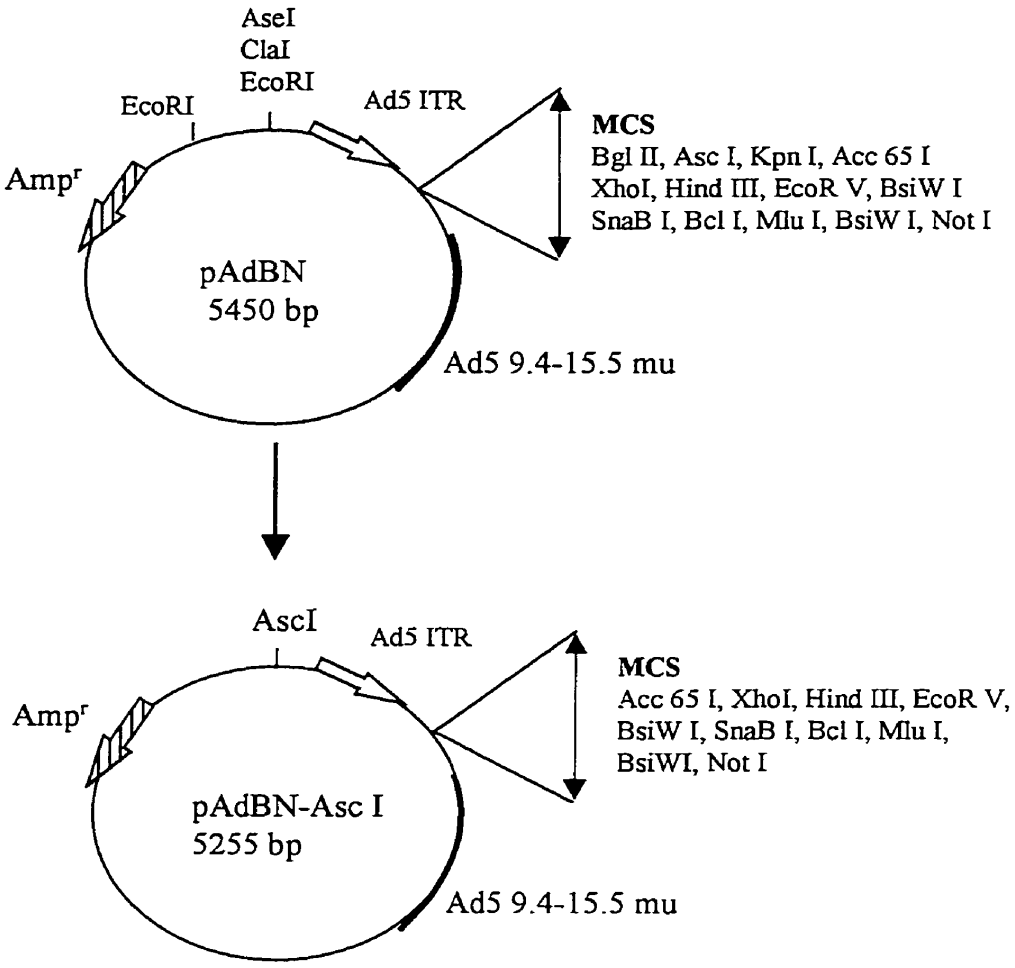
HK II 5.15 kb promoter
Ampr
29-1/Xba (pUC 18)
+1
XbaI
HK II ORF
pCAT basic XbaI
XbaI
HK II 5.15 kb promoter
Ampr
pHexII5150 CAT
+1
BamHI
BamHI
XbaI
BamHI
CAT ORF
BamHI partial and re-ligation
+1 = open reading frame (ORF) START
XbaI
HK II 4.5 kb promoter
Ampr
pHexII4557 CAT 8.9 kb
+1
BamHI
XbaI
BamHI
CAT ORF

B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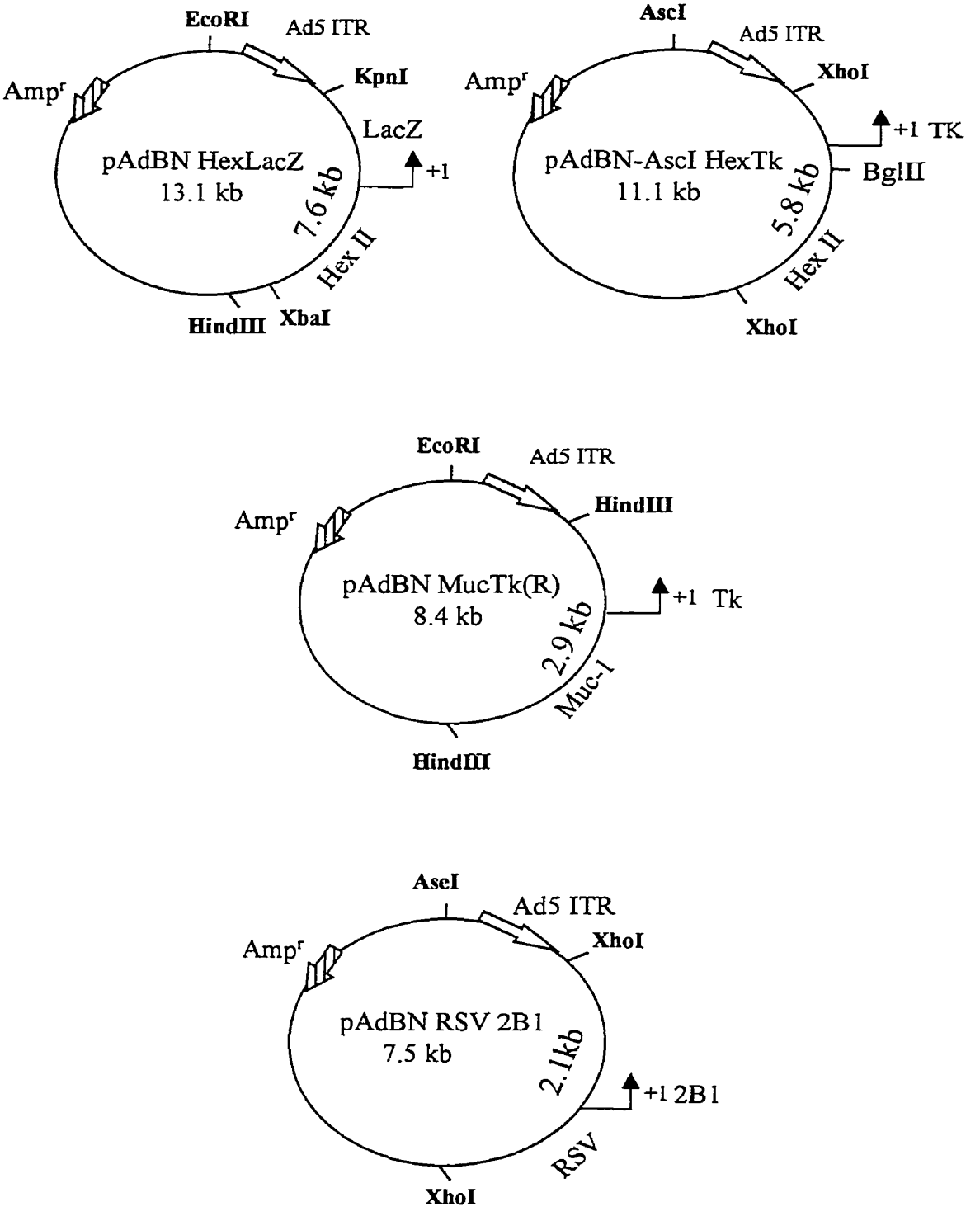
ClaI
EcoRI
EcoRI
Ad5 ITR
Amp^r
MCS
Bgl II, Asc I, Kpn I, Acc 65 I
XhoI, Hind III, EcoR V, BsiW I
SnaB I, Bcl I, Mlu I, BsiW I, Not I
pAdBN
5450 bp
Ad5 9.4-15.5 mu
AscI
Ad5 ITR
Amp^r
MCS
Acc 65 I, XhoI, Hind III, EcoR V,
BsiW I, SnaB I, Bcl I, Mlu I,
BsiWI, Not I
pAdBN-Asc I
5255 bp
Ad5 9.4-15.5 mu

C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Ad5 ITR
KpnI
Amp^r
LacZ
pAdBN HexLacZ
13.1 kb
+1
7.6 kb
Hex II
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XbaI
AscI
Ad5 ITR
XhoI
Amp^r
+1 TK
pAdBN-AscI HexTk
11.1 kb
BglII
5.8 kb
Hex II
XhoI
EcoRI
Ad5 ITR
HindIII
Amp^r
pAdBN MucTk(R)
8.4 kb
+1 Tk
2.9 kb
Muc-1
HindIII
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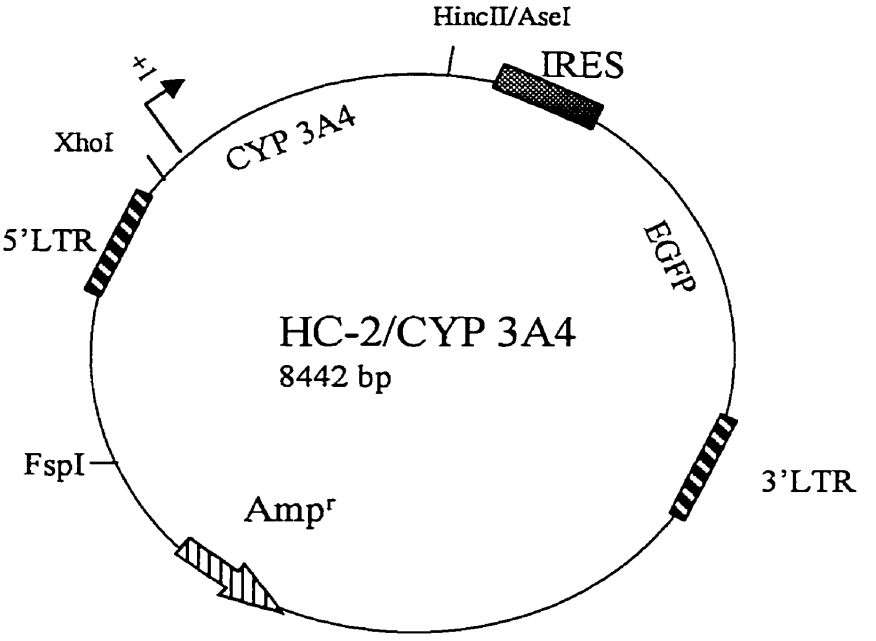
+1 2B1
RSV
XhoI

D


HincII/AseI
IRES
+1
XhoI
CYP 3A4
5'LTR
EGFP
HC-2/CYP 3A4
8442 bp
FspI
Amp^r
3'LTR

ending step and then a *BglII* digest. pUC/HexII4557 (see description above) was digested with *KpnI*, followed by a blunt-ending step and a *BglII* digest. The *BglII*/blunt-end fragment of vTk was ligated into pUC/HexII4557 to generate pUCHexTK. Unique sites were identified upstream and downstream of the HexTk expression cassette (*XbaI* and *EcoRI* respectively) and were modified with *XhoI* linkers. The expression cassette was then cloned into the *XhoI* of the pAdBN-AscI shuttle plasmid in the antisense orientation to the left ITR. The construct was linearized with *AscI* and co-transfected with Ad5 ΔE1/E3 linear viral DNA in 293 cells. AdHexTk positive clones were screened for Tk expression by performing cell killing assays (as described below) in DA-3 cells after infection with the different adenovirus clones and treatment with gancyclovir.

AdRSV2B1: the pSP450 plasmid encoding the rat cytochrome P450 2B1 isoform (CYP2B1) cDNA is a generous gift from Dr. Milton Adesnik (University of New York School of Medicine, NY, NY). To improve translation levels from the 2B1 cassette, it was modified with a Kozak sequence (292) of four GCC repeats (catgaattcgccgccgccgc) at the *NcoI* site immediately preceding the ATG codon. The modified CYP2B1 cDNA was transferred with an *EcoRI-SacI* digest to the cloning vector pBluescript (Promega, Madison, WI). The resulting cloning intermediate was cut with a *HindIII* partial digest and *HincII*. The 0.4 kb RSV (*NruI-HindIII*) promoter was isolated and inserted upstream of 2B1 in pBS/2B1 at the *HincII-HindIII* sites. An *XhoI* linker was used to create a site downstream of the RSV-2B1 cassette in Bluescript and the *XhoI-XhoI* expression cassette was cloned in the corresponding site of pAdBN in antisense orientation to the left ITR. The construct was linearized with *AseI* and co-transfected with Ad5 ΔE1/E3 linear viral DNA in 293 cells. Different adenoviral clones were used to infect 293 cells and whole

cell extracts were screened by immunoblotting as described below using a monoclonal anti-rat CYP2B1 antibody (Cedarlane Labs, Hornby, ON).

AdMucTk: the 1.55 kb *BglII-BamHI* fragment of pMC1Tk, containing the vTK cDNA was cloned into the *BamHI* site of pMUC1-1583 CAT. Subsequently, the 3.2 kb MucTk cassette was isolated using a *ClaI-BamHI* digest and ligated into the corresponding sites of pΔE1sp1B (Microbix Biosystems Inc., Toronto, ON). pΔE1sp1B is an adenoviral shuttle plasmid used to generate recombinant Ad5 particles by co-transfection in 293 cells with pBHG11 (Microbix), a circularized version of the adenoviral genome deleted in the E1 and E3 regions. This system for adenovirus production was used first in the laboratory before the adoption of the pAdBN system. The recombinant AdMucTk virus/ expresses the MucTk cassette in the sense orientation relative to the 5' ITR. Recombinant clones were selected by Southern blotting using the vTK cDNA as a probe.

AdMucTk(R): the *ClaI-BamHI* MucTk cassette from pΔE1sp1BMucTk (described above) was inserted in pUC18 (Pharmacia) in the *AccI-BamHI* sites to create pUCMucTk. The incompatible ends were converted to blunt ends using the Klenow polymerase. The *HindIII-HindIII* MucTk cassette from pUCMucTk was inserted in the corresponding site of pAdBN and a clone containing the expression cassette antisense to the left ITR was selected. The construct was linearized with *EcoRI* and co-transfected with Ad5 ΔE1/E3 linear viral DNA in 293 cells. Recombinant adenovirus clones were screened by performing cytotoxicity assays in T47D cells.

AdCMVLacZ, where the cytomegalovirus (CMV) promoter is driving the expression of the β-galactosidase gene, is a gift from Dr. B. Massie (BRI, Montreal, Canada) and was used as a positive control for histochemical marking studies. AdΔE1E3 is a gift from Dr.

Jack Gauldie (McMaster, Hamilton, Canada), and was used as a negative control in adenovirus infections experiments, since it is a replication defective adenovirus devoid of foreign genes. AdRSVTk was kindly provided by Dr. Savio Woo (Baylor University, Houston, TX), and was used as a positive control in gancyclovir sensitization experiments.

2.3 Construction of recombinant retroviruses

A retrovirus vector encoding the human cytochrome P450 3A4 isoform (CYP3A4) was constructed using HC-2. HC-2 is a retroviral plasmid based on the MSCV series (293), and modified to contain an internal ribosomal entry site (IRES) allowing for bicistronic expression cassettes to be made. Another feature of this cloning plasmid is the presence of the enhanced green fluorescent protein (EGFP) cDNA downstream of the IRES, allowing for quantitation of transduced cells in a target population by fluorescence activated cell sorting (FACS) analysis. A multiple cloning site upstream of the IRES facilitates the introduction of desired genes. HC-2 was a generous gift from Dr. Jacques Galipeau (McGill University, Montreal, QC).

The retroviral vector HC-2/CYP3A4 is outlined in fig.1D. The human CYP3A4 cDNA was obtained from Dr. Frank Gonzalez (National Cancer Institute, Bethesda, MD). It was released from the pGEM7 plasmid with a *XhoI-AseI* digest. The *AseI* site was first modified to generate a blunt-ended fragment. The resulting CYP3A4 fragment, devoid of a poly(A) signal was inserted in the *XhoI-HincII* sites of HC-2 upstream of the IRES. Elimination of the poly(A) signal is necessary for production of a bicistronic CYP3A4/GFP transcript.

AP-2 and AP-3 are retroviral vectors based on the ΔU3nlsLacZ vector backbone(129), where the enhancer-promoter region of the 5' LTR was replaced with the CMV immediate early promoter for increased viral titers after transient transfection in the 293GPG packaging cell line (129). They respectively encode for IRES/EGFP and vTk/IRES/EGFP. Both plasmids were a generous gift from Dr. Jacques Galipeau (McGill University, Montreal, QC). P450 reductase encoding retroviral plasmids, pBabe-puro-human reductase (HOR) and pBabe-hygro-rat reductase (RED) were a generous gift from Dr. David Waxman, (Boston University, Boston, MA).

293GPG cells were used to package VSV-G pseudotyped recombinant retroviral particles derived from the retroviral constructs described above. The cell line is derived from/ human embryonic kidney 293 cells, and is engineered for the stable expression of the vesicular stomatitis virus G (VSV-G) protein in an inducible fashion, using a tetracycline regulatable gene expression system, since constitutive expression of the VSV-G protein is toxic. It also expresses the necessary retroviral proteins for packaging of replication defective, infectious viral particles (129). VSV-G pseudotyped retroviruses have two major advantages over MuMLV viruses. They have a broad tropism range and are resistant to destruction during ultracentrifugation, thereby allowing for the concentration of retroviral stocks. 293GPG cells are also highly transfectable, and yield high viral titers after transient transfections of retroviral plasmids. Cells were transfected with retroviral plasmids using lipofectAmine (Canadian Life Technologies, Burlington, ON), according to the manufacturer's recommendations. Tetracycline was added to the transfection medium to suppress the production of the VSV-G protein. 6 hrs hours after transfection, the medium was replaced with RPMI 10%. 24 hrs after transfection, the medium was

replaced once more with RPMI 10%, and supernatant collection was started 48 hours later. Retrovirus supernatants were collected once a day for 6 consecutive days. The supernatants were pooled and concentrated by ultracentrifugation at 25,000 rpm, for 1 hour at 4°C. Viral stocks were stored at -80 °C.

2.4 Stable cell lines production by retroviral infection

To study the effect of pharmacological modulation of gap junctions on the bystander effect, murine mammary carcinoma DA3 cells stably expressing vTK were derived for use in mixing experiments. DA3 cells were transduced with AP-2, as a negative control, or AP-3, to generate vTK expressing cells. Briefly, 1-2 $x10^4$ cells were seeded in 24-well plates, and were infected with 50 ul of concentrated virus stock (approximate titer: 10^8/ cfu/ml) in a total volume of 500 ul. The infection cycle was carried out on three consecutive days for a total of 3 viral infections. Cells were allowed to recover in their normal growth medium for 24 hrs after the last infection. They were passaged into larger culture vessels to amplify the resulting population. Transduction efficiency was quantified by FACS analysis. The resulting polyclonal cell lines DA3-EGFP and DA3-vTKEGFP were 100% positive for GFP expression (data not shown).

To study the CYP 3A4 gene for prodrug activation based gene therapy, two human breast carcinoma cell lines were used. The parental MCF-7 and MDA-231 cells were transduced with HC-2/CYP 3A4 or AP-2, as a control for retroviral infection. The resulting MCF-7/CYP, MDA-231/CYP, MCF-7/AP-2 and MDA-231/AP-2 stable cell lines were in turn infected with P450 human or rat reductase expressing retroviruses, HOR or RED respectively, to derive cell lines expressing a combination of CYP3A4 and P540 reductase. The AP-2 derived cell lines were infected as a negative control for doubly

transduced cells and for the effect of the reductase gene alone on prodrug activation. The infection cycles were carried out as described above and stable cell lines were characterized for recombinant protein expression by western blotting of cellular extracts, and for green fluorescent protein expression by FACS analysis.

3. Transfections and reporter gene assays

Transient transfections were performed using lipofectAmine (Canadian Life Technologies, Burlington, ON), according to the manufacturer's recommendations. 70-80% confluent cells were transfected with 1 μg of the reporter plasmids pCAT, pMUC1-1583 CAT, pHexII4557 CAT or pRSV CAT (rous sarcoma virus long terminal repeat promoter) (ATCC, Rockville, MD). 1 μg of pSV_2-βgalactosidase (Promega, Madison, WI) was co-transfected as an internal control for variations in transfection efficiency in serum free D-MEM. The medium was replaced with fresh growth medium after six hours. In promoter regulation experiments, the cells were transfected in RPMI 1640 glucose deficient media. Six hours after transfection, the medium was replaced with RPMI 1640 glucose deficient media supplemented with 100 nM bovine insulin or 25 mM glucose or 10 μM glucagon (Sigma Aldrich Canada, Oakville, ON) or a combination of insulin and glucose, supplemented with 1 mM sodium pyruvate and a 100 μM glucose. Cells were harvested 24 to 48 hrs and lysed in 0.25 M Tris-HCl, pH7.5 by three successive freeze/thaw cycles. Protein concentration of the extracts was measured using the Bradford protein assay from Bio-Rad (Mississauga, ON), and equal amounts were assayed in the chloramphenicol acetyl transferase (CAT) and β-galactosidase assay, which were carried out as described by manufacturer (Promega, Madison,WI). Percent

acetylation was measured using the Bio-Rad Gelscan Phosphoimager and the Molecular Analyst software program.

4. Total RNA extraction

For regulation studies, cells were grown to subconfluence in T75 cm^2 flasks, starved in serum free, glucose-deficient RPMI 1640 media (Canadian Life Technologies, Burlington, ON) for 18hrs, and induced in glucose 25 mM, insulin 100 nM, or glucagon 10 μM (Sigma Aldrich Canada, Oakville,ON) for 12 hrs.

RNA was extracted from cells using RNAzol (TEL-TESTinc., Friendswood, TX) as described by manufacturer. Contaminating DNA was digested with 5-10 units of DNAse I (Pharmacia Biotech, Baie d'Urfe, QC) in 10 mM $MgCl_2$, 1 mM DTT, 20-40 units of RNA guard (Pharmacia Biotech, Baie d'Urfe, QC), 50-80 μl of total RNA at 1μg/μl for 30 min at 37 °C, followed by precipitation of total RNA.

5. Ribonuclease protection assay (RPA)

The HK II (0.1 fmoles) and human β-actin (2 fmoles) probes or the vTk and human β-actin probes were simultaneously hybridized to 20-30 μg of total RNA using the RPA II kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Probes were prepared by *in vitro* transcription using the MAXIscript kit (Ambion, Austin, TX) and α^{32}P UTP, 6000Ci/mmol, 40 mCi/ml. In order to generate the HK II probe template, a cDNA fragment corresponding to nucleotides 1500 to 1803 of full length HK II cDNA (GenBank Z46376) was inserted in the pDP18-T7/T3 vector (Ambion, Astin, TX). The template was cut with *KpnI* at the 5' end of the insert and transcribed towards that site from the T7 promoter to produce a 430 bp transcript. The HK II protected fragment is

303 bp. To generate the vTk probe template, the pMC1Tk plasmid, a generous gift from Mario Cappechi (NCI, Bethesda, MD) was cut with *EcoRV*. The resulting 104 bp fragment was aligned with the human thymidine kinase sequence and found to have no homology at 80 % and 90%. This alignment was done to demonstrate that the probe used to hybridize RNA in the RPA was recognizing the transduced viral thymidine kinase and not the endogenous one present in human cells. The blunt ended 104 bp Tk fragment was ligated in the *SmaI* site of the pDP18-T7/T3 vector. The resulting pDP18/Tk template was cut with *EcoRI* at the 5' end of the insert and transcribed toward that site with the T7 polymerase. The unprotected transcript is 167 bp long and the protected Tk transcript is 104 bp.

The human β-actin template pTRI-βactin human was purchased from Ambion and transcribed with the T7 polymerase to produce a 300 bp transcript which protects a 245 bp fragment. Ambion's Century marker templates were used to synthesize 100 to 500 bp long fragments. Electrophoresis was performed in 5% acrylamide/8M urea gels. Quantitation was performed using the NIH image 1.67b densitometry software.

6. Histochemical staining for β-galactosidase expression

Cells were plated at $2x10^5$ in 6 well plates, one day prior to virus infection. They were infected with viruses at a multiplicity of infection (MOI) of 10. Each condition was done in duplicate. After 48 hrs, they were fixed with 1% glutaraldehyde, washed with 0.02% NP40 in PBS and stained for 16 hrs with 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$, 0.01% sodium deoxycholate, 2 mM MgCl2, 1mM EGTA, 1 mg/ml X-gal in 0.02% NP40 PBS. Staining was scored by visualization under light microscopy. For in vivo marking studies, two different mouse models were used. For the mouse mammary carcinoma

model, DA-3 cells were grown as a subcutaneous tumor in syngeneic mice. For the human lung carcinoma model, NCI-H661 cells were grown as a subcutaneous tumor in nude mice. Cells were trypsinized and washed in ice-cold serum free medium less than 30 minutes before injections. $5x10^6$ cells, resuspended in 0.5 ml of medium were injected subcutaneously in the flank of the animals. Once tumors were palpable, after 3-4 days on average, intratumoral injections of $1x10^9$ plaque forming units (PFUs) of AdHexLacZ or AdΔE1E3 were done with a 25-gauge needle in a total volume of 0.2 ml, in viral storage buffer, at the same injection site. Two mice were injected with each virus, for each model. Animals were sacrificed 48 hrs after viral injections and tumors were excised and snap frozen in liquid nitrogen. Frozen sections of these tumors were mounted on/ microscope slides and fixed and stained with X-gal as described.

7. Cell viability assays, viral infections and prodrug treatments

Cell viability was determined, after the various treatments described below, using a colorimetric assay which measures the ability of viable cells to reduce MTT (Sigma), a soluble yellow tetrazolium salt to an insoluble purple formazan precipitate. The MTT assay was carried out as described (294) and readings were done in a Bio-Rad plate reader, model 450, at 570 nm. In the case of the CYPs activation studies, cell viability was also determined using another cell toxicity assay using crystal violets (Sigma) as described (295). Different prodrugs were used: gancyclovir or GCV (Cytovene™, Hoffman-La-Roche), cyclophosphamide or CPA (Sigma), R-ifosfamide or R-IFA and S-ifosfamide or S-IFA, a gift from Dr. Irving Wainer (McGill University, Montreal, QC), activated derivative of IFA, 4-hydroperoxy-IFA, a gift from Dr. David Waxman (Boston

University, Boston, MA), and ifosfamide or IFA (IFEX™, Rhone-Poulenc Rorer). R-IFA and S-IFA are the two enantiomers of IFA.

7.1 Evaluation of AdHexTk/GCV combination

To generate a dose-response curve for GCV toxicity at increasing multiplicities of infection (MOI), cells were seeded in 96-well plates at a cell density of 3-5 x 10^3, depending on the cell line on day 1. On day 2, they were infected with viral dilutions ranging between an MOI of 0 and 100, of either AdHexLacZ or AdHexTk. Gancyclovir (GCV) was added at a concentration of 25 ug/ml for the AdHexLacZ control and either 10 or 25 ug/ml for the AdHexTk infected cells. On day 4, culture medium was changed and a fresh dose of GCV was added. On day 6, an MTT assay was performed to measure cells viability. Each cell line was tested at least 3 times. Percent survival is determined by ratios of absorbance values from test conditions over absorbance values from non-infected cells (MOI 0). For killing curves, cells were seeded in T25 cm^2 flasks on day 1. On day 2, they were infected with the appropriate MOI (see fig.10 and table 1) with AdHexLacZ or AdHexTk. On day 3, they were trypsinized, counted and plated at $5x10^3$ cell/well in 96-well plates. GCV was added at concentrations ranging from 0 to 5000 ug/ml. On day 5, the culture medium was changed and a fresh dose of GCV. On day 7, the MTT was performed and cell survival percentages calculated as described above. Each cell line was tested at least 3 times.

7.2 Evaluation of AdMucTk/GCV combination

To generate a dose-response curve for GCV toxicity at increasing multiplicities of infection (MOI), cells were seeded in 96-well plates at a cell density of 1-4 $x10^3$, depending on the cell line on day 1. On day 2, they were infected with viral dilutions of

AdMucTk or AdMucTk(R) ranging between an MOI of 0 to 20. Gancyclovir (GCV) was added at a concentration of 0, 10 or 25 ug/ml. Each condition was carried out in quadruplicate. On day 4, culture medium was changed and a fresh dose of GCV was added. On day 6, an MTT assay was performed to measure cells viability. Synergistic killing due to a combination of virus and prodrug was scored as a percentage of proliferation of infected cells treated with GCV versus proliferation of uninfected cells treated with GCV minus the effects of virus alone. Each cell line was tested at least 3 times.

7.3 Evaluation of bystander effect in cytotoxicity/mixing experiments

A cytotoxicity assay utilizing either DA3 infected with AdRSVTk at an MOI of 10 or uninfected DA3 followed by exposure to the prodrug GCV was used to measure the effect of chemical induction of intercellular communication on suicide gene therapy *in vitro*. $6x10^5$ DA3 cells were plated in each of 4 x 75 cm^2 tissue culture flasks and allowed to adhere overnight. The following day, two flasks were treated with cAMP to a final concentration of 1 mM. AdRSVTk was used to infect one flask of treated and one of cells not treated with cAMP, at a multiplicity of infection of 10. Forty eight hours later (when the GJIC of cells treated with cAMP was induced according to FACS analysis studies), the treated cells were harvested with trypsin, washed once with Dulbecco's buffered saline solution and resuspended in a final concentration of 10^5 cells/ml. For each treatment, infected cells were mixed with uninfected DA3 cells to obtain a population of cells where 0, 1, 2, 5, 10, 20, 50 or 100% of the cells were transduced with AdRSVTk. The cells were then seeded at 10^4 cells per well in a 96-well tissue culture plate and treated with various doses of gancyclovir (one 96-well plate per cAMP-treatment and

gancyclovir concentration was used, and final gancyclovir concentrations were 10 and 20 μg/ml). The high cell density was selected to ensure cell-cell contact. After three days, the MTT assay was carried out to measure cell viability. Cell viability for each condition was determined as the ratio of cells (of the same infection-percentage and cAMP-treatment) treated with gancyclovir to gancyclovir-untreated cells. In order to control for cAMP and gancyclovir non-specific effects on cell viability and proliferation, the cell viability of uninfected cells of the same cAMP-treatment was used as a reference. Percent cell survival for each condition was scored as the ratio of cell viability divided by the cell viability of uninfected cells (0% infection) of the same treatment. Four replicates per condition, mixing percentage, and treatment were used, and the whole mixing experiment was repeated four times.

7.4 Evaluation of vTk/GCV resistance phenotype in selected cell cultures

To investigate the phenotype of resistance to activated GCV observed *in vitro* in cytotoxicity assays, killing assays for AdRSVTk or AdCMVLacZ (used as a control for non-specific viral effect) infected cells treated with GCV were done. Cells were seeded in T25 cm^2 flasks on day 1. On day 2, they were infected at an MOI of 10 or 50 with AdCMVLacZ or AdRSVTk. On day 3, they were trypsinized, counted and plated at $5x10^3$ cell/well in 96-well plates. GCV was added at concentrations ranging from 0 to 5000 ug/ml. On day 5, the culture medium was changed and a fresh dose of GCV. On day 7, the MTT was performed and cell survival percentages derived as described above. Each condition was done in quadruplicate and each cell line was tested at least 3 times. Cells stably expressing Tk were seeded in 96-well plates at $5x10^3$ and treated with GCV as described.

7.5 Evaluation of CYP3A4 and CYP2B1 prodrug activation efficacy

Cells expressing CYP 3A4 and/or P450 reductase were seeded in 96-well plates at 2.5-5 $x10^2$ cells per well. The next day, CPA, R-IFA, S-IFA or 4-hydroperoxy-IFA, were added to the culture medium at dilutions ranging from 50 uM to 2 mM. Cells were exposed to the drug for a period of 4-5 days. Cell survival was determined with the MTT assay as described above, or with a crystal violet cell viability assay (295). Each condition was done in quadruplicate and each experiment repeated at least three times. To test the CYP2B1/CPA combination, parental MCF-7 or MDA-231 cells, or derivatives of these two cell lines expressing the P450 reductase gene, were seeded in T-25cm^2 flasks and infected with a recombinant adenovirus expressing CYP2B1 at an MOI of 50 or 100. The next day, cells were trypsinized and seeded in 96-well plates at $1x10^3$ cells per well, treated with CPA for 4 days and cell survival determined as described above.

8. Chemical induction of GJIC

8-bromoadenosine 3':5'-cyclic monophosphate (cAMP) was obtained from Sigma Chemical Co. (St. Louis, MO). Calcein acetoxymethylester (Calcein AM) and 1,1'-dioctadecyl-3,3,3,3'-tetramethylondocarbocyanine perchlorate (DiI) were obtained from Molecular Probes Inc (Eugene, OR).

8.1 Immunofluorescent studies for connexin 43

The presence of Cx43, one of the predominant gap junction proteins expressed in breast cells and one shown to be reduced during carcinogenesis, was examined in the DA3 mouse mammary cancer cell lines. Untreated or cAMP-treated cells with 1 mM cAMP for 48 hrs were grown onto cover slips, and then fixed in a 80:20 ratio by volume of methanol and acetone, for 10-20 minutes at 20°C, blocked with 2% BSA in PBS for 1

hour and incubated with a rabbit antibody directed against residues 360-382 of Cx43 (265) for 1 hour. After washing, the samples were incubated with goat anti-rabbit antibody conjugated to rhodamine. The cells were mounted on coverslips and imaged with a 25x 0.8 NA lens under identical conditions using a Zeiss LSM confocal microscope. Transmitted light and fluorescent images were printed on a Kodak XSL8300 printer.

8.2 Functional Assay of Gap Junctional Intercellular Communication (GJIC).

Fluorescence Activated Cell Sorting (FACS) analysis, as described by Kiang (296), with minor modifications, was used. Cells were trypsinized, washed in PBS, and stained for 15 minutes with either 4 μM DiI to mark the recipient cells, or 0.5 μM Calcein AM to load the donor cells. Cells were then washed with PBS and equal numbers of donor and recipient cells were mixed and incubated for 3.5 hours in 12-well plates. In order to maintain the same degree of cell density and contact in different experiments, cells were always plated at the same density of 500,000/well in 24-well plates. After incubation, cells were washed with PBS, trypsinized, resuspended in PBS and then analyzed by flow cytometry. Ten thousand cells were counted by two color sorting in a Coulter Epics XL-MCL apparatus. The excitation wavelength used was 488 nm, and the emission wavelengths for Calcein AM and DiI were 525 nm and 575 nm respectively. Gap junctional intercellular communication is defined as the percentage of recipient cells that received Calcein AM from donor cells (only possible through gap junctions, (270)), corrected for background using a non-incubated control. Four replicates per condition were used, and the whole assay was performed twice.

9. Hirt lysis and infectivity controls

Episomal DNA was isolated using the Hirt lysis protocol. Cells were harvested by trypsinization and pellets washed twice in ice-cold PBS. After resuspension in TE buffer, cell pellets were disrupted by three cycles of freeze/thaw. An equal volume of Triton-X100 was added and samples were incubated at 0°C for 10 minutes. NaCl at a final concentration of 0.3 M was added and samples were incubated at 0°C for 2 hours. Samples were then centrifuged for 5 minutes at 12,000g and supernatants were incubated with self-digested pronase at 1 mg/ml for 1 hour at 37°C. Finally, RNase A was added at a final concentration of 20 μg/ml and samples were incubated for 30 minutes at 37°C. DNA was resuspended in a minimal volume after phenol:chloroform extraction followed by ethanol precipitation. Equal amounts of DNA were immobilized on a nylon membrane using a dot blot apparatus (Bio-Rad). Blots were probed with a radiolabelled a 500 bp vTK cDNA fragment.

Two experiments were carried out in the context of the cytotoxicity/mixing studies. The first was to test the effect of the activated cAMP-dependent pathway on infectivity after cAMP treatment. $2x10^5$ DA3 were seeded in two 25 cm^2 plates and incubated overnight. The following day, cAMP was added in one of the two plates, at a final concentration of 1 mM. Cells were infected with Ad-RSV-vTK (MOI of 10) 48 hours post-treatment with cAMP. At 12 hours post-infection, cells were lysed as described above. The second experiment was intended to see the effect of cAMP on infectivity and viral DNA stability and expression in the mixing assay conditions. The precise conditions of the cell mixing assay, as described previously, were replicated, and cells were collected and lysed at 24 and 48 hours post infection.

To test for differences in infectivity in T47D and ZR75 cells infected with AdRSVTk, cells were plated at $3x10^5$ in 25 cm^2 flasks, infected the next day at an MOI of 20 with AdRSVTk or AdΔE1E3, used as a negative control. Cells were harvested by trypsinization after 24 hours and lysed as described.

10. GCV uptake studies

Cells were seeded in 24-well plates at $8x10^4$ cells/well. Forty-eight hours later, they were washed with PBS and the medium was replaced with [8-^{3}H]-GCV (Moravek Biochemicals Inc., Brea, CA), at a concentration of 0.5 ug/ml. Cells were harvested by trypsinization at 1, 5, 15, 30, 60, 120, 240 minutes and 24 hours. Cell pellets were resuspended in PBS and GCV content was determined by counting samples in a Wallace scintillation counter. In parallel to the cells seeded for the GCV uptake experiment, replicate wells were seeded under the same conditions and harvested at the same time points. Cell were lysed by three successive freeze/thaw cycles and cell extracts were used to determine protein concentration of each sample using the Bradford protein assay reagent from Bio-Rad. Radioactivity measurements were normalized to protein contents. The experiment was repeated three times.

11. Microsomes preparation and immunoblotting

For preparation of microsomes, cells were grown in 100mm dishes to subconfluence. Monolayers were washed with ice-cold 50 mM potassium phosphate buffer, pH 7.4-1mM EDTA. The buffer was made by mixing appropriate volumes of monobasic and dibasic potassium phosphate buffers, both at the same molarity, so that the final pH is 7.4. Cells were scraped off the dishes (4 dishes were used for each cell line) in 8 ml of the same

buffer and collected in 15 ml Corex tubes. They were lysed by sonication in 6 cycles of 7 seconds sonication, at 7 microns, and 14 seconds breaks in between. The cell lysate was centrifuged for 20 min at 12,000 x *g*, 4°C. Supernatants were transferred to 14 ml polypropylene tubes (Beckman) and ultracentrifuged in a SW41 rotor at 30,000 rpm for 60 min at 4°C. Microsomes were resuspended by sonication in 50 mM potassium phosphate buffer, 1mM EDTA, 20% glycerol and assayed for protein content using the Bradford protein assay from Bio-Rad. 40 ug protein from each cell sample were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. 0.2 to 1 pmoles of recombinant human P450 reductase (Cedarlane Labs, Hornby, ON) and lymphoblast expressed recombinant CYP3A4 (Gentest Corporation, Woburn, MA) were used as standards to evaluate sample contents for each of these proteins. Blots were probed with rabbit anti-human cytochrome P450 reductase polyclonal antibody or rabbit polyclonal CYP3A4 antibody (Cedarlane Labs). Detection of antigen-antibody complexes was performed using ECL western blotting detection reagents (Amersham) according to the manufacturer's instructions.

12. Immunofluorescent labeling

Cells were seeded in 6 well plates. Sterilized cover slips were laid at the bottom of each well and covered with 2 mls of medium. 2-3 x 10^4 cells were seeded in each well, 2 days prior to the immunolabeling procedure. Cells were then washed with PBS, fixed with 3% paraformaldehyde for 5 minutes at room temperature (R.T.), washed once with PBS, then fixed with cold methanol for 15 minutes at –20°C. They were then washed thoroughly with PBS and incubated at 4°C for 12-18 hours. Cells were fixed in 2% bovine serum albumin, 0.2% gelatin in PBS for 1 hour at R.T.. They were incubated with either rabbit

anti-human cytochrome P450 reductase polyclonal antibody, diluted at 1:300 in blocking solution, or rabbit polyclonal CYP3A4 antibody, diluted at 1: 500 (Cedarlane Labs), for one hour at R.T. Control wells for non-specific secondary antibody reactivity did not receive any primary antibody. Cells were washed thoroughly with 0.2% BSA in PBS and incubated for 30 minutes at R.T. in the dark with a goat anti rabbit antibody conjugated to Texas Red. After repeated washing, the cells were finally mounted on microscope slides in Gelvatol (Air Product and Chemical Inc., Allentown, PA) and viewed in a Zeiss Axiophot fluorescent microscope equipped with a 63x Plan Apochromt objective and selective filters. Images were photographed using a Kodak Elite Chrome Tungsten 160T film.

13. Statistical analysis

One-factor analysis of variance ANOVA, t-tests with Bonferroni's correction for multiple comparisons and standard deviation were calculated for the appropriate experiments using Microsoft Excel.

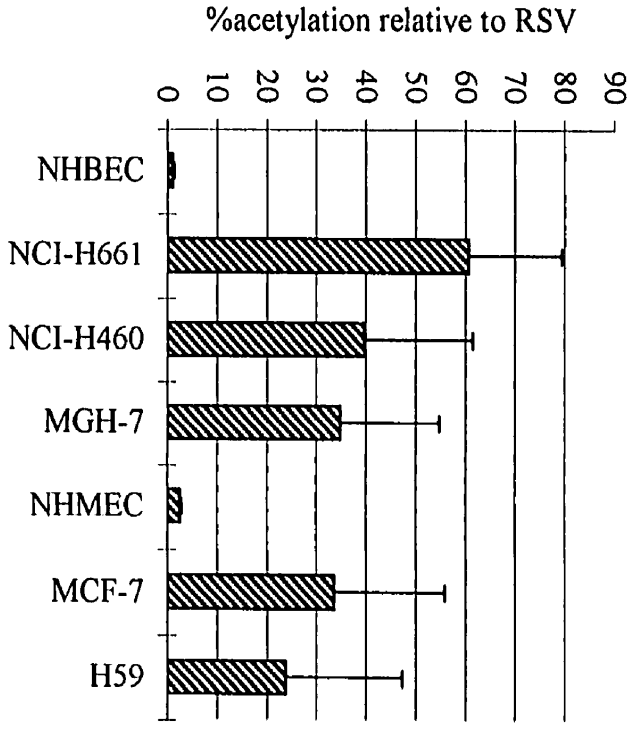
CHAPTER III: Hexokinase II, a Tumor Specific Promoter for Human Gene Therapy

1. Promoter activation studies

1.1 Relative activity of the HK II promoter in human normal and tumor cells

The rat hexokinase type II promoter was subcloned in the pCAT basic vector, in order to study promoter activation using the chloramphenicol acetyl transferase reporter gene (CAT) (fig.1A). The pHexII4557 CAT reporter gene construct was transiently transfected in a panel of normal and tumor human cells to determine the level of activation of the hexokinase type II promoter. To allow for comparisons amongst the various cell lines, acetylation in pHexII4557 CAT transfected cells was corrected for background acetylation observed in cells transfected with pCAT alone. Acetylation values were normalized for variations in transfection efficiency using β-galactosidase activity. The same amount of protein was used for both CAT and β-galactosidase assays, making it unnecessary to express values relative to mg of proteins. Finally, acetylation in pHexII4557 CAT transfectants is expressed relative to the one in pRSV CAT transfectants, where the constitutive Rous Sarcoma Virus LTR promoter is driving CAT expression (fig.2). HK II promoter activity was higher in all tumor cell lines tested than in primary normal cells of the same origin. In lung derived cells, relative CAT activity in NCI-H661 cells was the highest at 61% of RSV CAT, followed by NCI-H460 at 40% and MGH-7 at 35%. In contrast, it was low at 0.9% in NHBEC originating from two different donors. A similar pattern was observed in mammary tissue where CAT activity was at 20% in MCF-7, as opposed to NHMEC where it was 2.4% of pRSV CAT activity. In H59 cells, derived from a Lewis lung carcinoma, HK II activation was 30% of RSV (ANOVA P-value: 0.04).

Fig.2 Promoter activation studies in a panel of normal and tumor cells expressed as % acetylation in pHexII4557 CAT transfectants relative to % acetylation in pRSV CAT transfectants. Background acetylation from pCAT transfectants was subtracted and results were normalized to β-galactosidase activity used as an internal control. Results are the average of 3-4 independent experiments with each condition done in duplicate. Error bars represent standard deviations. Analysis of variance was performed on these groups; ANOVA P=0.04.


%acetylation relative to RSV
0
10
20
30
40
50
60
70
80
90
NHBEC
NCI-H661
NCI-H460
MGH-7
NHMEC
MCF-7
H59

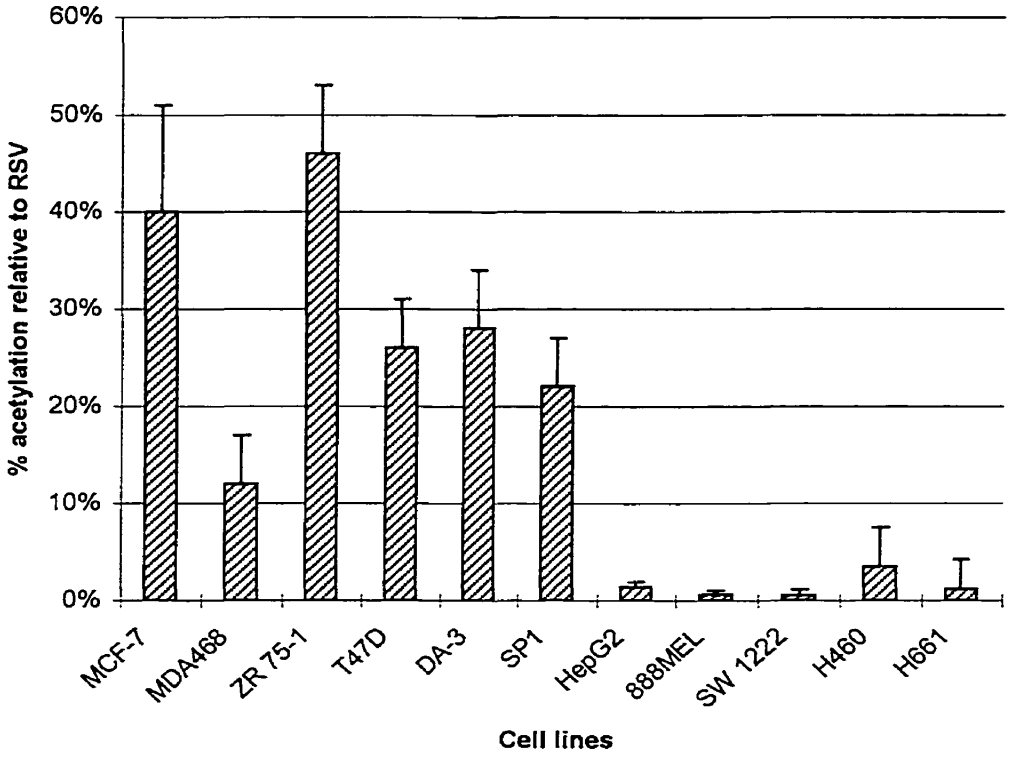
1.2 Relative activity of the MUC-1 promoter in mammary tumor cells

In order to determine the tissue specificity of the MUC1 mucin promoter, expression assays were carried out using a chloramphenicol acetyl transferase (CAT) reporter gene driven by the 1.6 kb MUC1 promoter, from -1583 bp to +33 bp, relative to the transcriptional start site. Various cell lines were transfected with pGCATA-MUC1-1583/+33CAT (pMuc-1 CAT) and cell extracts were measured for CAT activity (fig.3). Two human mammary adenocarcinoma lines, MCF7 and MDA-MB-468, were found to be permissive for the MUC1 promoter. Compared to the constitutive RSV LTR promoter, the MUC1 promoter is expressed in MCF7 at a level of 40%. In MDA-MB-468, MUC1 is expressed at 12%, compared to RSV. In ZR-75-1 and T-47D, two human ductal/ carcinomas, MUC1 promoter activation is 46% and 26%, respectively. In DA3 and SP1, two murine mammary carcinoma lines, MUC1 promoter activity is 28% and 22% respectively, compared to RSV. These findings confirm previous reports that the MUC1 promoter is very highly active in mammary cell lines (200).

HEPG2, a human hepatoblastoma, was non-permissive for the MUC1 promoter with expression levels of 1.4% compared with RSV. As well, 888MEL, a human malignant melanoma, was also found to be non-permissive for the MUC1 promoter, activation being 0.6% compared with RSV. Furthermore, the human colon adenocarcinoma, SW1222, and the human large cell lung carcinoma, H460, were also shown to be only very slightly permissive for the MUC1 promoter; the levels of MUC1 promoter activity were 0.5% and 3.5%, respectively.

These studies of the efficacy and the tissue specificity of the MUC1 mucin promoter using the CAT reporter gene confirm that the MUC1 promoter is very strongly active in

Fig.3 Promoter activation studies in a panel of tumor cells expressed as % acetylation in pMUC1-1583 CAT transfectants relative to % acetylation in pRSV CAT transfectants. Background acetylation from pCAT transfectants was subtracted and results were normalized to β-galactosidase activity used as an internal control. Results are the average of 3-4 independent experiments with each condition done in duplicate. Error bars represent standard deviations. Analysis of variance was performed on these groups; ANOVA P=0.03.


60%
50%
40%
30%
20%
10%
0%
% acetylation relative to RSV
MCF-7
MDA468
ZR 75-1
T47D
DA-3
SP1
HepG2
888MEL
SW 1222
H460
H661
Cell lines

mammary carcinoma cell lines and is specific for these cell lines as well. Activation of the MUC1 mucin promoter does not occur in the hepatic, epithelial, colon or pulmonary carcinoma cell lines tested.

1.3 HK II and Muc-1 comparisons

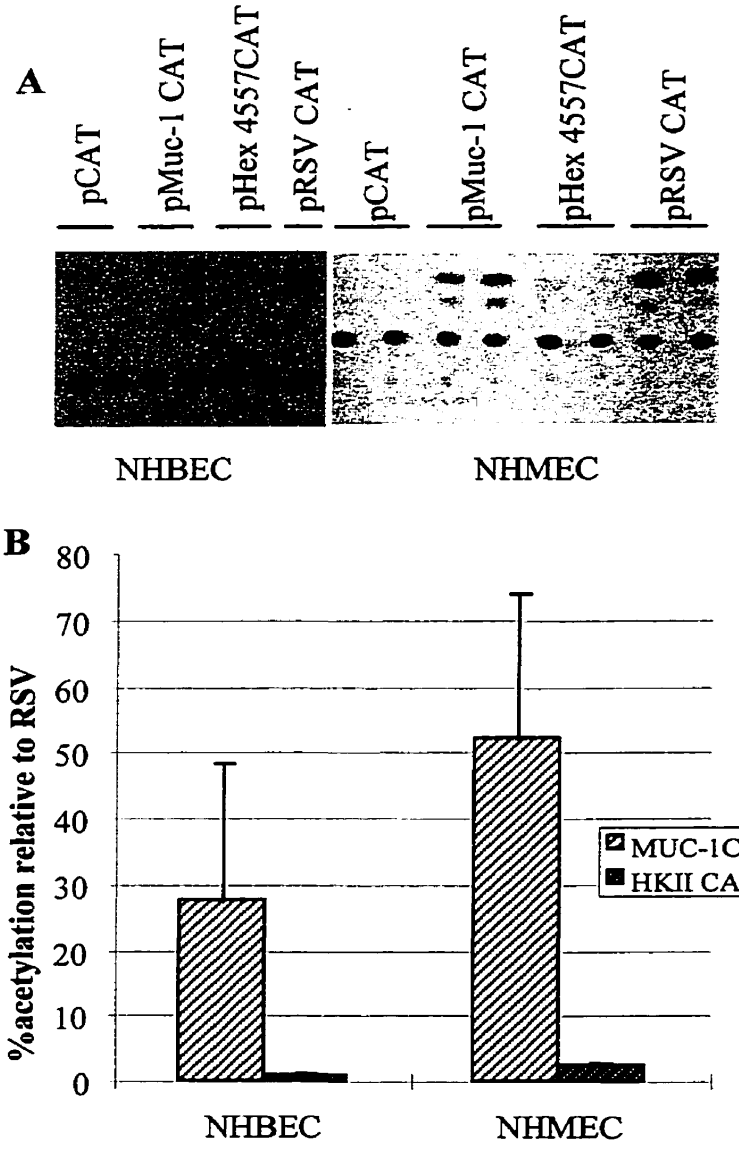
In order to confirm the tumor versus normal tissue activation of the HK II promoter we compared it to the tissue specific mucin-1 promoter (MUC-1), which was studied previously in our laboratory and others' (202). The difference in activation between these two promoters in primary normal human cells of bronchial or mammary origin is striking (fig.4A,4B). NHBEC transfected with pMUC1 CAT exhibited a relative activity of 28% while the same cells transfected with pHexII4557 CAT had only 0.9% activity. Similarly, NHMEC transfected with pMUC1 CAT exhibited CAT activity of 52% whereas the same cells transfected with pHex 4557 CAT had 2.4% activity. These results show the selectivity of HK II activation in tumor against normal cells and demonstrate the differences between the use of a tumor-specific promoter and a tissue-specific one.

2. Hexokinase II regulation studies

2.1 Modulation of HK II promoter activity with regulators of glucose metabolism

Transcription of the hexokinase type II gene has been studied in rat skeletal muscle tissue and in rat hepatocytes and was found to be up-regulated in response to glucose and insulin and down-regulated in response to glucagon (217). In chemically transformed rat hepatocytes however, regulation is altered. While insulin and glucose still stimulate HK II activation, the suppression effect of glucagon is lost. Therefore, we were interested to see if this tight regulation was maintained for the rat tumor HK II promoter when

Fig.4 Promoter activation studies as % acetylation in pHexII4557 CAT or pMUC1-1583 CAT transfectants relative to % acetylation in pRSV CAT transfectants. **A**: representative CAT assay in normal cells; **B**: comparison between HKII and MUC-1 promoter activation in NHBEC and NHMEC. Background acetylation from pCAT transfectants was subtracted and results were normalized to β-galactosidase activity used as an internal control. Results are the average of 3-4 independent experiments with each condition done in duplicate. Error bars represent standard deviations. Student's t test was used to compare results from the two promoter constructs in NHBEC (P=0.03) and in NHMEC (P=0.01).


A
pCAT
pMuc-1 CAT
pHex 4557CAT
pRSV CAT
pCAT
pMuc-1 CAT
pHex 4557CAT
pRSV CAT
NHBEC
NHMEC
B
%acetylation relative to RSV
80
70
60
50
40
30
20
10
0
MUC-1CAT
HKII CAT
NHBEC
NHMEC

transfected in human cells. In human lung carcinoma cells H661 (fig.5), promoter activation was greatest with both glucose (25mM) and insulin (100nM) with a two-fold increase over basal. While the differences between groups were significant (ANOVA P=0.01), incubation with glucose, insulin, and glucagon did not lead to dramatic differences in HK II levels. These results demonstrate the same induction effect of glucose and insulin, but also the same lack of suppressive effect for glucagon observed for the HK II promoter in transformed rat hepatocytes.

2.2 Modulation of HK II mRNA levels in normal and tumor cells

The use of a reporter gene system to characterize the modulation of HK II in primary normal bronchial cells NHBEC was not possible since we have shown that NHBEC do not activate HK II to induce CAT activity (figs. 2 and 4). Instead, endogenous HK II levels in these cells were measured in order to gain insight into the possible mechanism of HK II regulation (fig.6). In NHBEC, there was a significant induction over basal levels with glucose, (P=0.005), while glucagon repressed HK II expression (P=0.05). This regulation was clearly lost in NCI-H460 and NCI-H661, where levels of HK II mRNA show no significant variation under all conditions. These results demonstrate differences in HK II regulation between human tumor and normal cells.

3. Activation of HK II in adenoviral vectors

3.1 *In vitro* studies of HK II promoter specificity

To test the feasibility of using the HK II promoter in adenoviral vectors for targeted gene therapy, recombinant adenoviral particles carrying the HK II promoter and β-galactosidase marker gene were generated, based on the pAdBN-HexLacZ shuttle vector

Fig.5 Regulation of HK II promoter studied in NCI-H661 cells. Cells were transfected with pHexII4557 CAT as described and treated with 25 mM glucose, or 100 nM insulin, or 10 μM glucagon or a combination of glucose and insulin. Results are the average of 3 independent experiments with each condition done in duplicate. Error bars represent standard deviations. Analysis of variance was performed on these groups; ANOVA P=0.014.

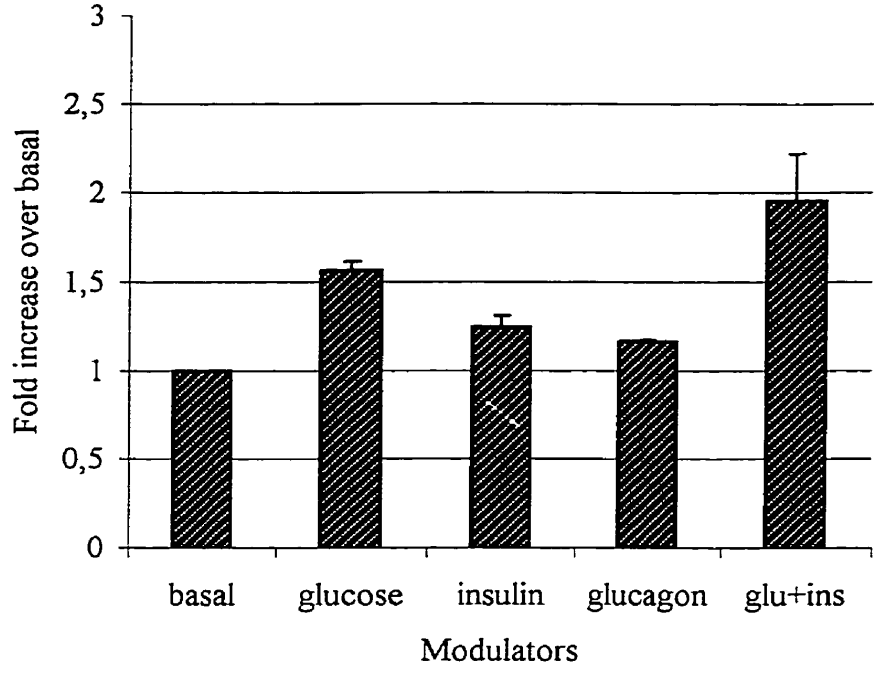
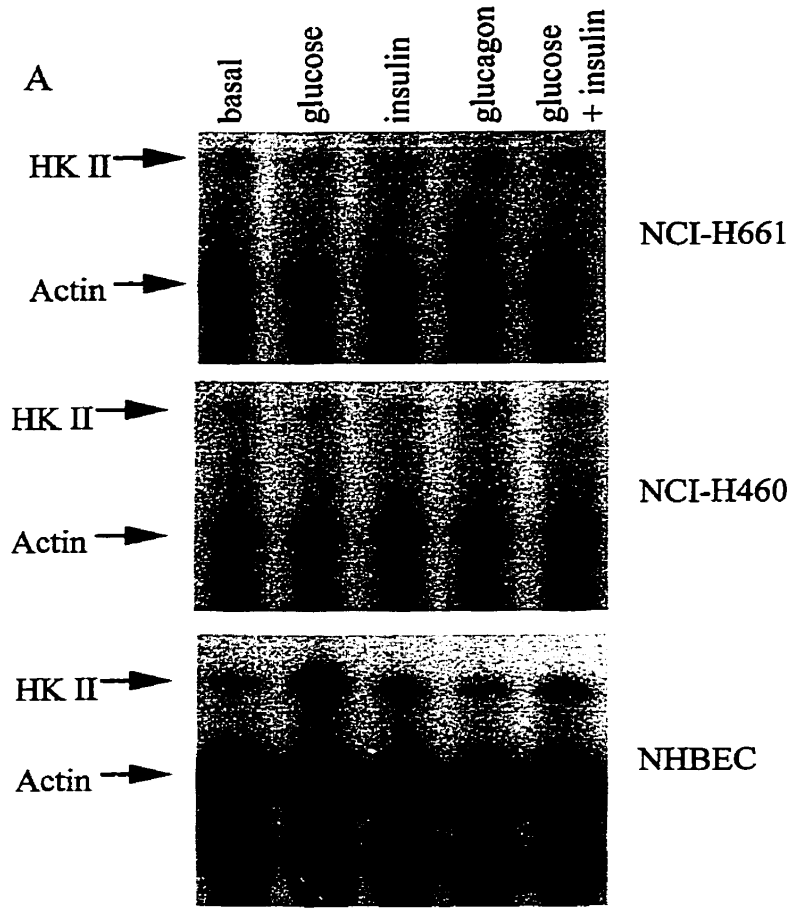
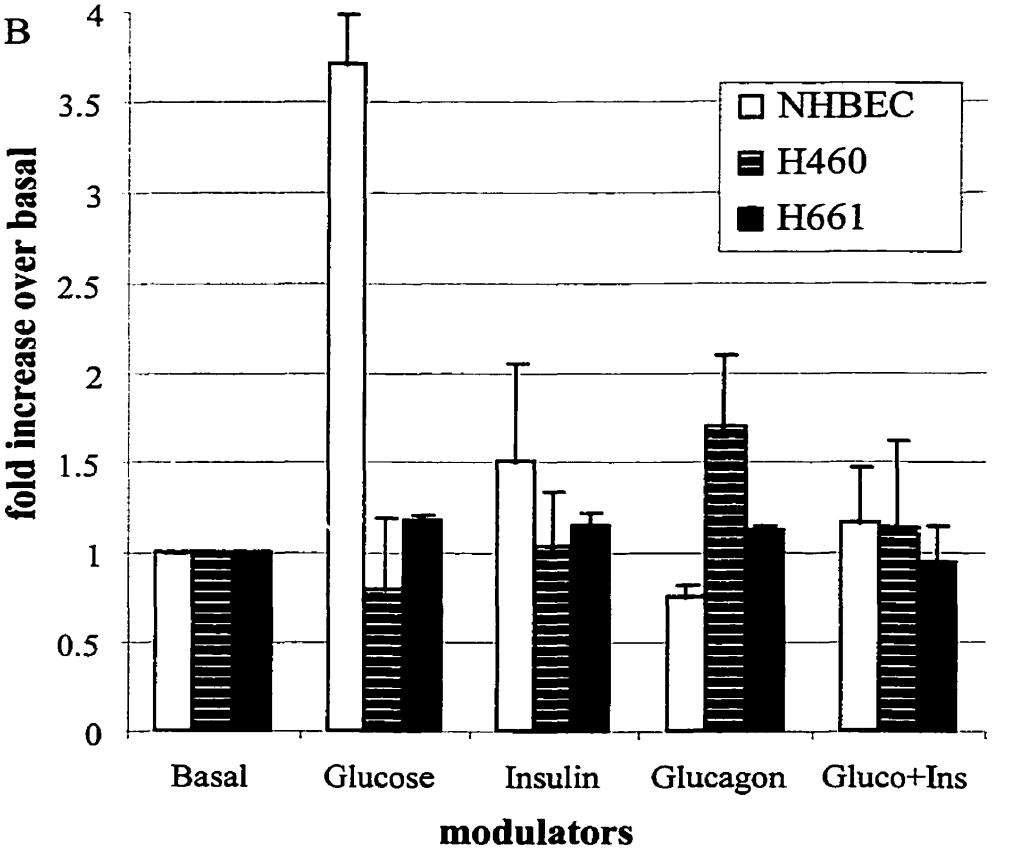

3
2,5
2
1,5
1
0,5
0
Fold increase over basal
basal
glucose
insulin
glucagon
glu+ins
Modulators

Fig.6 Regulation of HK II promoter in normal and transformed cells. **A**: Relative levels of HK II mRNA in NHBEC, NCI-H460, and NCI-H661. Ribonuclease protection assays were performed using simultaneous hybridization with the HK II probe and the human β-actin probe used as an internal control. **B**: quantitation of ribonuclease protection assays; Values represent HK II mRNA levels normalized to β-actin and expressed as ratios over basal from cells grown under basal conditions (control) or induced with glucose, insulin, glucagon, or a combination of insulin and glucose. Results are the average of 3 independent experiments. Error bars represent standard deviations. Analysis of variance was performed on these groups in each of NHBEC ($P=0.001$), H460 and H661; in NHBEC, comparisons of different modulators to levels under basal using t-tests show a significant difference with glucose ($P=0.005$) and glucagon ($P=0.05$).


A
basal
glucose
insulin
glucagon
glucose + insulin
HK II
Actin
NCI-H661
HK II
Actin
NCI-H460
HK II
Actin
NHBEC

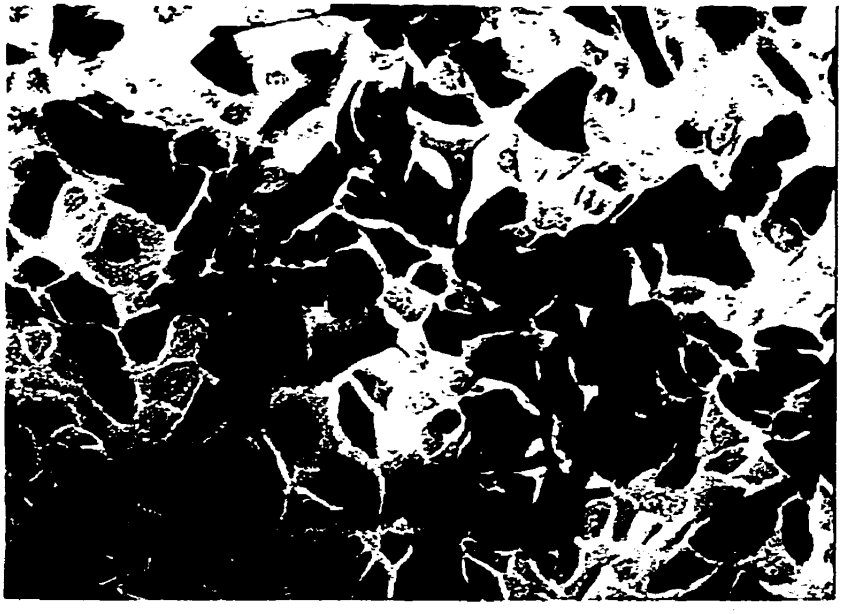

B
fold increase over basal
4
3.5
3
2.5
2
1.5
1
0.5
0
NHBEC
H460
H661
Basal
Glucose
Insulin
Glucagon
Gluco+Ins
modulators

(fig.1C). The adenovirus AdHexLacZ was used to infect a variety of cell lines at an MOI of 10. Levels of X-gal staining were compared for each cell line with AdCMVLacZ infected cells used as a positive control and AdΔE1E3 infected cells as a negative control, at the same MOI (fig.7). In cell lines of bronchial origin, NCI-H661 transduced with AdHexLacZ (fig.7F) showed the highest level of staining. It was at 95% of AdCMV LacZ transduced cells (fig.7E), while level of staining in AdHexLacZ infected NCI-H460 (fig.7I) was 10% of AdCMV LacZ infected cells (fig.7H). In NHBEC, staining in AdHexLacZ (fig.7C) infected cells was 1% of AdCMVLacZ (fig.7B) infected cells. Staining in NCI-H661, NCI-H460, and NHBEC infected with AdΔE1E3 (fig. 7D, 7G, and 7A, respectively) was negative. Other cell types tested include human hepatoblastoma HepG2 and primary cultures of normal mouse hepatocytes. In HepG2, staining in AdHexLacZ (fig.7O) infected cells was 30-40% of AdCMVLacZ (fig.7N) infected cells. In mouse hepatocytes cultures, staining in AdHexLacZ (fig.7L) infected cells was 1% or less than AdCMVLacZ (fig.7K) infected cells. All infections were repeated at least three times to confirm reported results. Pictures shown are taken from representative experiments. These results demonstrate the validity of using HK II as a tumor specific promoter in adenoviral vectors, since it is activated in transformed cells only and not in normal ones. However, all infections described previously were done at an MOI of 10. To test if specificity of the promoter for tumor cells is retained at a wider range of MOIs, NHBEC were infected with AdHexLacZ at MOIs of 10, 50 or 100. At an MOI of 10, no activation was observed, as shown before (fig.7C). At an MOI of 50 (fig.8A), 30-40 % of the cells stained positive relative to the positive control infection

Fig.7 Histochemical staining for β-galactosidase expression. All infections are done at an MOI of 10. Panels A, B, and C: NHBEC infected with AdΔE1E3, AdCMVLacZ and AdHexLacZ, respectively; panels D,E, and F: NCI-H661 infected with AdΔE1E3, AdCMVLacZ and AdHexLacZ, respectively; panels G, H, and I: NCI-H460 infected with AdΔE1E3, AdCMVLacZ and AdHexLacZ, respectively; panels J, K, and L: primary mouse hepatocytes infected with AdΔE1E3, AdCMVLacZ and AdHexLacZ, respectively; panels M, N, and O: HepG2 infected with AdΔE1E3, AdCMVLacZ and AdHexLacZ, respectively.

A

B

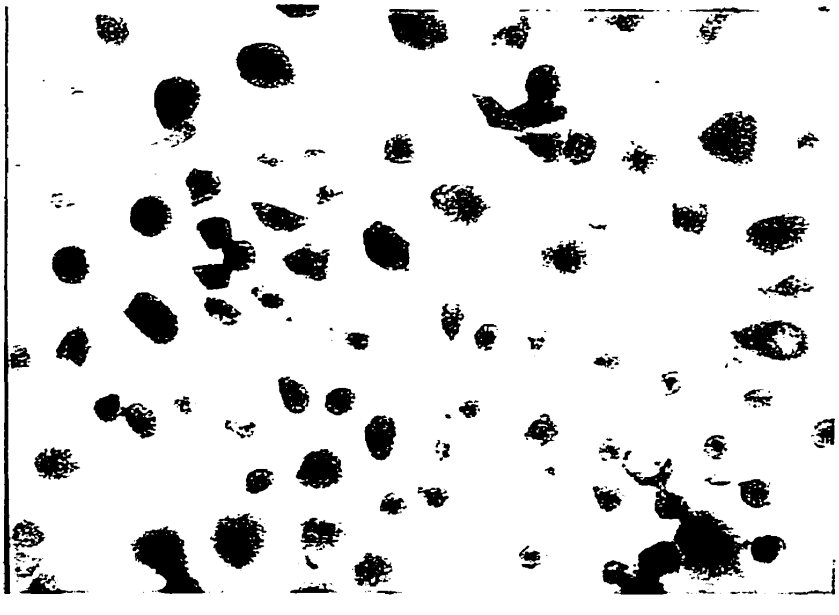


C

D

E

F

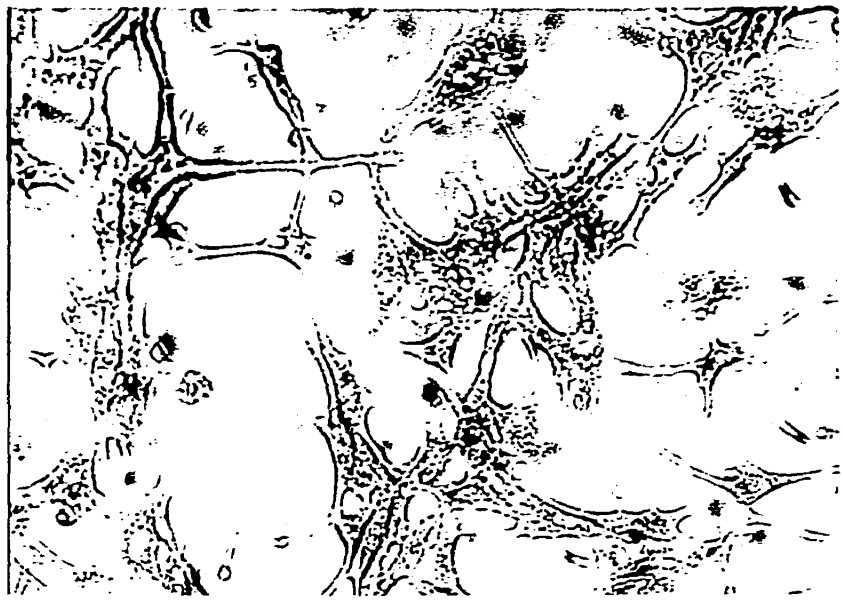


G

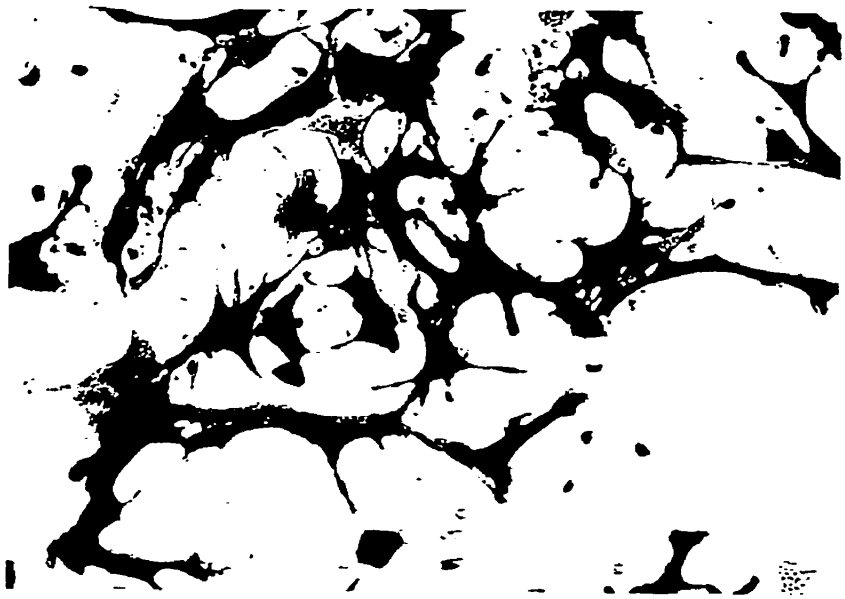
H

I

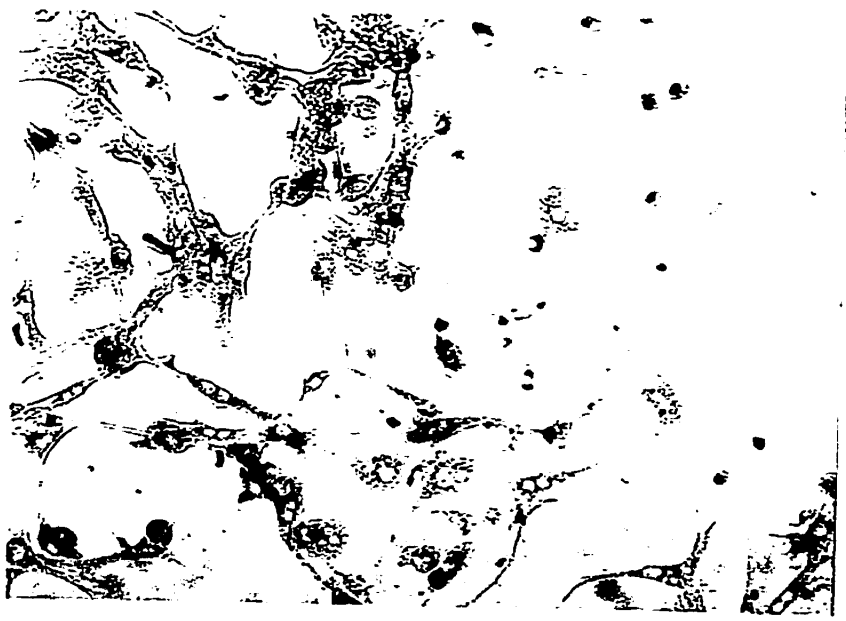
J



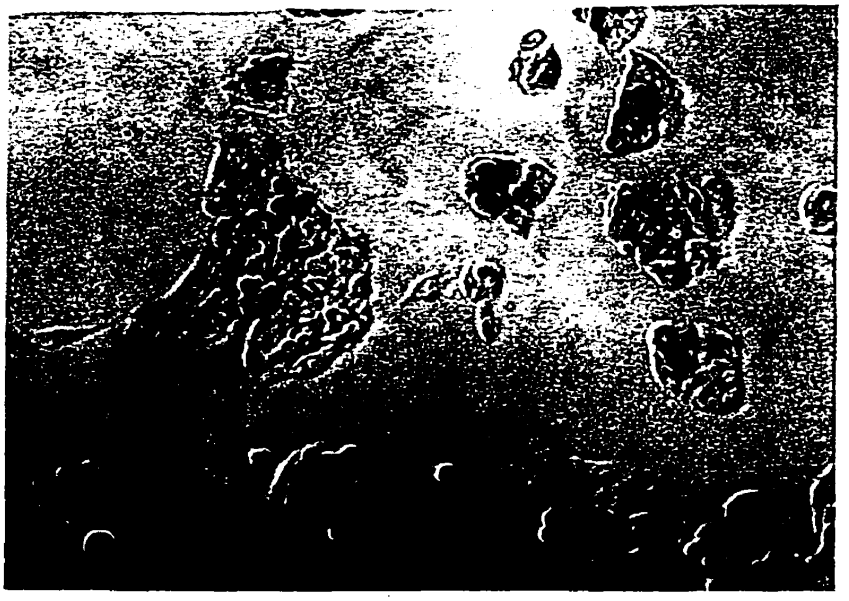
K



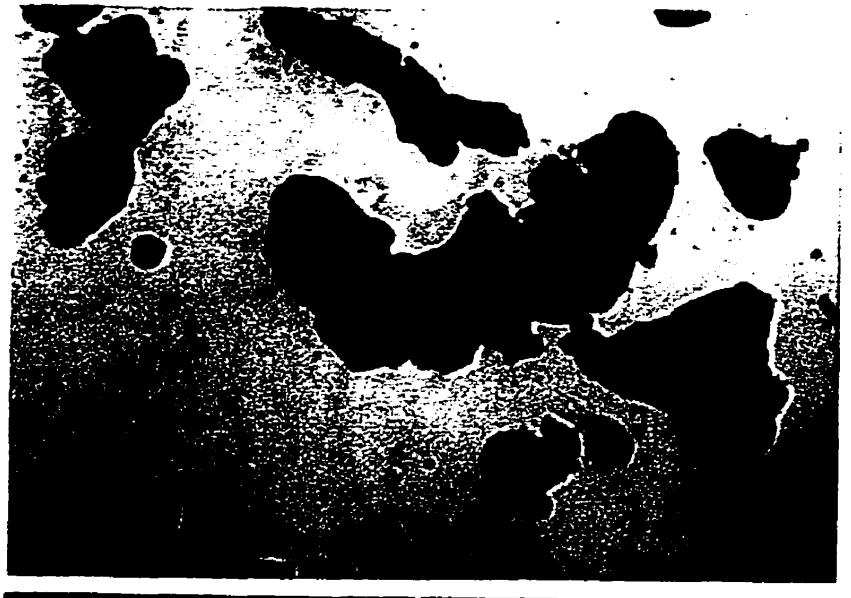
L



M



N



O

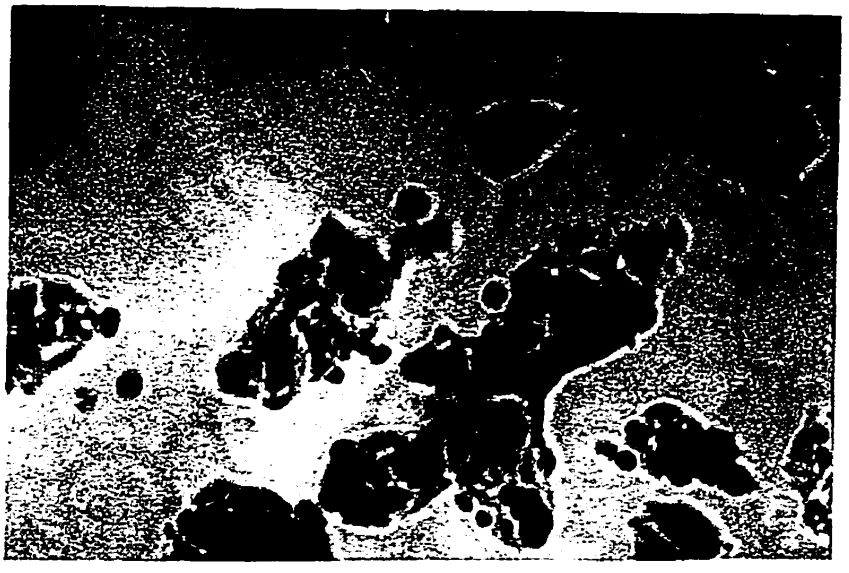
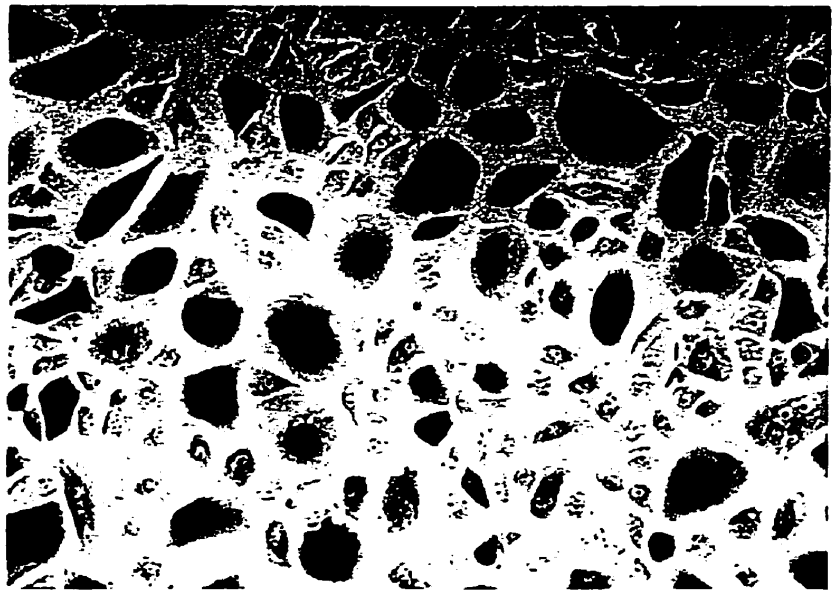
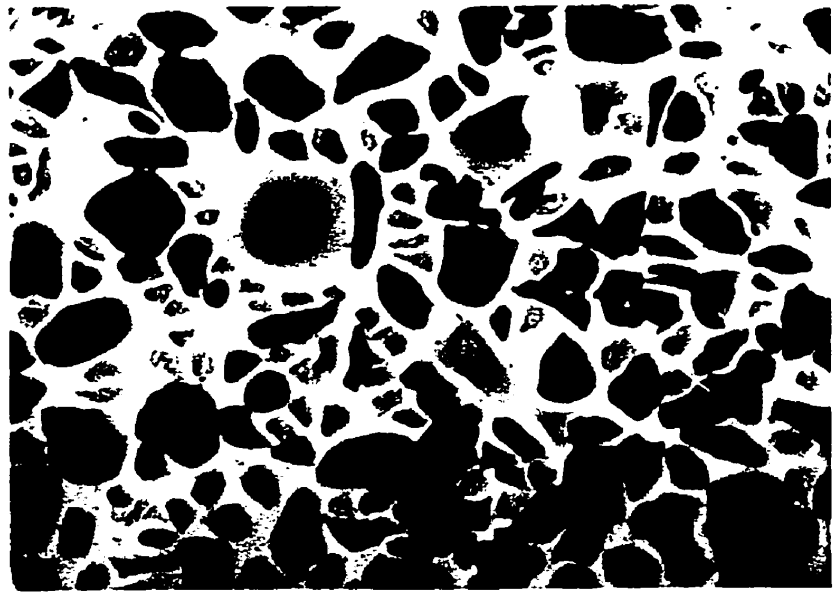


Fig.8 Histochemical staining for β-galactosidase expression. **A** : NHBEC infected with AdHexLacZ at an MOI of 50. **B**: NHBEC infected with AdCMVLacZ at an MOI of 50.

A



B



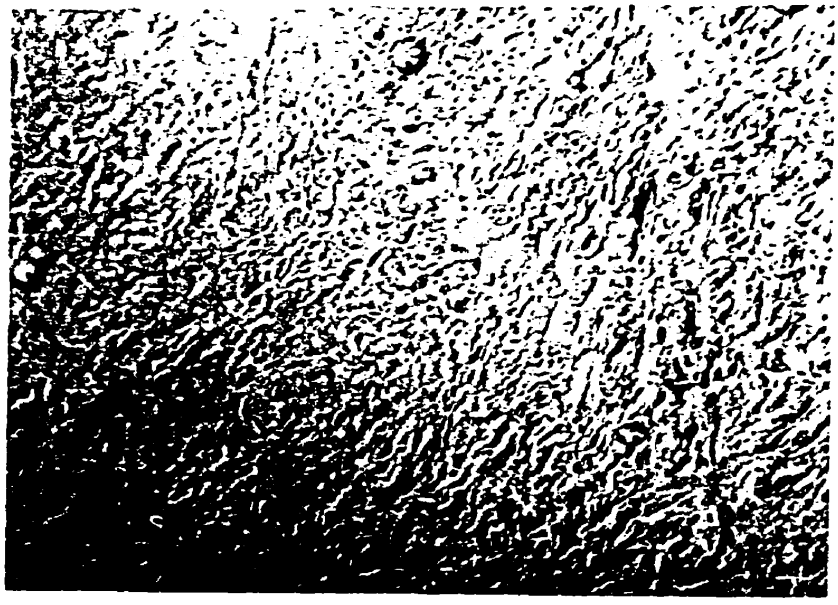
done with AdCMVLacZ (fig.8B) at the same MOI. At an MOI of 100, 50-70 % stained positive relative to the positive control (data not shown). Cells infected with AdΔE1E3 at the same MOIs were negative for X-gal staining (not shown). This observation suggests that the specificity of the promoter is dependent upon the number of copies of the construct transferred into each cell. Since this critical copy number is different for normal and tumor cells, it is possible to use the HK II promoter as a targeting tool for suicide gene therapy.

3.2 *in vivo* studies of HK II directed gene transfer

To validate the use of recombinant AdHexLacZ as an efficient marking tool for gene transfer studies *in vivo*, intratumoral injections were done in the DA-3 and NCI-H661 subcutaneous tumor models. Tumors were injected as described in chapter II, section 6, with AdHexLacZ or AdΔE1E3, which was used as a negative control, and frozen sections of tumor samples were stained with X-gal. In the DA-3 model, AdHexLacZ injected samples showed a patchy X-gal staining (fig.9A) distributed throughout the tissue, representing nearly 30 % of the tumor sample. In the H661 model (fig.9E), the same pattern was observed, and staining represented 10-20 % of the tumor sample. Tumors from both models (fig. 9A and 9E) injected with the AdΔE1E3 virus did not show any positive staining with X-gal. Hematoxylin and eosin staining of the same samples shows cellular content of the sections (fig. 9C, 9D, 9G et 9H).

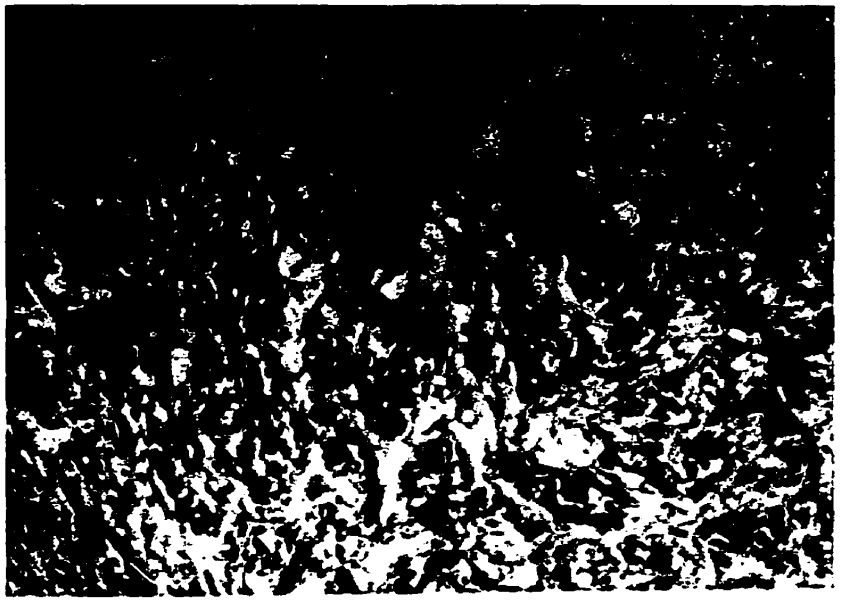
Fig.9 Histochemical staining on tissue sections of tumor samples. **A, B** : X-gal staining for β-galactosidase expression in DA-3 cells infected in vivo with AdΔE1E3 and AdHexLacZ, respectively. **C, D** : Hematoxylin and eosin staining of sections from the same samples as in A and B, respectively. **E, F** : X-gal staining for β-galactosidase expression in NCI-H661 cells infected in vivo with AdΔE1E3 and AdHexLacZ, respectively. **G, H** : hematoxylin and eosin staining of sections from the same samples as in E and F, respectively.

A

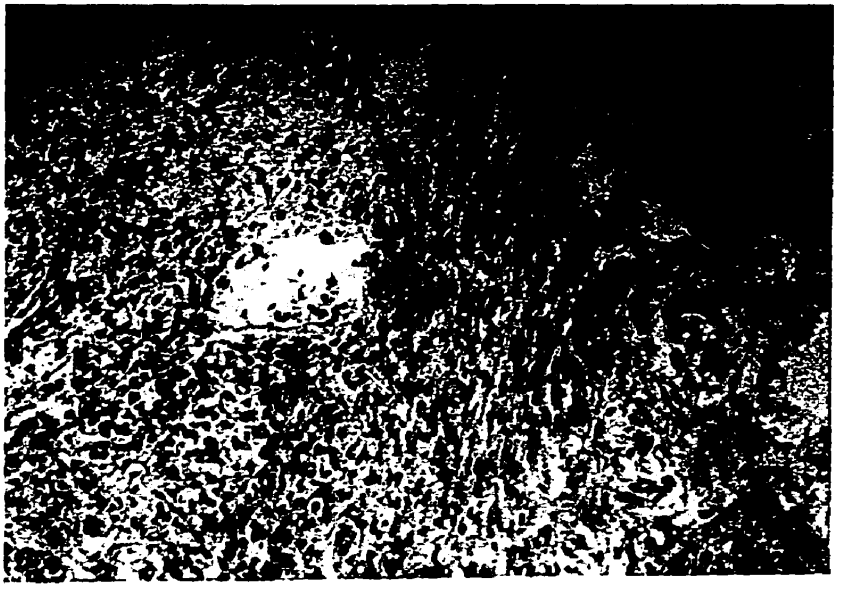


B

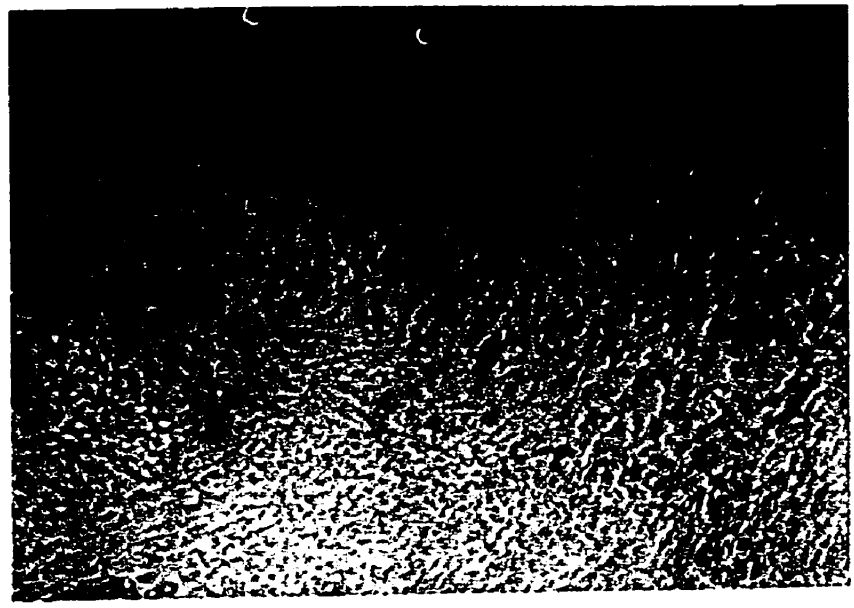
C



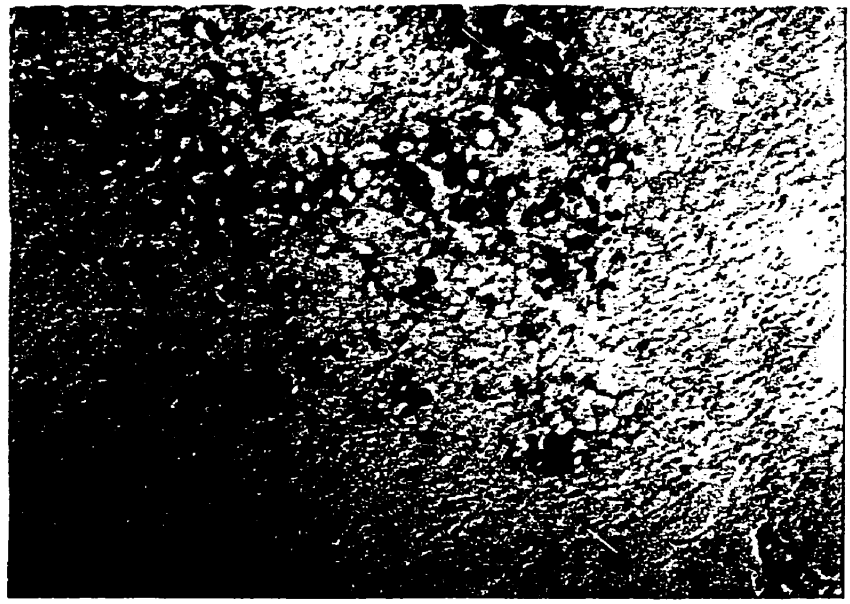
D



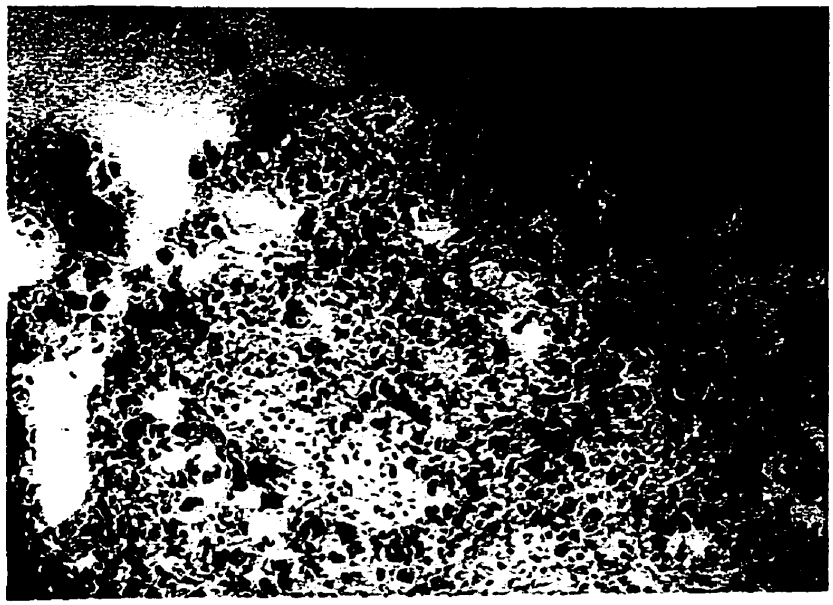
E



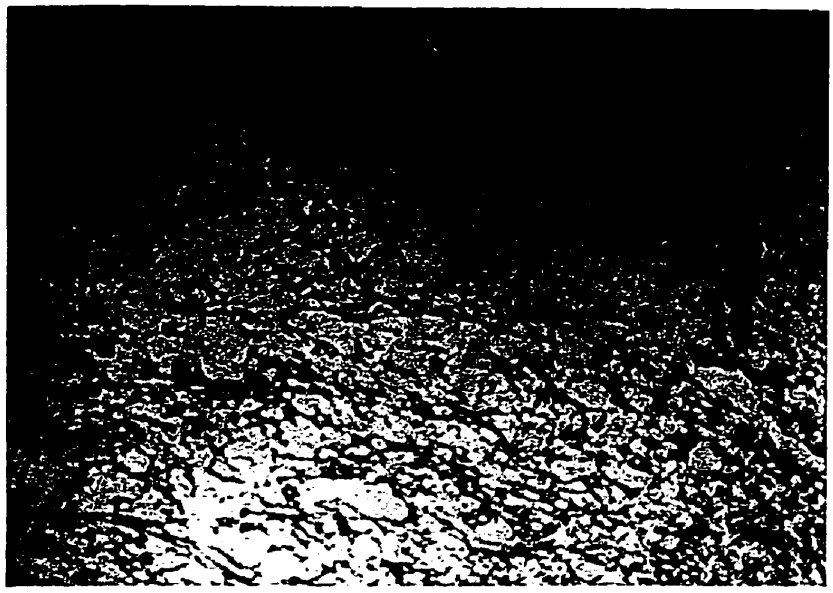
F



G



H



CHAPTER IV: Viral Thymidine Kinase as a Suicide Gene

1. Promoters for tumor specific killing using the vTK/gancyclovir combination

1.1 HK II directed vTK/ganciclovir (GCV) killing using adenoviral vectors

To test the use of the HK II promoter in adenoviral vectors for targeted suicide gene therapy, recombinant adenoviral particles carrying the HK II promoter and viral thymidine kinase were generated, based on the pAdBN-HexTk shuttle vector (fig.1C). Cell survival after the virus/GCV treatment was determined using an MTT assay. To control for possible differences between cell lines with respect to adenoviral infectivity, we performed dose-response curves of viral dose relative to cell killing. We varied MOI of AdHexTk and looked at cell killing at two doses of GCV, 10 and 25 μg/ml, for each of the six cell lines studied. From these experiments, we defined an optimal MOI for each cell line in the linear range of the curve to prevent saturation of infection. The results are summarized in table 1. The choice was also based on maximal cell killing in AdHexTk transduced cells, along with minimal toxicity in AdHexLacZ transduced ones upon GCV treatment. In NCI-H661, shown as an example in fig.10, an MOI of 20 resulted in 7% killing with the AdHexLacZ control while it resulted in 54% killing with AdHexTk (P=0.0025, average of four experiments). Moreover, cell killing continued to increase at an MOI of 50, showing there was no saturation at 20. For NHMEC and NHBEC, there was minimal killing across a 10-fold increase in MOI. Therefore we selected the one that also resulted in minimal toxicity in control conditions. We have shown that these two cell lines are transducible by adenoviruses by X-gal staining after AdCMVLacZ infection (data not shown and fig.7, respectively). Using the optimal MOI, we performed cell killing assays over a range of GCV concentrations in AdHexTk transduced cells. The

Fig.10 Dose-response diagram of increasing AdHexTk or AdHexLacZ MOI vs. cell killing using two different doses of GCV. Experiments were carried out in quadruplicates for each condition with at least three repeats for every cell line. The results shown were obtained in NCI-H661 cells. Error bars represent standard deviations. * represents a statistically significant result ($P<0.01$) using ANOVA to compare between the three different treatments and t-tests to compare HexTk to HexLacZ.

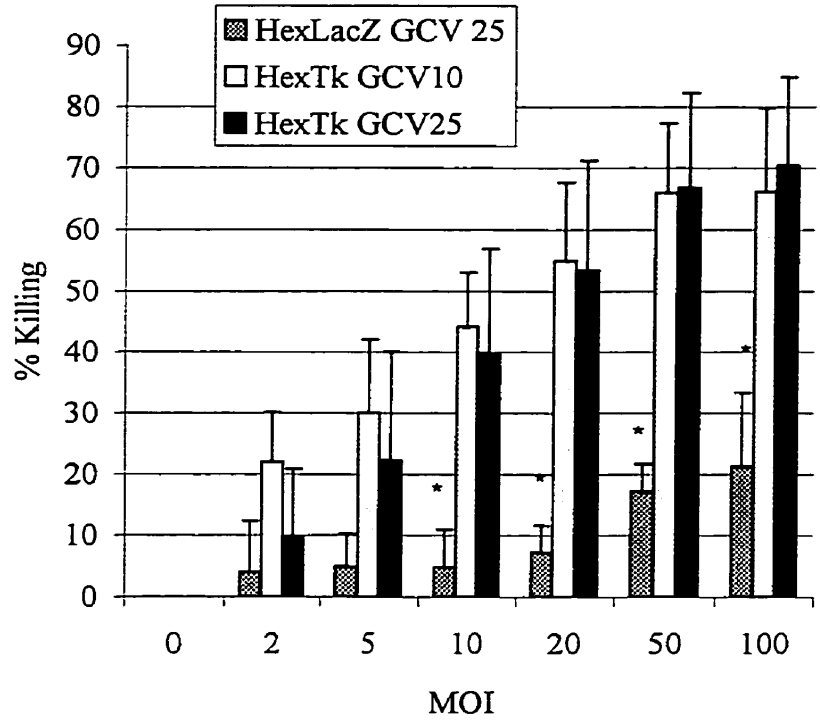

HexLacZ GCV 25
HexTk GCV10
HexTk GCV25
% Killing
90
80
70
60
50
40
30
20
10
0
0
2
5
10
20
50
100
MOI

Table 1: Summary of AdHexTk viral dilutions and cytotoxicity in various cell lines

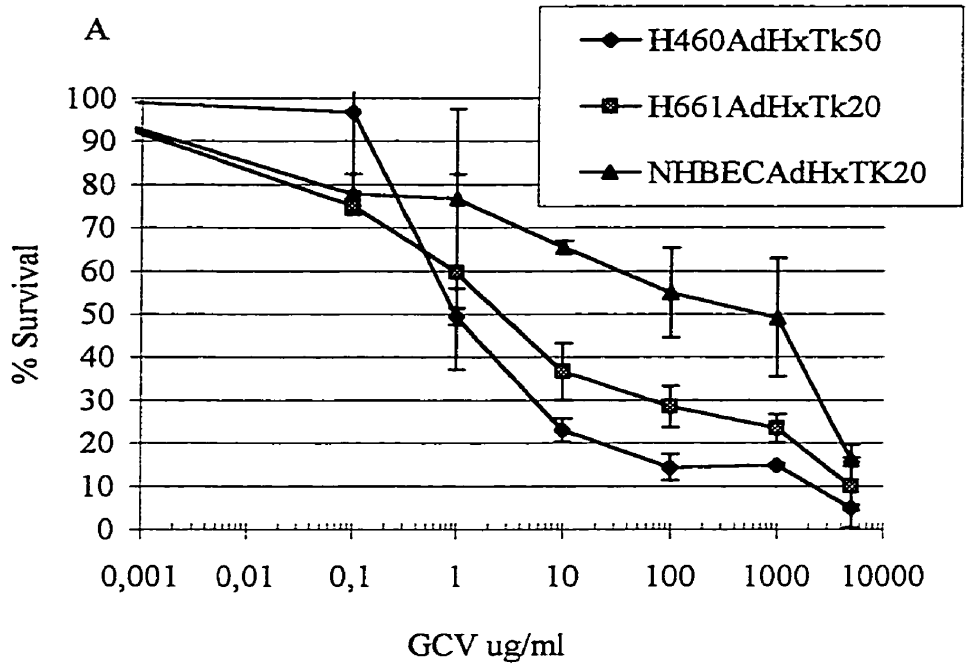
Cell line	*Optimal MOI*	*IC_{50} GCV ug/ml*
NHBEC	20	100
NCI-H460	50	1
NCI-H661	20	1
NHMEC	50	>1000
MCF-7	50	10
HepG2	50	1

curves are shown in fig.11 and the IC_{50} values are summarized in table 1. Results demonstrate selectivity in toxicity with a 10-100 fold increase in IC_{50} between lung cancer cells H661 and H460 respectively and NHBEC (fig. 11A). There was also a 100-fold increase in IC_{50} in NHMEC relative to breast carcinoma cells MCF-7. In HepG2 cells, an IC_{50} of 1 μg/ml was observed (fig.11B) comparable to other tumor cell lines.

1.2 MUC-1 directed vTK/ganciclovir (GCV) killing using adenoviral vectors

The MUC-1 promoter was also tested as a tissue specific targeting tool in adenoviral vectors for suicide gene therapy. Recombinant adenoviral particles carrying the MUC-1 promoter and viral thymidine kinase in the sense orientation to the left ITR were generated, based on the pΔE1sp1BMucTk shuttle vector. The killing efficiency of Ad-MUC-vTK/GCV was measured using the MTT cytotoxicity assay. Various cell lines were infected with the recombinant virus at increasing MOIs and incubated in the presence or absence of GCV. Data shown in table 2 are the summary of toxicities of Ad-MUC-vTK infection at an MOI of 50, in combination with 10 ug/ml GCV, expressed as percent killing relative to the uninfected control, also treated with GCV. Amongst the four cell lines derived from human mammary tumors, T47D showed the highest sensitivity at 42% killing. The other mammary carcinoma cell lines were all sensitive to the treatment but to varying degrees. In MCF7 cells, killing in the test conditions was 32%, 27% in MDA-MB-468 and 18% in ZR-75. These results confirm that the MUC1 promoter in Ad-MUC-vTK virus is active in breast cancer cell lines, although there is a large variability in the amount of killing seen between lines. In contrast, the proliferation of 888MEL, a human melanoma, was reduced by 1.4%. Confirmation that this cell line was efficiently infected with recombinant adenovirus was done by infecting cells with

Fig.11 A and B: Cell killing curves in several cell lines showing average toxicity over a range of GCV doses in AdHexTK infected cells. Experiments were carried out in quadruplicates for each condition with at least three repeats for every cell line. Error bars represent standard deviations.


A
H460AdHxTk50
H661AdHxTk20
NHBECAdHxTK20
% Survival
100
90
80
70
60
50
40
30
20
10
0
0,001
0,01
0,1
1
10
100
1000
10000
GCV ug/ml

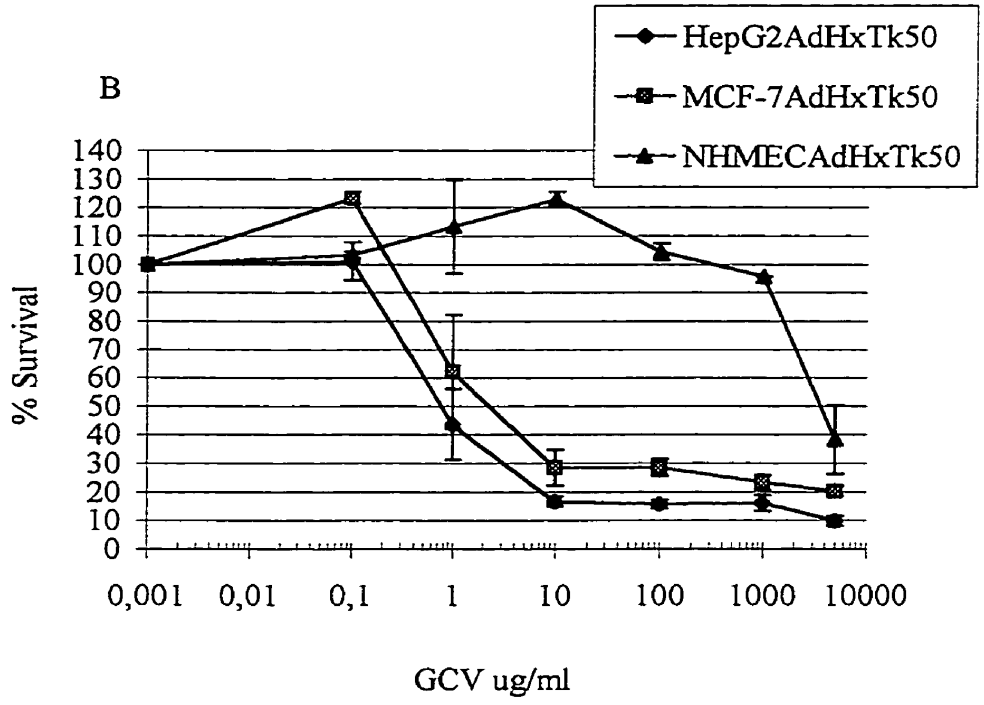

B
HepG2AdHxTk50
MCF-7AdHxTk50
NHMECAdHxTk50
% Survival
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0,001
0,01
0,1
1
10
100
1000
10000
GCV ug/ml

Table 2 : Cytotoxicity of AdMUCTK/GCV in various cell lines.

Cell Line	*% Killing AdMucTk/GCV*	*% Killing AdMucTk (R)/GCV*
T47 D	42 ± 14	42 ± 9
MCF-7	32 ± 11	-
MDA-MB-468	27 ± 12	-
ZR-75-1	18 ± 5	-
888 MEL	1.4 ± 2	-
HepG2	30.4 ± 0.7	34 ± 12
H460	54 ± 13	-

AdCMVlacZ and determining recombinant β-gal protein expression by staining with X-gal. However, H460, a human non-small cell lung carcinoma and HepG2, a human hepatoblastoma, were also killed at 54% and 30%, respectively. In the promoter studies described in fig.3, the MUC1 promoter showed very little activity in HEPG2, 1.4% compared to pRSVCAT, as well as in H460, where acetylation was 3.5% of pRSVCAT transfectants. To eliminate the possibility of interference from the left portion of Ad5, which contains the E1A enhancer, another recombinant adenovirus AdMucTk(R) where the expression cassette is found in the anti-sense orientation to the left ITR, was generated. The killing efficiency of AdMucTk(R)/GCV was measured using the same assay as before, in two cell lines, T47D and HepG2. The same cytotoxicity was observed/ as before, with 42% killing in T47D, and 34% in HepG2 (table 2).

2. Pharmacological modulation of the bystander effect

2.1 GJIC communication, connexin 43 and cAMP

Connexin 43 (Cx43) is the predominant gap junction protein in breast tissue. DA-3, mouse mammary carcinoma cells, were studied as a model of breast cancer and connexins localization in these cells was shown to be modified following cAMP treatment. The effect of cAMP on the spatial localization of Cx43 in DA-3 breast adenocarcinoma cells is shown in figure 12. Untreated (A,B) or cAMP-treated (C,D) DA3 cells were immuno-labeled for Cx43 and transmitted light (A,C) or fluorescent (B,D) images were collected under identical conditions for direct comparison. Cx43 assembly into gap junction plaques is clearly more evident after cAMP treatment (panel D). A functional assay was performed to measure the increase in GJIC following treatment with cAMP. As shown in figure 13, there is a significant increase in the GJIC

Fig.12 A and B : Untreated DA-3 cells under light and fluorescence microscopy, respectively. C and D : DA-3 cells treated with 1 mM cAMP for 48 hrs. Fluorescent label reveals Cx43 distribution.

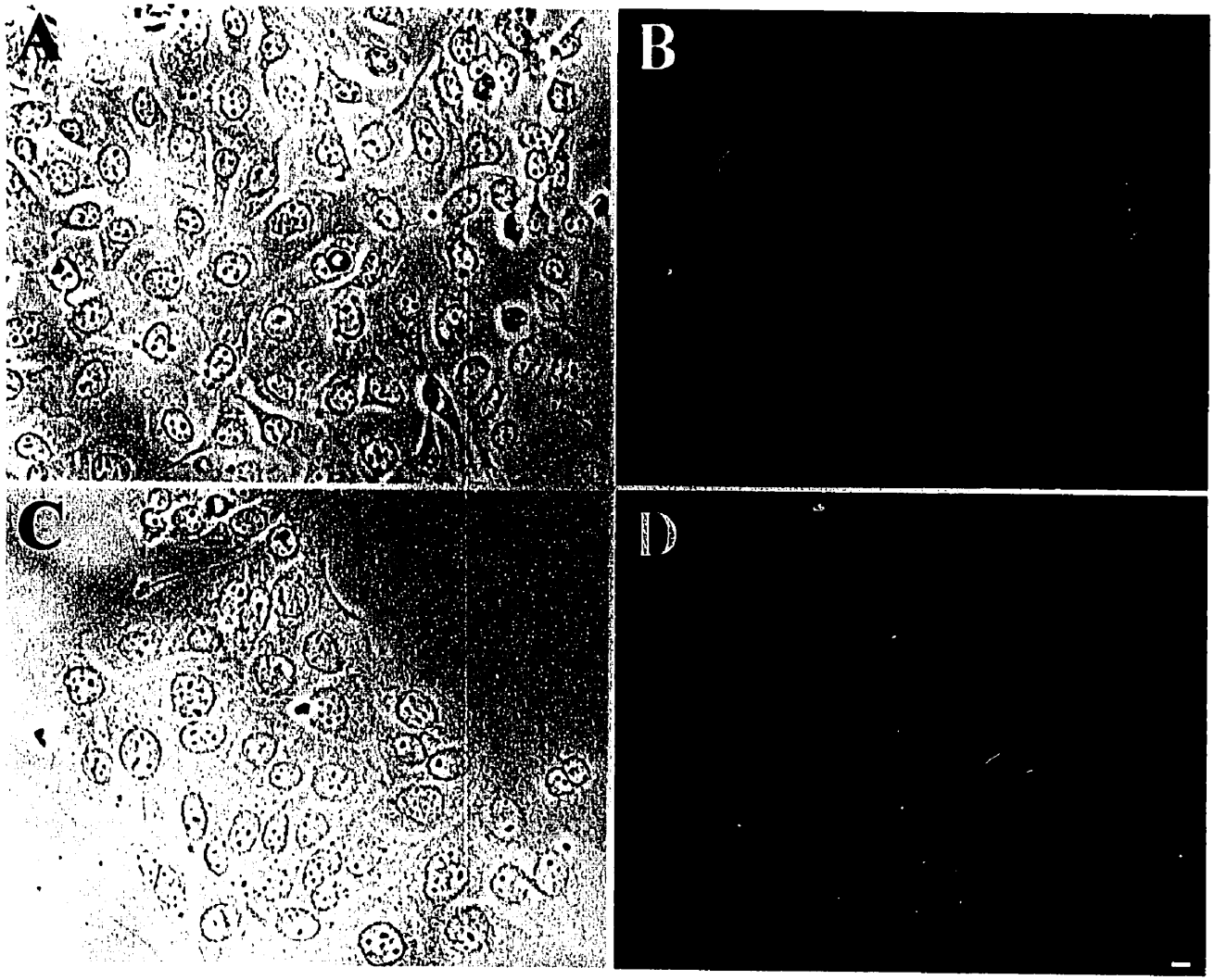
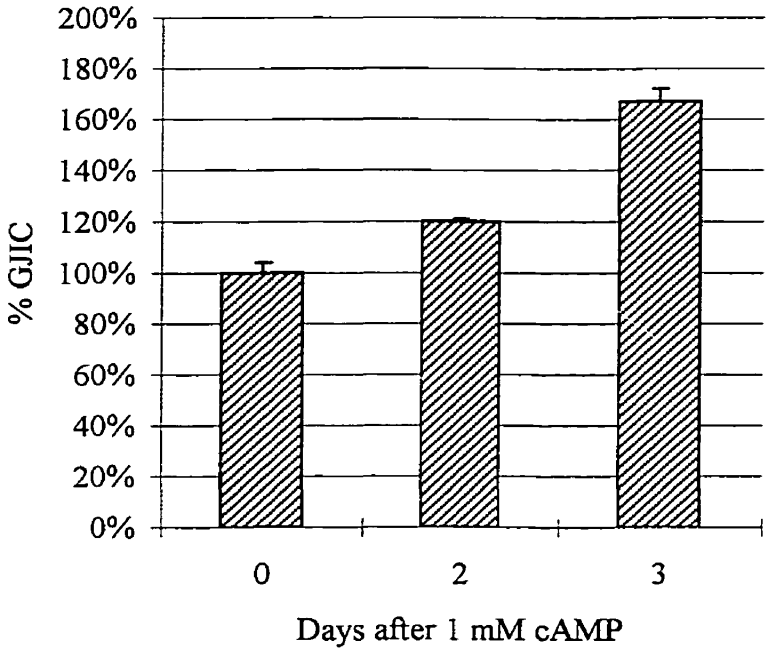

A
B
C
D

Fig.13 Percent GJIC as measured by a dye transfer assay followed by FACS analysis in DA-3 cells treated with 1 mM cAMP for 48 or 72 hrs. Experiments were carried out in duplicates for each condition. Results shown are the average of three independent experiments. Error bars represent standard deviations.


200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
% GJIC
0
2
3
Days after 1 mM cAMP

in DA3 cells after exposure to cAMP under these conditions. Together these assays demonstrate that cAMP is effective at increasing the amount of functional Cx43 that results in induced GJIC.

2.1 Cytotoxicity in cell mixing assays

The bystander effect has been demonstrated to play a role in mediating toxicity seen after treatment of cells with the TK/GCV combination. To further investigate this contribution and test whether this effect can be pharmacologically enhanced, cells were transduced with TK with two different vector systems, adenoviruses and retroviruses. The recombinant AdRSVTk was used to infect cells at an MOI of 10. This MOI was shown to be adequate for infection of DA-3 cells in a uniform fashion leading to about 95% of cells being positive (data not shown). AdRSVTk infected were mixed with uninfected cells at proportions covering 0, 1, 2, 5, 10, 20, 50, and 100% and treated with GCV at 10 or 20 ug/ml and cell killing was measured (fig.14 A and 14 B). Survival curves showed a significant increase in toxicity in cells treated with cAMP. This effect was especially striking when lower proportions of transduced cells were present in the mixture. The results in fig.14 C demonstrate that the number of cells infected with AdRSVTk in a cell population that is required to achieve 50% killing is roughly decreased by half when cAMP is present.

In retrovirally transduced DA-3 cells the same trend was observed (fig.15 A and 15 B) where cAMP treatment enhanced the efficacy of GCV at cell killing when only 2-10% of the cells were stably expressing Tk. The number of Tk-transduced cells in a cell population required to achieve 50% killing was significantly decreased (fig 15 C) when the cell mixture was treated with cAMP. Reproducibility of the results with cAMP on

Fig.14 **A**: Percent survival after treatment with 10 ug/ml of GCV in mixed cell populations of AdRSVTk-infected cells and non-infected cells relative to the percent of infected cells in the mixture, before and after cAMP treatment. **B** : same as A, after treatment with 20 ug/ml of GCV. **C** : Results from cell survival assays shown as proportion of infected cells present in the mixture required to achieve 50% killing, in cAMP treated and untreated populations. Mixing experiments were carried out in quadruplicates for each condition with three separate repeats. Error bars represent standard deviations.

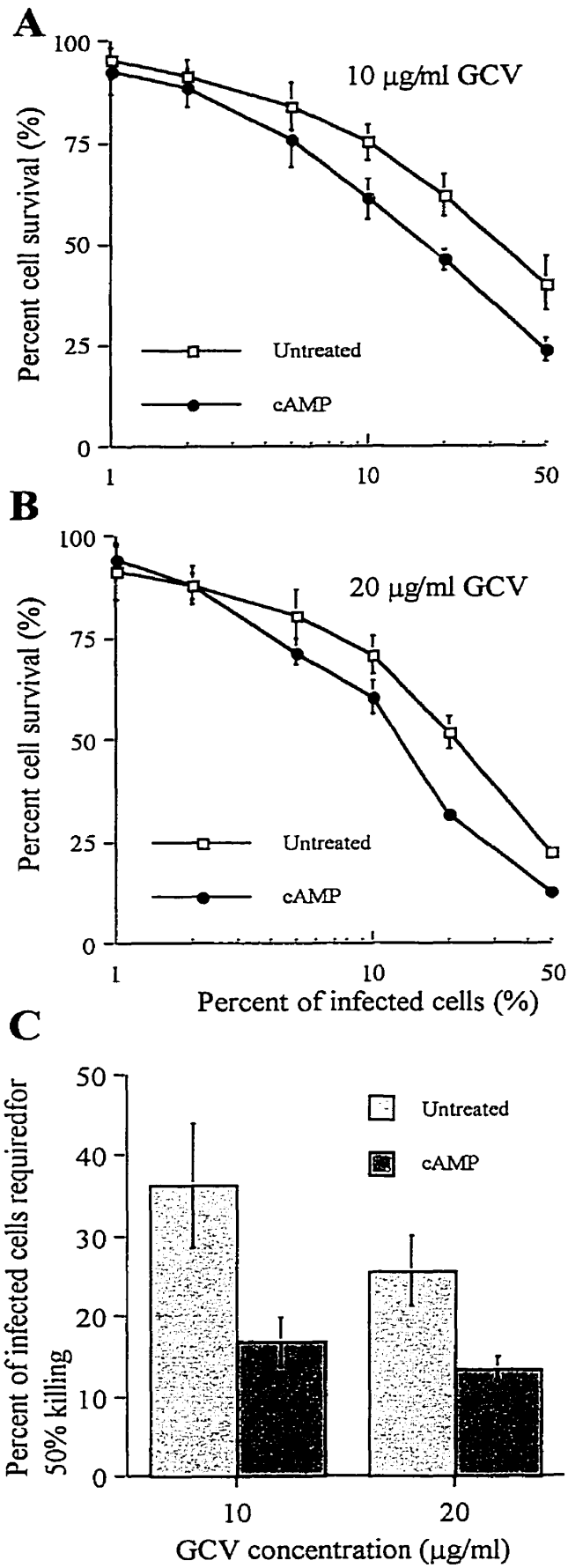
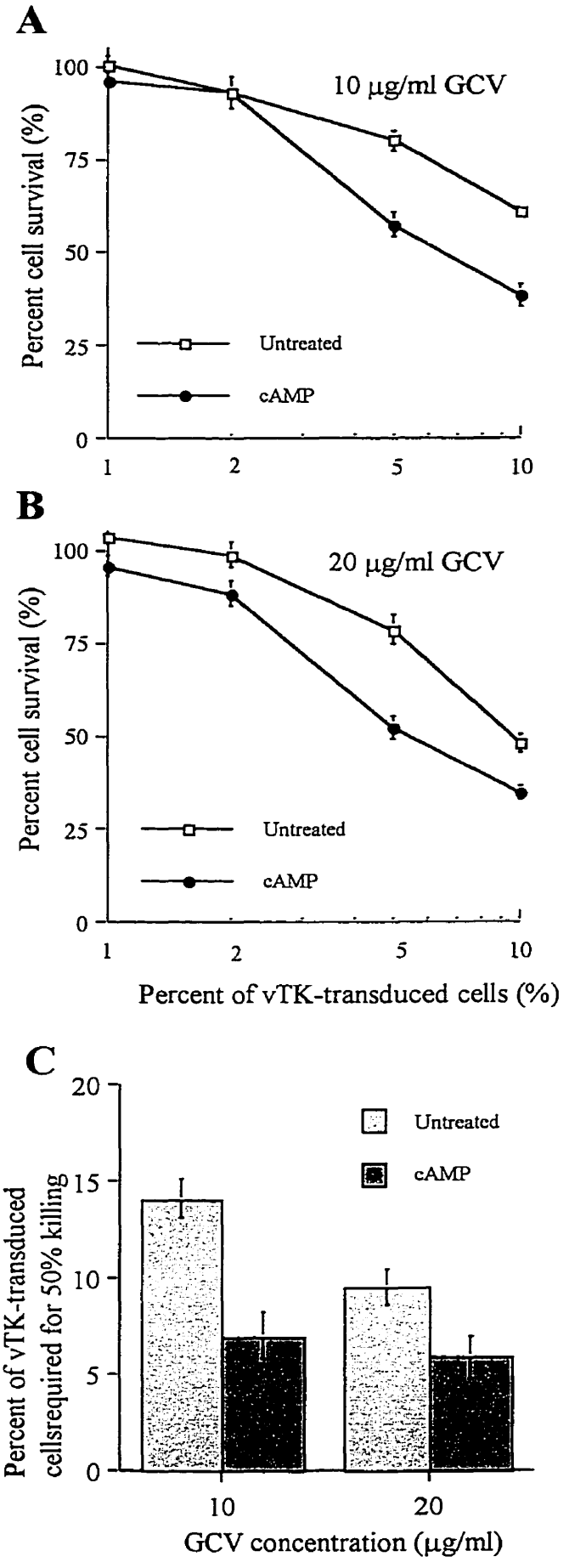

A
10 µg/ml GCV
Percent cell survival (%)
100
75
50
25
0
Untreated
cAMP
1
10
50
B
20 µg/ml GCV
Percent cell survival (%)
100
75
50
25
0
Untreated
cAMP
1
10
50
Percent of infected cells (%)
C
Percent of infected cells requiredfor 50% killing
50
40
30
20
10
0
Untreated
cAMP
10
20
GCV concentration (µg/ml)

Fig.15 **A**: Percent survival after treatment with 10 ug/ml of GCV in mixed cell populations of AP3-transduced (retroviral construct encoding HSVTk gene in a bicistronic construct with GFP) and non-transduced cells relative to the percent of Tk expressing cells in the mixture, before and after cAMP treatment. **B** : same as A, after treatment with 20 ug/ml of GCV. **C** : Results from cell survival assays shown as proportion of infected cells present in the mixture required to achieve 50% killing, in cAMP treated and untreated populations. Mixing experiments were carried out in quadruplicates for each condition with three separate repeats. Error bars represent standard deviations.


A
10 µg/ml GCV
Percent cell survival (%)
100
75
50
25
0
Untreated
cAMP
1
2
5
10
B
20 µg/ml GCV
Percent cell survival (%)
100
75
50
25
0
Untreated
cAMP
1
2
5
10
Percent of vTK-transduced cells (%)
C
Percent of vTK-transduced cellsrequired for 50% killing
20
15
10
5
0
Untreated
cAMP
10
20
GCV concentration (µg/ml)

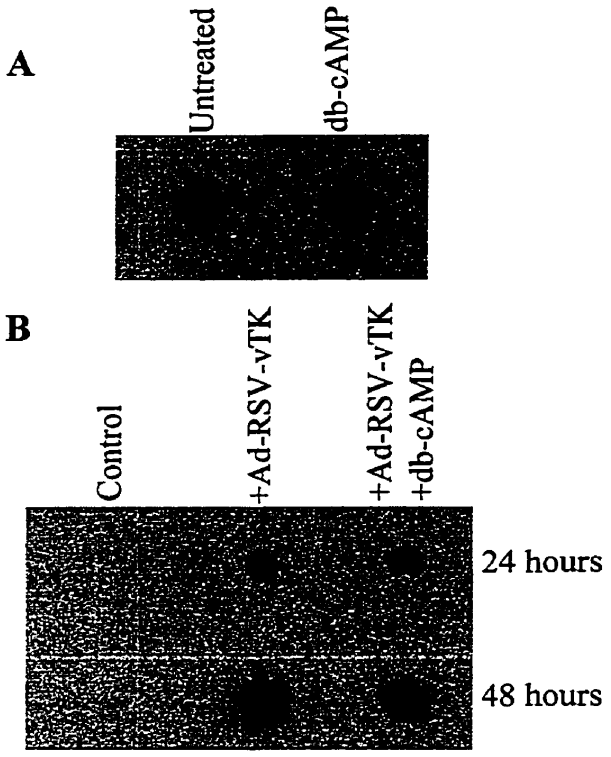
increasing toxicity of the vTK/GCV combination independently of the delivery system used further strengthens the evidence in favor of a direct effect of cAMP on intercellular communication, which itself has been shown to be an important element in mediating the bystander effect.

2.3 Controls for side-effects of cAMP

Some of the issues related to gene transfer using adenoviruses as delivery vehicles include susceptibility to infection of certain cell types and the effect of a specific treatment on a cell's infectivity. To determine the effect of increasing cellular communication on infectivity, cells were treated with cAMP for 48 hours, under the same conditions that are used prior to the mixing experiments. Since cAMP affects cell growth, a parallel set of plates was used to assess cell number, and cells were infected with AdRSVTk accordingly with an MOI of 10, taking into account the difference in cell number. DNA extracted by Hirt lysis 12 hrs later was analyzed (fig.16 A). There was no difference in the amount of adenoviral DNA present in treated and untreated cell populations.

The direct effect of using cAMP on adenoviral infectivity or TK transcription was assessed by simultaneously treating with cAMP and carrying out the infections. DNA was collected 24 and 48 hours later, and no difference was detected (fig. 16 B) between treated and untreated cell populations at the level of amount of adenoviral DNA present. Finally the possibility that cAMP could act synergistically with GCV to affect cell survival was looked at. There are no differences in untransduced cell survival between cAMP treated and un-treated cells after gancyclovir administration (data not shown).

Fig.16 **A** : Tk dot blot with DNA extracted from DA-3 cells treated with cAMP 48 hrs prior to infection with AdRSVTk. **B** : Tk dot blot with DNA extracted from DA-3 cells 24 or 48 hrs after infection with AdRSVTk and/or treatment with cAMP. The blots are representative of results obtained on four different occasions.


A
Untreated
db-cAMP
B
Control
+Ad-RSV-vTK
+Ad-RSV-vTK
+db-cAMP
24 hours
48 hours

3. Cellular resistance to vTK/GCV killing

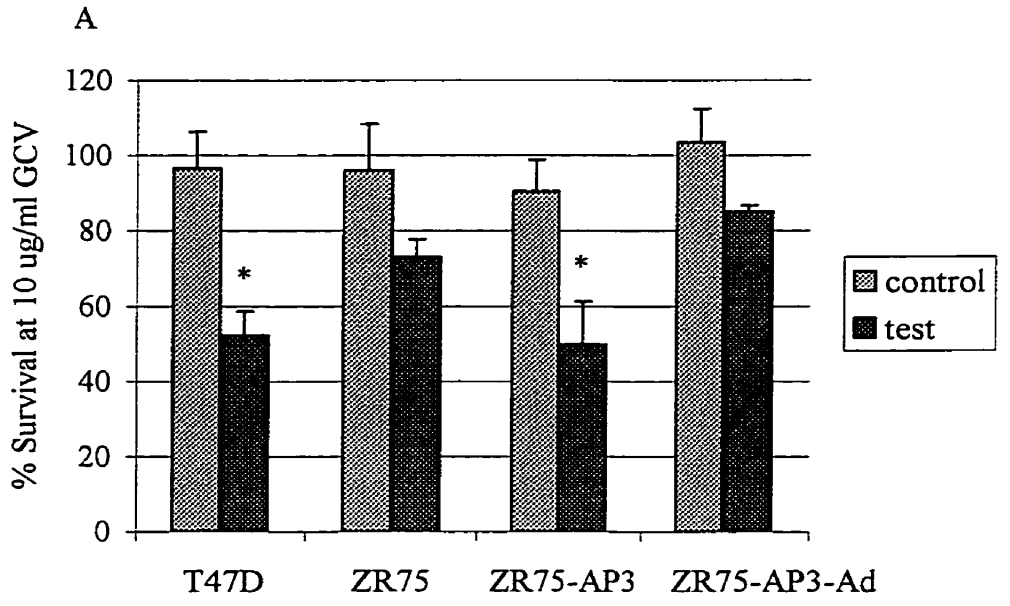
We have examined the phenomenon of resistance to vTK/GCV killing observed most strikingly in ZR75, by looking at several parameters that are likely to play a role in this suicide gene strategy. Our goal was to determine whether it is an intrinsic resistance to the activated GCV metabolite or a specific problem related to the use of adenoviruses as delivery vehicles.

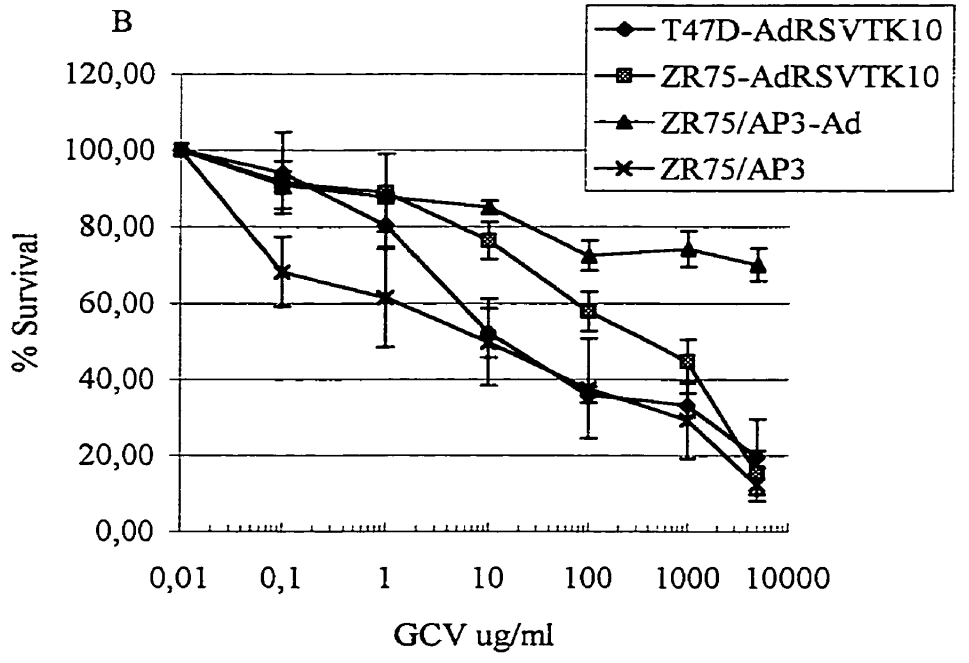
3.1 Spectrum of sensitivity is vector-dependent

Cytotoxicity studies with the Tk/GCV suicide gene system have demonstrated the existence of a spectrum of sensitivity to the activated drug regardless of promoter/ activation status (table 2), in our lab, and in others' (250; 251). ZR75 and T47D were selected as a system to study Tk/GCV sensitivity and identify parameters that are important in determining sensitivity. This choice was based on the observation reported in fig.17 A. The cells were infected with AdRSVTk at an MOI of 10 to express the Tk gene under the control of a strong constitutive promoter. When treated with GCV at 10 ug/ml, percent survival was decreased by half in T47D cells ($p=0.005$), while there was a 20% decrease in ZR75, relative to control cells infected with AdCMVLacZ. To confirm our observation concerning the resistance of ZR75 cells to Tk/GCV combination, we tried to reproduce it in retrovirally transduced cells. The recombinant retrovirus AP-3 was used to stably transduce ZR75 cells with the Tk gene, and AP-2 was used to generate a control population expressing the GFP alone. Cytotoxicity assays were performed to test the sensitivity of ZR75/AP-2 and ZR75/AP-3 to GCV at 10 ug/ml. Percent survival was significantly decreased by 40% ($p=0.035$).

Fig.17 **A** : Percent survival in different cell lines exposed to GCV at 10 ug/ml. T47 D and ZR75 were infected with AdRSVTk at an MOI of 10. Control condition is AdCMVLacZ for adenoviral infections at the same MOI. ZR75-AP were transduced with AP-3 (Tk/GFP bicistronic retrovirus). Control condition is AP-2 infections (GFP only retrovirus) for retroviral transduction. ZR75-AP-Ad are the same as ZR75-AP, but they were infected with AdCMVLacZ at an MOI of 50 prior to GCV exposure. **B** : Cell killing curves in several cell lines showing toxicity over a range of GCV doses in TK infected cells, under various conditions. Results are the average of 3 independent experiments with each condition done in quadruplicate. Error bars represent standard deviations.

* represents a statistically significant result ($P<0.05$) using t-tests to compare between test and control conditions.

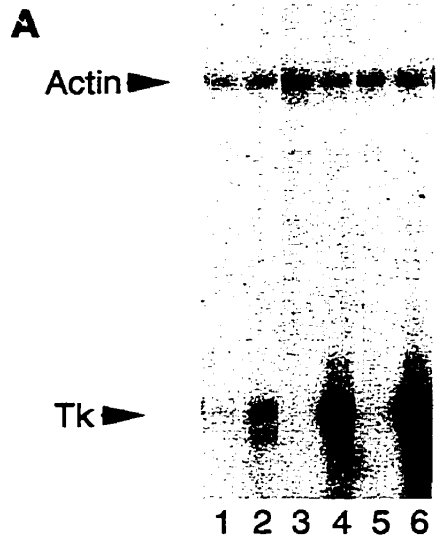

A
120
100
80
60
40
20
0
% Survival at 10 ug/ml GCV
*
*
control
test
T47D
ZR75
ZR75-AP3
ZR75-AP3-Ad


B
T47D-AdRSVTK10
ZR75-AdRSVTK10
ZR75/AP3-Ad
ZR75/AP3
120,00
100,00
80,00
60,00
40,00
20,00
0,00
% Survival
0,01
0,1
1
10
100
1000
10000
GCV ug/ml

One reason for vector-dependence of Tk/GCV sensitivity in ZR75 cells could be that adenoviral proteins that are being expressed in these cells along with the transgene are interfering with ZR75's sensitivity to GCV. To test this hypothesis, ZR 75 cells, and the two stable derivative cell lines ZR75/AP-2 and ZR 75/AP-3, which express Tk, were each infected with AdCMVLacZ at an MOI of 50 (ZR75-AP-Ad), and the infected cell population was then exposed to increasing concentrations of GCV. At a dose of 10 ug/ml, decrease in survival was only 20% in the AP-3 transduced cells. Sensitization was not observed anymore in these cells.

Dose ranging experiments were done on the previously described cells and IC_{50} values for these cell lines reflected the same changes in sensitivities for ZR75, depending on the context of Tk gene delivery. The infected cell population was then exposed to different concentrations of CGV. After a 5 days exposure, cell survival was determined with an MTT assay, as described. The results shown in fig.17 B demonstrate a 10- to 100-fold difference in GCV dose required to decrease survival by 50% between the sensitive and resistant populations. T47D cells infected with AdRSVTk at an MOI of 10 had an IC_{50} of 10 to 20 ug/ml, while ZR75 cells infected with AdRSVTk under similar conditions had an IC_{50} of approximately 500 ug/ml. ZR75 cells transduced with a retrovirus expressing Tk gene, ZR75-AP3, had an IC_{50} of 10 to 20 ug/ml while ZR75-AP3 cells infected with AdCMVLacZ at an MOI of 50 prior to GCV exposure were dramatically resistant to GCV toxicity.

Fig.18 **A**: RNase protection assays (RPAs) show Tk RNA levels in different cells lines. Lane 1: ZR75-AP2; lane 2: ZR75-AP3; lane 3: ZR75-AdΔE1/E3; lane 4: ZR75-AdRSVTk; lane 5: T47D-AdΔE1/E3; lane 6: T47D- AdRSVTk. **B** : Dot blot of Tk cDNA content in ZR75 and T47D cells after infection with AdRSVTk under the same conditions that were used for RPAs. The blots are representative of results obtained on four different occasions.


A
Actin
Tk
1 2 3 4 5 6
B
ZR 75
T47D

3.2 Thymidine kinase expression levels

The level of suicide gene expressed in target cells can have an influence on the level of sensitization observed. Comparisons at the RNA level were made for vTK mRNA using RNase protection assays (fig.18A). There was no statistically significant difference in vTK mRNA between AdRSVTk infected ZR75 (lane 4) and T47D cells (lane 6) at an MOI of 10. When normalized to actin, relative levels of Tk in ZR75 are 28 ± 8.2 and in T47D 29 ± 9.7. The experiment was repeated at least three times. Differences in infectivity by adenoviruses between the two cell lines were also ruled out by extracting episomal DNA by Hirt lysis and looking at TK cDNA levels (fig.18B) in adenovirally infected cells. It was also demonstrated by AdCMV LacZ infection at increasing MOI followed by X-gal staining in T47D and ZR75 cells that both are equally well infected by adenoviruses (data not shown). Finally, levels of expression in retrovirally transduced ZR75 (ZR75-AP3, lane 2) were even lower than those observed in adenovirally transduced ZR75 cells (lane 4), yet the former were more resistant to GCV treatment. GFP levels in ZR75-AP3, reflecting the percent of cells in that population having integrated a functional copy of the Tk gene is 75-80%, as detected by FACS analysis (data not shown).

3.3 Intercellular communication and cell cycling

Other confounding variables for cell cycle dependent drugs such as ganciclovir are cell cycling. However ZR75 and T47D have roughly equivalent doubling times. The bystander effect is also important for the Tk/GCV paradigm. A functional assay for measuring gap junctional intercellular communication (GJIC) was done to evaluate differences between ZR 75 and T47D. The latter exhibited more efficient GJIC with

50%, while ZR 75 had 20% communication between donor and receiver populations (fig.19). However, under the conditions of the cell killing assays, levels of Tk expression in the tested populations were very high in both T47D and ZR75 (fig.18A). In this case, the bystander effect is not expected to play a role in the level of sensitivity of cell population where a high proportion of cells is expressing the transgene (see results in section 2.2 of chapter 4).

3.4 GCV uptake

In an attempt to measure differences in prodrug accumulation, if any, between the two human mammary carcinoma cell lines with different susceptibilities to the vTk/GCV combination, we performed GCV uptake studies. Although GCV is known to be a highly lipophilic drug, an active uptake mechanism was shown to play a role in increasing the intracellular concentration of this nucleoside (297). T47D and ZR75 were exposed to ^{3}H-GCV for increasing time periods and GCV content was measured and normalized for cell number (reflected by protein amounts). There was no significant difference in ^{3}H-GCV/mg of protein content between the two cell lines, as shown in fig.20, except after 24 hrs exposure, where ZR 75 cells had accumulated more ^{3}H-GCV than T47D. This was therefore not a limiting factor in the phenotype observed.

Fig.19 Percent GJIC in T47D and ZR75 cells as measured by a dye transfer assay followed by FACS analysis. Experiments were carried out in duplicates for each condition. Results shown are the average of three independent experiments. Error bars represent standard deviations.

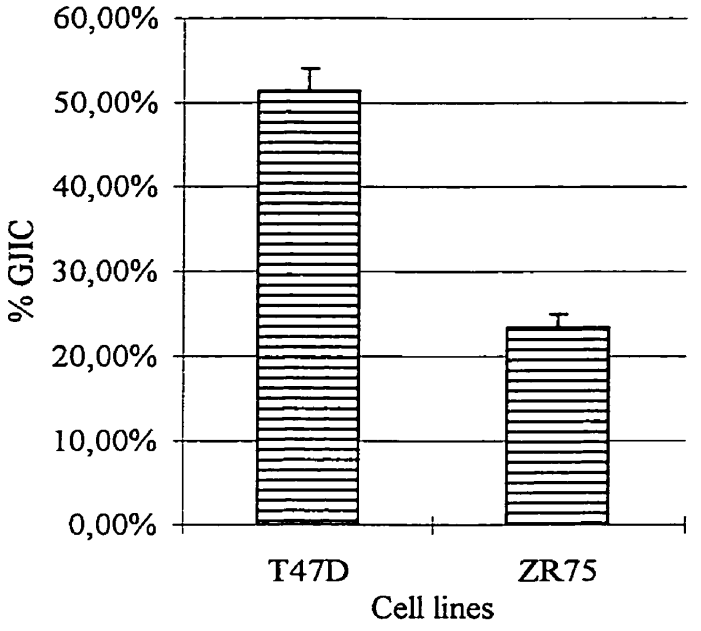
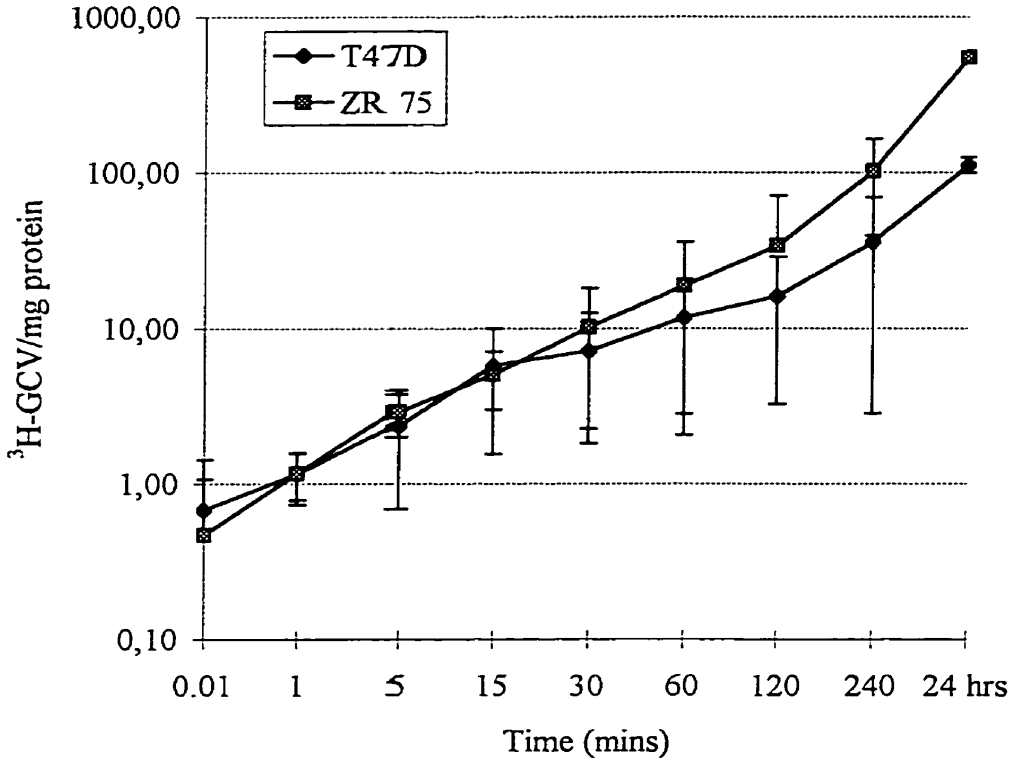

60,00%
50,00%
40,00%
30,00%
20,00%
10,00%
0,00%
% GJIC
T47D
ZR75
Cell lines

Fig.20 Specific ^{3}H-GCV content in T47D and ZR75 cells at different time points after exposure to radiolabelled GCV at a concentration of 1 ug/ml. Experiments were carried out in duplicates for each condition. Results shown are the average of three independent experiments. Error bars represent standard deviations.


1000,00
100,00
10,00
1,00
0,10
T47D
ZR 75
^{3}H-GCV/mg protein
0.01
1
5
15
30
60
120
240
24 hrs
Time (mins)

CHAPTER V: Cytochrome P450s as Suicide Genes

1. CYP3A4 and P450 reductase and CYP2B1 expression levels in human breast carcinoma cell lines

The P450 3A4 isoform was stably expressed in MDA-231 and MCF-7, two breast carcinoma cell lines, after transduction of these two cell lines with the VSV-G pseudotyped HC-2/CYP3A4 retrovirus. No selection of the transduced cell population was carried out. The percentage of cells expressing the green fluorescent protein (GFP) was determined by FACS analysis and was 90% for MDA-231/CYP cells and 35% for MCF-7/CYP (data not shown). The control cells MDA-231/AP-2 and MCF-7/AP-2 also expressed the GFP at 90% and 40%, respectively. These four cell lines were then infected with either RED (rat isoform) or HOR (human isoform) recombinant retroviruses, to derive new cell lines expressing both CYP3A4 and P450 reductase, with the appropriate negative controls. Reductase and 3A4 protein levels were determined by Western analysis of microsomal preparations from these cell lines, as shown in fig. 21. Using the standards shown on the same gel, semi-quantitative results for microsomal P450 and reductase contents of the different lines are summarized in table 3. Microsomal CYP3A4 contents in MCF-7, originally at 1.43 pmol/mg were nearly doubled in the CYP transduced derivatives, and varied between 2.46 and 3.03 pmol/mg. P450 reductase in the same cell was expressed at high levels even in the parental cells at 34 pmol/mg, and its expression was increased to about 42-48 pmol/mg in all retrovirally transduced cells, even the AP-2 controls. In MDA-231, CYP3A4 expression was increased 9-fold in CYP transduced cells where it was at 17-20 pmol/mg, compared to the parental uninfected cells where it was 1.59 pmol/mg. P450 reductase was expressed at low levels in the parental cells, and in AP-2 controls. But it was also expressed at low

Fig.21 **A** : microsomal content of P450 reductase protein in MCF-7 and MDA-231 cells and derived cell lines. Order of lanes is the same for MCF-7 and MDA-231 cells. Lane 1 : parental cells (MDA-231 or MCF-7); lane 2 : MCF-7/AP2; lane 3 :MCF-7/CYP; lane 4 : MCF-7/AP2/RED; lane 5 : MCF-7/CYP/RED; lane 6 : MCF-7/AP2/HOR; lane 7 : MCF-7/CYP/HOR; lanes 8-11 : P450 reductase standards of 0.2, 0.4, 0.6, and 1 pmoles, respectively. **B** : microsomal content of CYP 3A4 protein in MCF-7 and MDA-231 cells and derived cell lines. Order of lanes is the same for MCF-7 and MDA-231 cells. Lane 1-4 : CYP3A4 standards of 0.2, 0.4, 0.6, and 1 pmoles, respectively; lane 5 : parental cells (MDA-231 or MCF-7); lane 6 : MCF-7/AP2; lane 7 :MCF-7/CYP; lane 8 : MCF-7/AP2/RED; lane 9 : MCF-7/CYP/RED; lane 10 : MCF-7/AP2/HOR; lane 11 : MCF-7/CYP/HOR. 40 ug of microsomes were loaded in each lane.

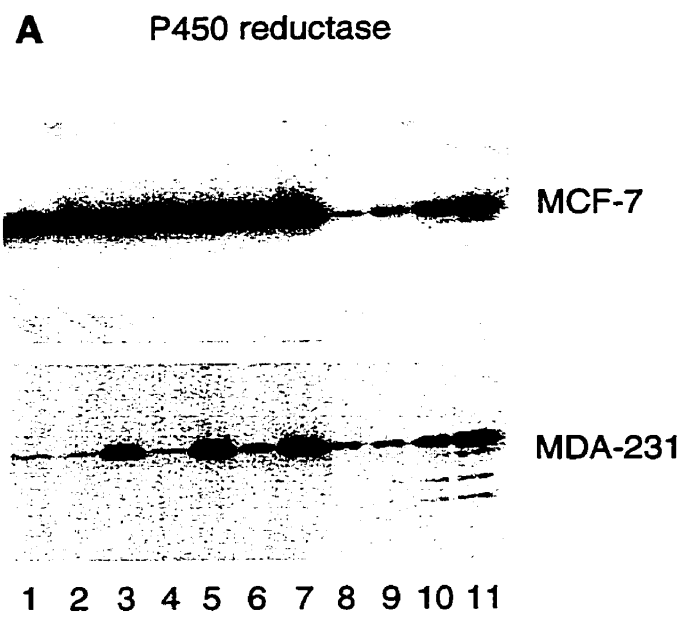

A
P450 reductase
MCF-7
MDA-231
1 2 3 4 5 6 7 8 9 10 11
B
CYP3A4
MCF-7
MDA-231
1 2 3 4 5 6 7 8 9 10 11

Table 3: Recombinant protein content in stable cell lines

Retrovirally transduced cell lines	*Specific P450 content (pmol/mg of microsomal protein)*	*Specific P450 reductase content (pmol/mg of microsomal protein)*
MCF-7	1.43	33.86
MCF-7/AP-2	1.43	41.45
MCF-7/CYP	2.46	44.44
MCF-7/AP-2/RED	1.43	45.33
MCF-7/CYP/RED	2.46	43.24
MCF-7/AP-2/HOR	1.43	42.94
MCF-7/CYP/HOR	3.03	47.15
MDA-231	1.59	6.92
MDA-231/AP-2	1.59	6.92
MDA231/CYP	17.28	23.44
MDA-231/AP-2/RED	1.59	6.94
MDA-231/CYP/RED	20.14	31
MDA-231/AP-2/HOR	1.59	10.54
MDA-231/CYP/HOR	16.9	33.28

levels in AP-2/RED and AP-2/HOR in the range of 7-10 pmol/mg. Its expression is induced in the CYP/RED and CYP/HOR derivatives to about 31-33 pmol/mg.

Immunofluorescence studies (fig. 22) done on these cell lines are summarized in table 4. They show a profile similar to the protein content determined by Western blot analysis. There is a background level of both CYP3A4 and P450 reductase in cells transduced with AP-2. The same basal level is also seen in parental MDA-231 cells. There is a visible increase in the intensity of red label when cells were transduced with recombinant retroviruses encoding these proteins. In MCF-7 cells, similar results were observed (data not shown). It was not possible to double label for CYP3A4 and reductase for technical reasons, to confirm the induction of reductase by CYP 3A4 expression. Control/ conditions with secondary antibody only incubations did not reveal any background label due to secondary antibody only.

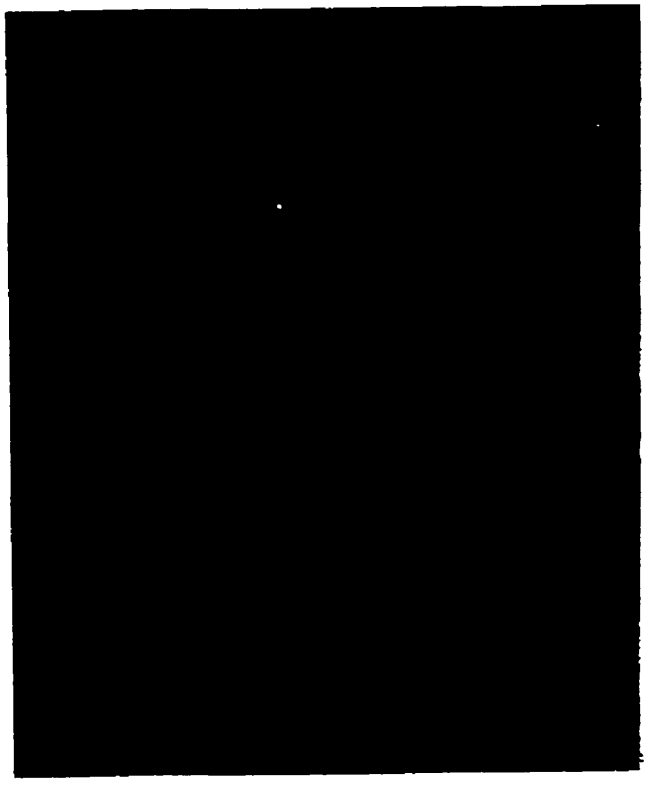
2. Sensitivity to activated metabolites

Sensitivity of MDA-231 and MCF-7 and their derivatives to the activated metabolite of ifosfamide, 4-hydroperoxy ifosfamide (4-OOH-IFA), was tested to verify that the lack of toxicity seen was not due to an intrinsic resistance in these cells to activated ifosfamide metabolites. Cells were seeded as before and exposed to 4-OOH-IFA continuously for 4 days at concentrations ranging from 1 nM to 50 uM. In both cell lines, complete cell killing was seen at a concentration of 5 uM (fig. 23, B and D), with IC_{50} values between 500nM and 50uM. In contrast toxicity to IFEX, the parent ifosfamide compound containing both isomers was not efficient at cell killing (fig.23, A and C) as expected.

Fig. 22 Immufluorescence studies. **A**: the primary antibody is anti-human CYP3A4 polyclonal and the secondary is goat anti-rabbit conjugated to a Texas Red fluorescent label. 1 and 3: MDA-231/AP2 cells under red and green fluorescence, respectively. 2 and 4: MDA-231/CYP cells under red and green fluorescence, respectively. **B**: the primary antibody is anti-rat P450 reductase polyclonal and the secondary is goat anti-rabbit conjugated to a Texas Red fluorescent label. 1 and 3: MDA-231/AP2 cells under red and green fluorescence, respectively. 2 and 4: MDA-231/AP2/HOR cells under red and green fluorescence, respectively.

A

1



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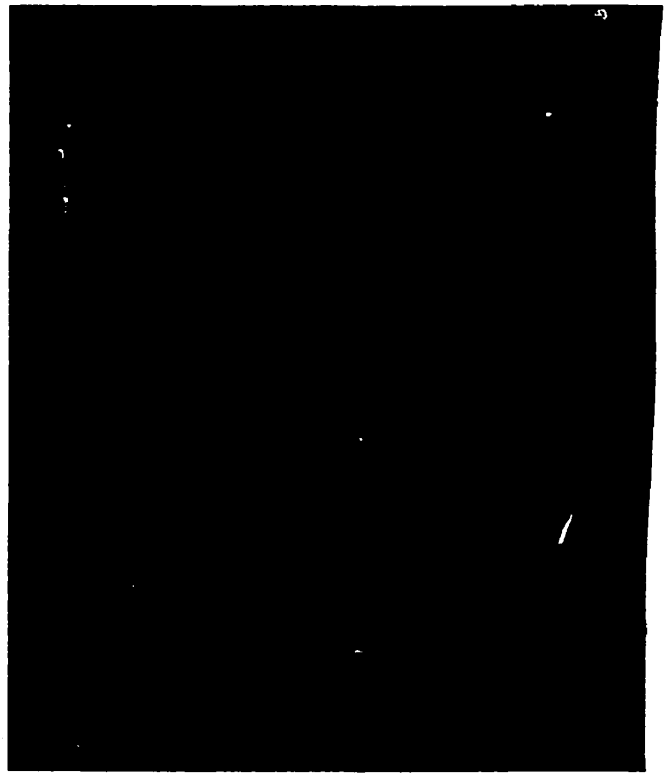

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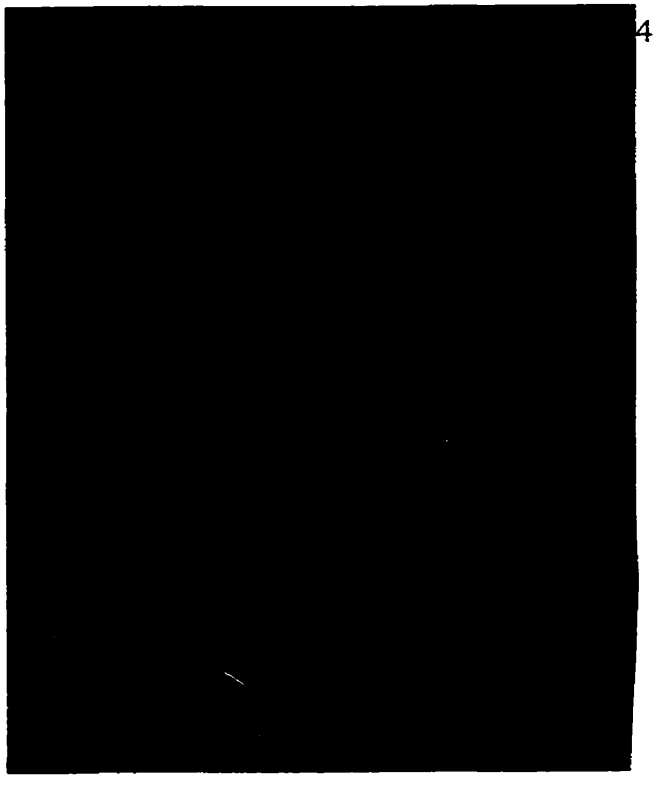

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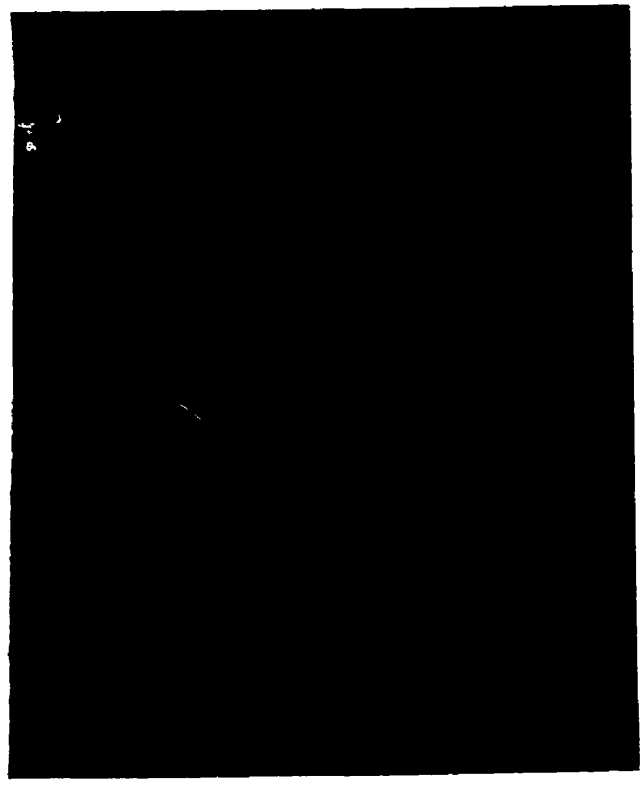
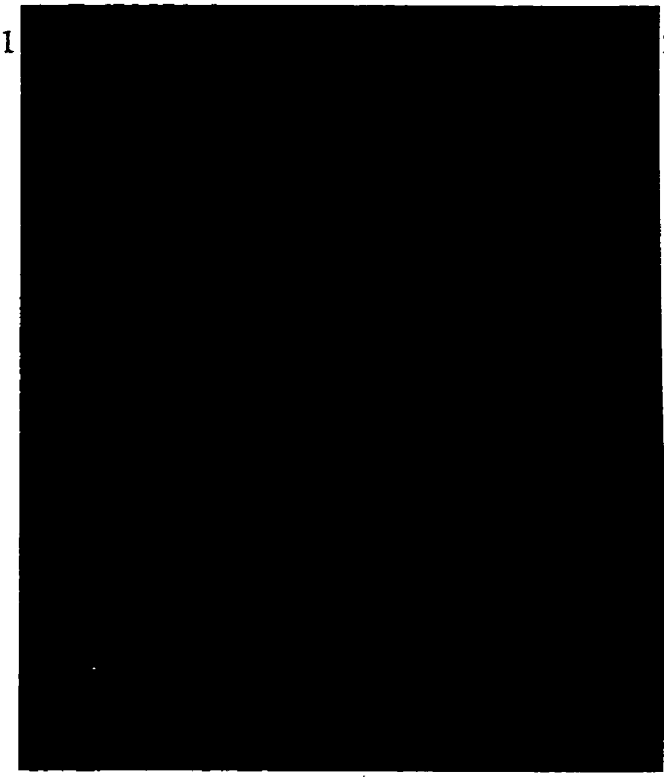
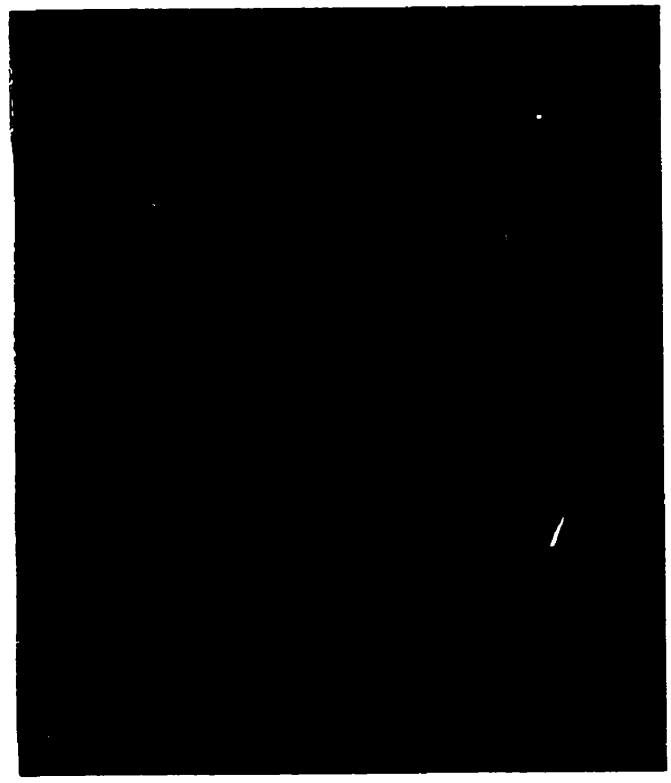


B

1



2



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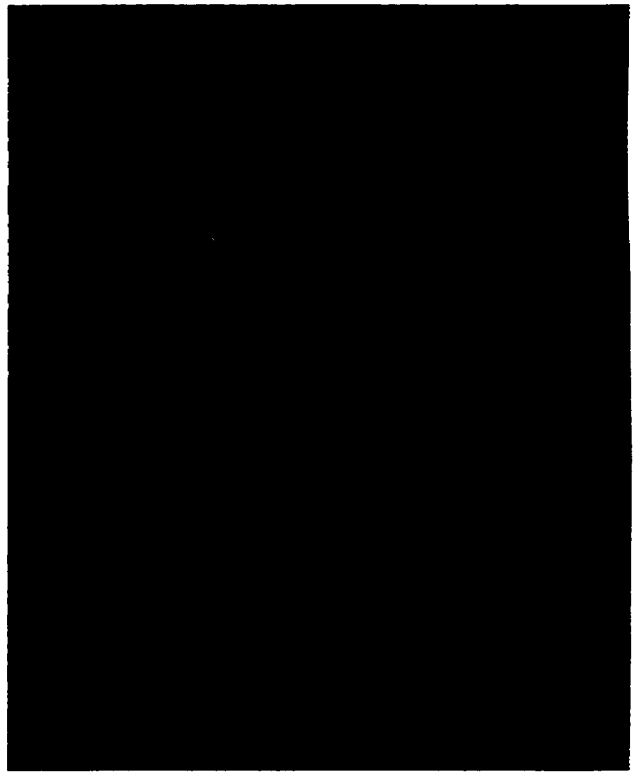

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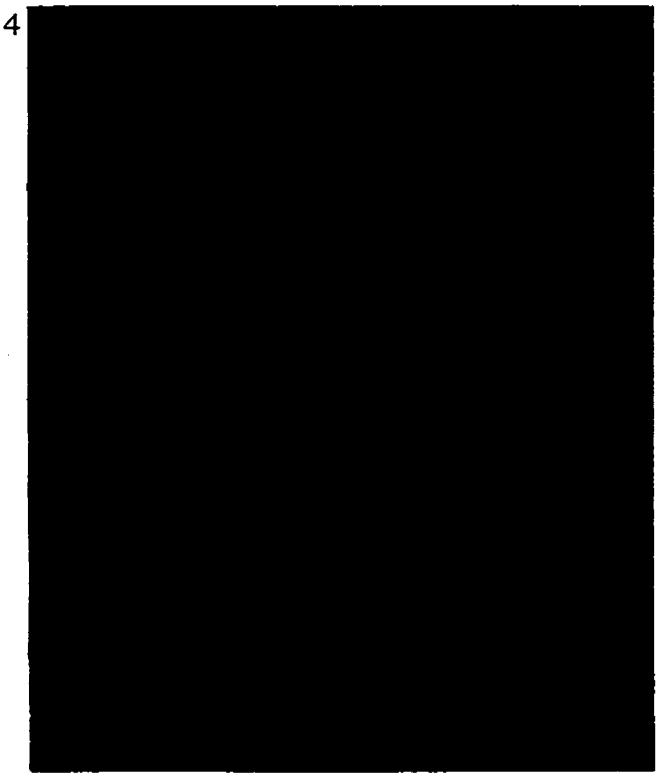
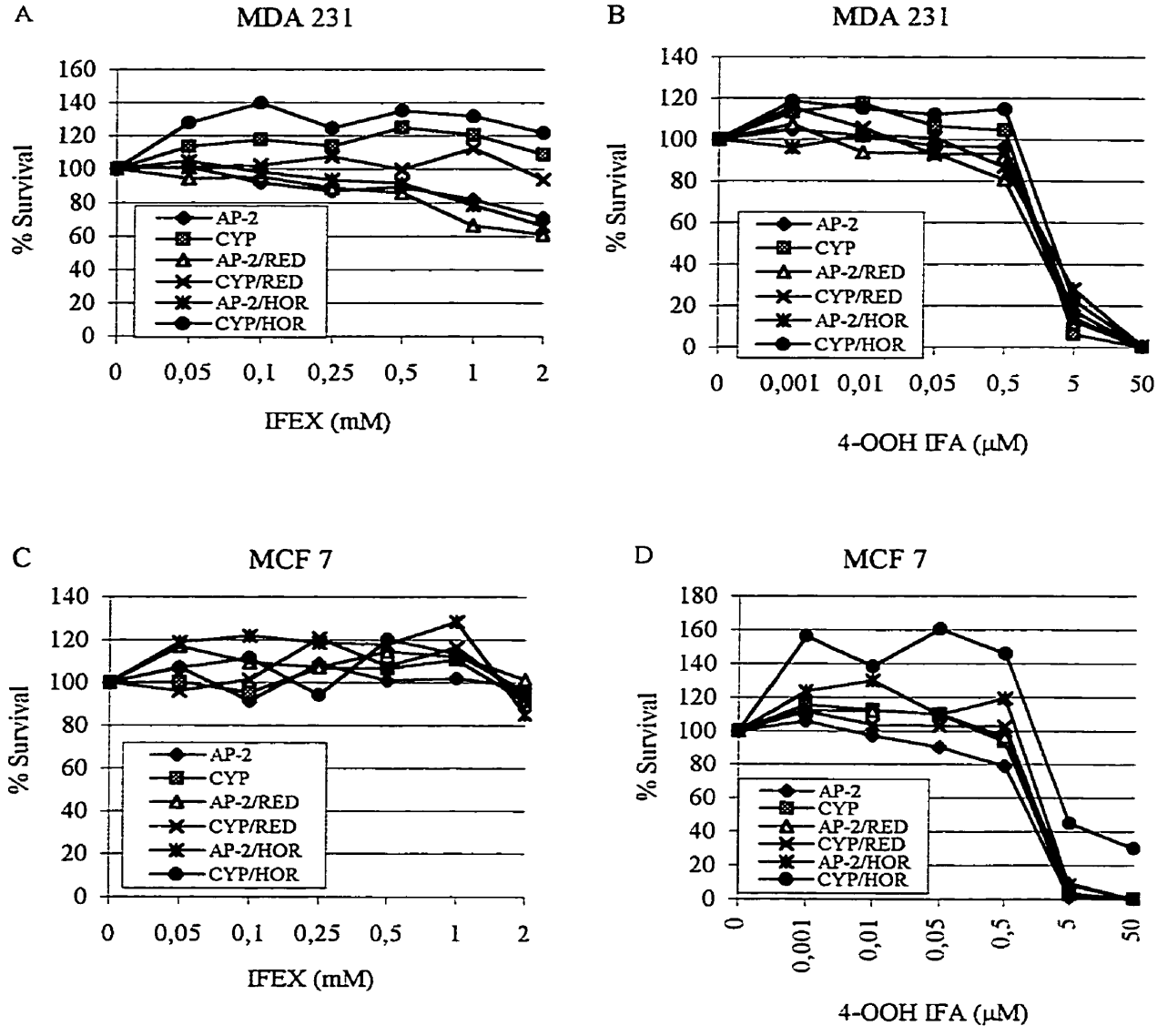


Table 4: Immunofluorescence staining of recombinant protein in stable cell lines

Cell lines	*GFP*	*CYP3A4*	*P450 reductase*
MCF-7	-	+	++
MCF-7/AP-2	+	++	++
MCF-7/CYP	-	+++	N/A
MCF-7/AP-2/RED	+	N/A	+++
MCF-7/AP-2/HOR	+	N/A	+++
MDA-231	-	+	+
MDA-231/AP-2	+	+	+
MDA231/CYP	+	+++	N/A
MDA-231/AP-2/RED	++	N/A	+++
MDA-231/AP-2/HOR	++	N/A	+++

Fig.23 Intrinsic sensitivity in MDA-231 and MCF-7 to activated ifosphamide metabolites. **A** and **C**: stable derivatives from these two cell lines expressing CYP3A4, P450 reductase, a combination of these two and the related controls were exposed to increasing concentrations of IFEX, between 0.05 and 2 mM. **B** and **D**: stable derivatives from these two cell lines expressing CYP3A4, P450 reductase, a combination of these two and the related controls were exposed to increasing concentrations of 4-OOH-IFA, the activated metabolite of IFEX, ranging between 1 nM and 50 μM. Experiments were carried out in quadruplicates for each condition with at least three repeats for every cell line. Error bars represent standard deviations.


A
MDA 231
% Survival
IFEX (mM)
AP-2
CYP
AP-2/RED
CYP/RED
AP-2/HOR
CYP/HOR
B
MDA 231
% Survival
4-OOH IFA (µM)
C
MCF 7
% Survival
IFEX (mM)
D
MCF 7
% Survival
4-OOH IFA (µM)

3. Cytotoxicity assays with CYP P450s

Cytotoxicity in MCF-7, MDA231 and their derivatives expressing CYP3A4 was evaluated upon exposure to CPA, S-IFA, or R-IFA. R-IFA is the active enantiomer of ifosfamide. No toxicity was observed in MCF-7 (fig.25 A,B, and C) or in MDA-231 (fig.24 A,B,and C) in the AP-2 or CYP infected cell lines. Cell survival was evaluated using the MTT assay, for the results shown in fig 24 and 25. The same experiments were carried out and cell survival was determined with the crystal violet assay, as described. Results are summarized in table 5. In this case, cell survival decreased to 50% at the highest concentration of drugs, but there was no difference between CYP expressing cells and the negative control condition of AP-2 transduced cells. Therefore the toxicity seen was not specific to activation of prodrug by CYP3A4.

4. Cytotoxicity assays with CYP P450s and reductase

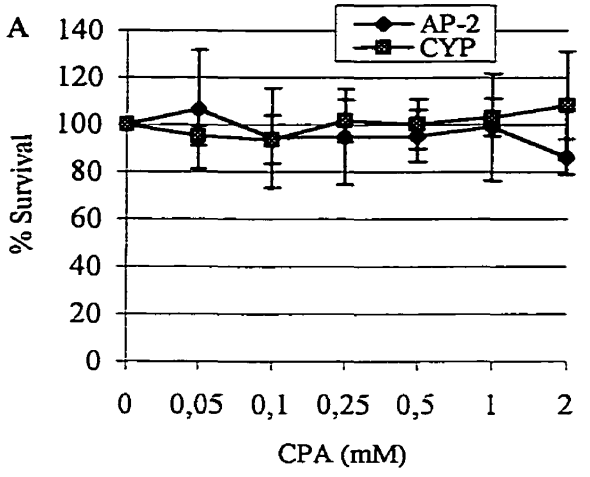
Cytotoxicity in MCF-7, MDA231 and their derivatives expressing CYP3A4 and P450 reductase, CYP/RED and CYP/HOR expressing the rat and human reductase genes respectively, was evaluated upon exposure to CPA, S-IFA, or R-IFA. R-IFA is the active isomer of ifosfamide. Cells infected with a combination of AP-2/RED or AP-2/HOR, expressing the GFP and the rat or human reductase, respectively, were used as a negative control for double infections with a retrovirus. No toxicity was observed in MCF-7 (fig.25 D,E, and F) or in MDA-231 (fig.24 D, E,and F) in the AP-2 or CYP infected cell lines. Cell survival was evaluated using the MTT assay, for the results shown in fig 25 and 26. The same experiments were carried out and cell survival was determined with the crystal violet assay, as described. The results are summarized in table 5. In this case, cell

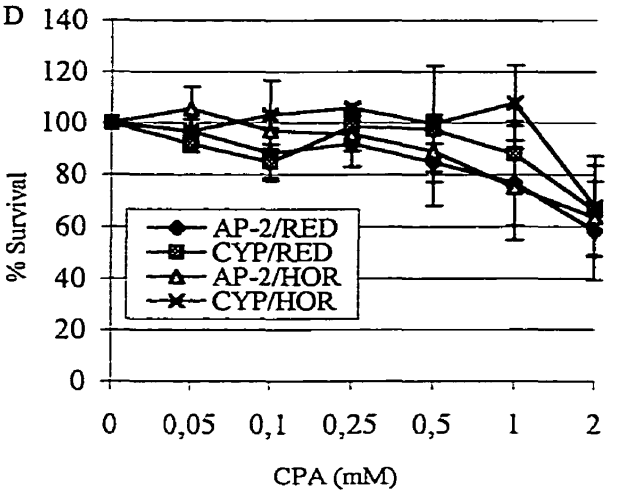
survival decreased to 50% at the highest concentration of drugs, but there was no difference between CYP/reductase expressing cells and the negative control condition of AP-2/reductase transduced cells. Therefore the toxicity seen was not specific to activation of prodrug by CYP3A4.

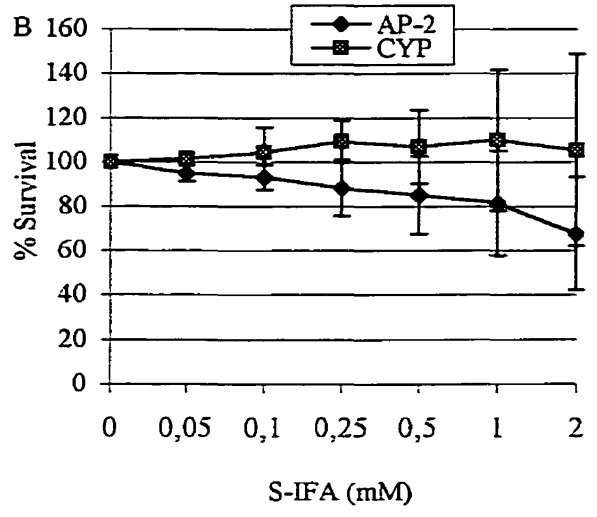
5. Activity of expressed enzymes

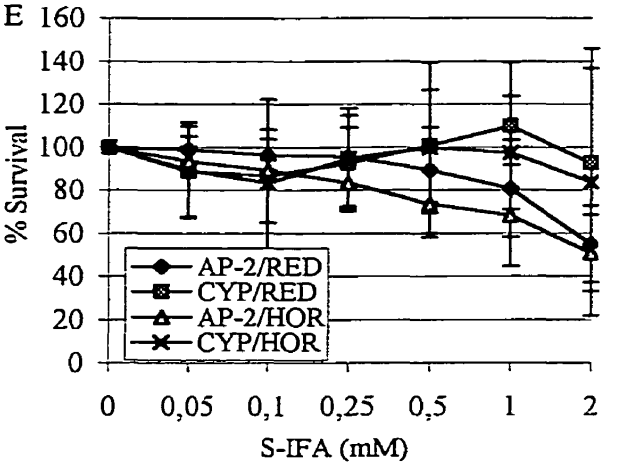
Activity assays for P450 reductase were done by monitoring the NADPH-dependent reduction of cytochrome C at 550 nm at 30°C. Results are expressed as nmol cytochrome C reduced/min/mg. In MDA-231 and MDA-231/AP2 cells, reductase activity was ~0.03, while in MDA-231/CYP it was increased 10-fold to 0.3. In MDA-231/AP-2/HOR, it was 0.5, and in MDA-231 CYP/HOR it was 0.4. In MCF-7, basal reductase activity was higher, at 0.1. In MCF-7/CYP, MCF-7 AP-2/HOR and MCF-7 CYP/HOR it was increased by 4-5 fold, and was between 0.4 and 0.5. These results confirm the reductase enzyme is functional. They also show that its activity is increased by CYP3A4 expression, as seen in MDA-231/CYP and MCF-7/CYP microsomal content by Western blot analysis.

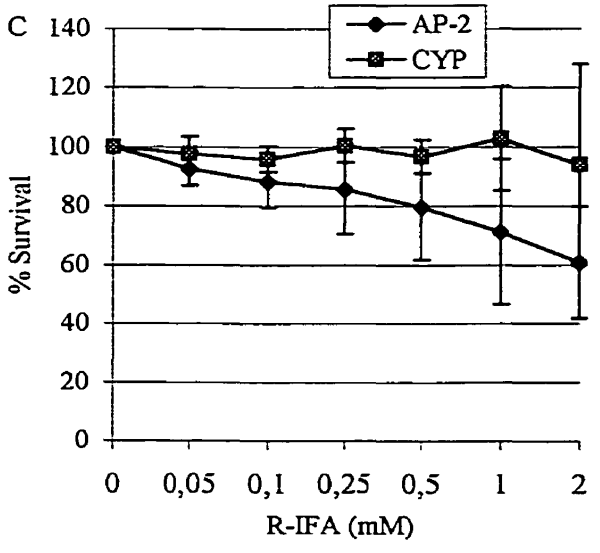
Fig.24 Cytotoxicity studies in MDA-231 cells as determined by MTT assays. **A**,**B**, and **C**: Percent survival in stable derivatives of MDA-231 expressing CYP3A4 and the control cells infected with a GFP-expressing retrovirus (AP-2), exposed to increasing concentration of CPA, S-IFA and R-IFA, respectively, relative to untreated controls. **D**,**E**, and **F**: Percent survival in stable derivatives of MDA-231 expressing CYP3A4 and P450 reductase, exposed to increasing concentration of CPA, S-IFA and R-IFA, respectively, relative to untreated controls. The control cells AP-2/HOR and AP-2/RED are expressing GFP and P450 reductase. Experiments were carried out in quadruplicates for each condition with at least three repeats for every cell line. Error bars represent standard deviations.


A
AP-2
CYP
% Survival
0
20
40
60
80
100
120
140
0
0,05
0,1
0,25
0,5
1
2
CPA (mM)


D
AP-2/RED
CYP/RED
AP-2/HOR
CYP/HOR
% Survival
0
20
40
60
80
100
120
140
0
0,05
0,1
0,25
0,5
1
2
CPA (mM)


B
AP-2
CYP
% Survival
0
20
40
60
80
100
120
140
160
0
0,05
0,1
0,25
0,5
1
2
S-IFA (mM)


E
AP-2/RED
CYP/RED
AP-2/HOR
CYP/HOR
% Survival
0
20
40
60
80
100
120
140
160
0
0,05
0,1
0,25
0,5
1
2
S-IFA (mM)


C
AP-2
CYP
% Survival
0
20
40
60
80
100
120
140
0
0,05
0,1
0,25
0,5
1
2
R-IFA (mM)

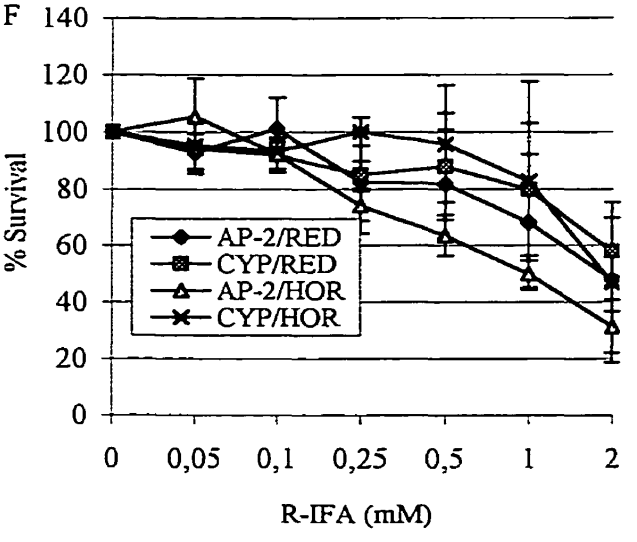
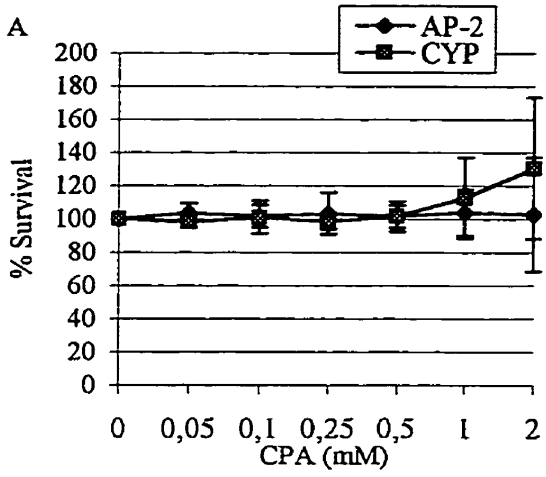
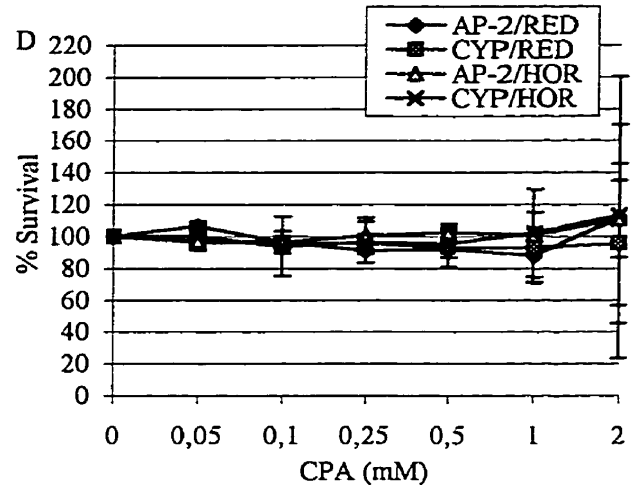
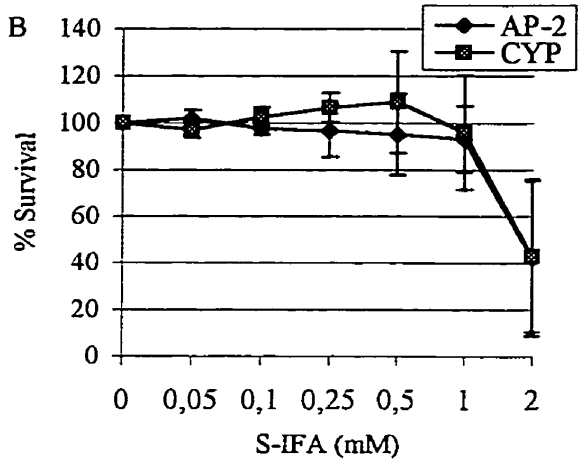
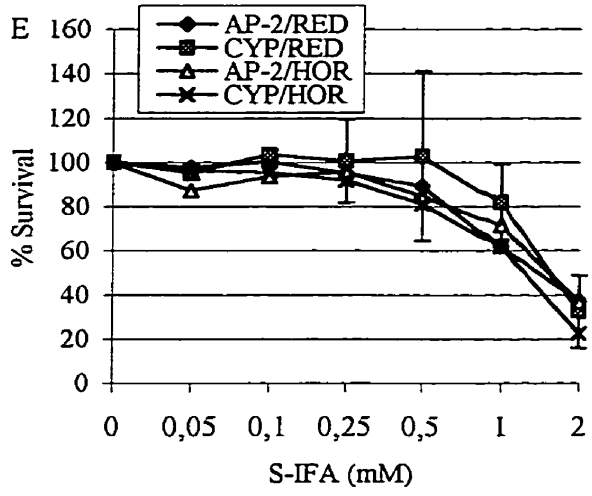

F
AP-2/RED
CYP/RED
AP-2/HOR
CYP/HOR
% Survival
0
20
40
60
80
100
120
140
0
0,05
0,1
0,25
0,5
1
2
R-IFA (mM)

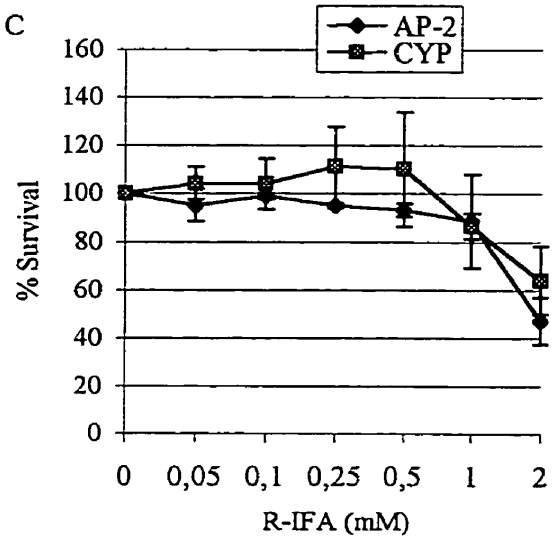
Fig.25 Cytotoxicity studies in MCF-7 cells as determined by MTT assays. Cytotoxicity studies in MDA-231 cells as determined by MTT assays. **A**,**B**, and **C**: Percent survival in stable derivatives of MDA-231 expressing CYP3A4 and the control cells infected with a GFP-expressing retrovirus (AP-2), exposed to increasing concentration of CPA, S-IFA and R-IFA, respectively, relative to untreated controls. **D**,**E**, and **F**: Percent survival in stable derivatives of MDA-231 expressing CYP3A4 and P450 reductase, exposed to increasing concentration of CPA, S-IFA and R-IFA, respectively, relative to untreated controls. The control cells AP-2/HOR and AP-2/RED are expressing GFP and P450 reductase. Experiments were carried out in quadruplicates for each condition with at least three repeats for every cell line. Error bars represent standard deviations.


A
AP-2
CYP
% Survival
0
20
40
60
80
100
120
140
160
180
200
0
0,05
0,1
0,25
0,5
1
2
CPA (mM)


D
AP-2/RED
CYP/RED
AP-2/HOR
CYP/HOR
% Survival
0
20
40
60
80
100
120
140
160
180
200
220
0
0,05
0,1
0,25
0,5
1
2
CPA (mM)


B
AP-2
CYP
% Survival
0
20
40
60
80
100
120
140
0
0,05
0,1
0,25
0,5
1
2
S-IFA (mM)


E
AP-2/RED
CYP/RED
AP-2/HOR
CYP/HOR
% Survival
0
20
40
60
80
100
120
140
160
0
0,05
0,1
0,25
0,5
1
2
S-IFA (mM)


C
AP-2
CYP
% Survival
0
20
40
60
80
100
120
140
160
0
0,05
0,1
0,25
0,5
1
2
R-IFA (mM)

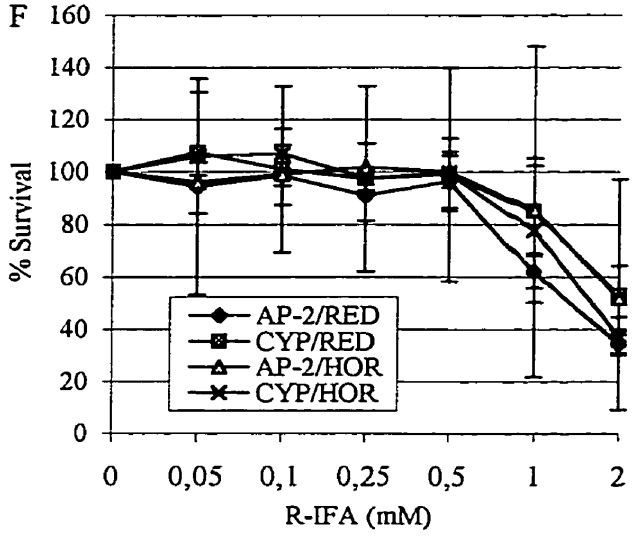

F
AP-2/RED
CYP/RED
AP-2/HOR
CYP/HOR
% Survival
0
20
40
60
80
100
120
140
160
0
0,05
0,1
0,25
0,5
1
2
R-IFA (mM)

Table 5: Cytotoxicity in stable cell lines exposed to Cyclophosphamide, S-ifosphamide or R-ifosphamide as determined by a crystal violet growth inhibition assay

Cell Line	*IC_{50} mM*		
	CPA	S-IFA	R-IFA
MDA-231 AP-2	>2	1.5	1.5
MDA-231 CYP	>2	1.5	1.8
MDA-231 AP-2/RED	>2	1	1
MDA-231 CYP/RED	>2	1.8	1.5
MDA-231 AP-2/HOR	>2	2	0.5
MDA-231 CYP/HOR	>2	1.5	0.75
MCF-7 AP-2	>2	1	1.8
MCF-7 CYP	>2	2	0.75
MCF-7 AP-2/RED	>2	1.8	1
MCF-7 CYP/RED	>2	1.8	>2
MCF-7 AP-2/HOR	>2	1.2	1
MCF-7 CYP/HOR	>2	1.5	1

CHAPTER VI: DISCUSSION

Hexokinase II as a tumor specific promoter

We report the use of hexokinase II promoter for transcriptional targeting of human tumor cells. It has long been known that tumor cells have an abnormally high aerobic glycolytic phenotype (207). Other groups have tried to identify the elements in glucose metabolism that are responsible. Several reports have been published showing hexokinase type II is involved and is transcriptionally upregulated in tumors, reviewed in (208). Our promoter activation studies confirm this finding and show high activity of the promoter in human tumor cell lines from bronchial and mammary origins. Under the same conditions, activation in normal primary cell cultures of the same origin was very low.

To examine differences between normal and transformed phenotypes, regulation of HK II expression was studied using modulators of glucose metabolism such as glucose, insulin, and glucagon. While the same physiological regulation was observed in normal human bronchial cells tested as it was reported in normal rat hepatocytes (217), inhibition of HK II by glucagon was lost in the tumor cell lines NCI-H460 and NCI-H661. In normal peripheral cells, glucagon signals through cAMP, and the ensuing cascade results in glycogen degradation to glucose-6-phosphate, an increase in glycolysis, and ATP production. However this pathway bypasses the HK II step altogether since HK II only plays a role in phosphorylating exogenous glucose (208). In the presence of glucagon, exogenous glucose is low. HK II is only activated again when cells need to replenish their glycogen stocks, after glucagon signaling stops. cAMP responsive elements have been found and characterized in tumor HK II promoter (219). However, in tumor cells, cAMP has been shown to activate the tumor HK II promoter in a glucose dependent or independent manner (220). The synergy of cAMP and glucose which normally indicate

opposing metabolic states allows tumor cells to keep proliferating even under limiting conditions in poorly vascularized tumors, by keeping HK II active and driving glycolysis. As for insulin stimulation, there is not a clear description yet of the coordinated events leading to increased HK II transcription upon cellular exposure to insulin. However there is evidence for the involvement of the PI-3 kinase dependent pathway and not the Ras/MAP kinase pathway in rat muscle cells (218; 298). In the case of glucose, which is a substrate for HK II, it has been shown that there is a direct involvement in promoter activation, ruling out a role for other glycolysis intermediates or glucose metabolites(220).

HK II has been shown to be expressed in some tissues in humans such as adipose tissue./ However, there is a discrepancy between various reports on HK II distribution since it is relatively unstable and is responsive to hormonal status(209). HK II mRNA was detectable in NHBEC although promoter studies show very little if any HK II promoter activation in normal cells. The promoter under study is isolated from a highly glycolytic rat hepatoma whereas cells and tissues explored are of human origin. Changes at the level of transcription factors or signaling pathways in transformed cells may account for the lack of discrimination between the rat and human promoter. The promoter for human HK II has not been isolated or characterized yet to allow for a comparison between the two. This may be one explanation to discrepancies between HK II promoter activation studies in NHBEC and HK II mRNA levels. Moreover, when looking at endogenous levels, there is an added level of complexity compared to promoter studies done with chimeric reporter gene constructs. The protein encoded by the HK II gene can have a role in its own regulation and in maintaining its mRNA at a predetermined level.

The pattern of differential regulation between normal and tumor cells observed with endogenous HK II mRNA levels in the ribonuclease protection assay was confirmed in rat HK II promoter activation studies performed on human lung carcinomas. This difference in regulation could be exploited in gene targeting since it adds a feature of pharmacological targeting in addition to the locational one.

It has been suggested that the pattern of HK I and HK II expression depends on the stage of malignancy (210). As progression is made to a less differentiated state and metastatic disease, the expression of HK II predominates (211). This is reflected in the variability of response between the different tumor cell lines. A direct interaction between the rat HK II promoter and mutant p53 has recently been reported (299) and provides a possible link between genetic cellular transformation, cell cycle control, and the most common phenotype of tumor cells, that is their ability to catalyze at high rates and exhibit rapid proliferation. There is a correlation between p53 status in the cell lines we tested and the strength of the HK II promoter. NCI-H661 has a mutant p53 and shows the highest activation levels, whereas NCI-H460 and MCF-7 have wild type p53(62) and show lower levels of promoter activation. There are no other studies providing a direct linking mechanism, such as activation of HK II in the presence of a mutant p53, between the malignant genetic transformations and selective activation of the HK II promoter in tumor cells. A speculative model for a mechanism of selective HK II promoter activation in tumor cells is that the promoter is under a general state of repression in normal cells, maintained by a balance of repressor and activator molecules. Repression is lost in tumor cells due to the absence or abnormal state of transcription factors acting as tumor suppressors.

One of the observations we made with the HK II promoter is that its capacity to activate LacZ expression in normal cells (NHBEC) is increased as MOI of AdHexLacZ is increased. At an MOI of 50, 30-40% of NHBEC cells infected with AdHexLacZ were positively stained with X-gal. Tumor cells infected with AdHexLacZ showed an activation of nearly 100% at an MOI of 10. While this shows there still is a margin of selectivity between normal and tumor cells, it also confirms the involvement of a repressor in normal cells. This repression could be overcome by saturating the system in vitro with promoter elements, as it was done at higher multiplicities of infection. Whether the same effect will be seen in vivo remains to be determined as dose-ranging studies with AdHexLacZ or AdHexTk are done to ensure specificity is retained at MOIs high enough to activate suicide genes in tumor cells.

Transcriptional targeting in adenoviral vectors has been problematic in some cases where selectivity was lost once the promoter/cDNA expression cassette was cloned in the E1 or E3 region of an adenoviral vectors(223). Our construct was made with the expression cassette in the antisense orientation to the left ITR to avoid any interference from viral enhancers and promoters and to improve expression of the transgene. This was effective in maintaining the selectivity of the promoter. In summary, we have demonstrated the rat HK II promoter is activated in human tumor cells but not normal ones. Previously described promoters for transcriptional targeting were mostly tissue-specific. In contrast, our results demonstrate tumor tissue specificity. Since the phenotype resulting from HK II overexpression is common to different types of cancers, the HK II promoter has the advantage of being versatile and lending itself to applications in different types of tumors.

Need for targeting and requirements for its success

For transcriptional targeting to be successful, promoters need to be both strong and tumor specific. In the case of suicide gene therapy, the level of suicide gene expressed in the cell has to be high enough to activate prodrugs efficiently. The drawback of utilizing cellular promoters is their weakness, relative to non-specific viral promoters. As described in section 4 of the introduction, methods to improve transcriptional targeting and achieve selectivity for tumor cell killing have been explored. Integration of a specific promoter in more intricate strategies, such as regulatable promoters, can be useful to amplify gene activation while restricting it to tumor cells. In this case, transactivators can be expressed from specific promoters to confer tumor specificity. Activation is amplified by having a suicide gene expressed from the promoter responding to that transactivator at very high levels.

Targeting can also be useful in eliminating immune responses to adenoviral vectors. A study from Wilson's group (174) shows the importance of antigen presenting cells (APCs), and more specifically dendritic cells in activation of a T cell response. When these cells are infected with adenoviruses expressing transgenes from strong constitutive promoters, transgene product recognition and cell elimination from infected tissues is going to be equally strong. However if specific promoters limit transgene expression in APCs, then immune response problems are likely to be reduced. This is in agreement with the study done by Lusky and colleagues (173) which shows that immune responses in the case of adenoviruses are due to reactions to the transgene and not only to reactions to viral proteins.

Moreover, in the case of a suicide gene approach, targeting is crucial to avoid toxicities in normal organs that readily take up the viral construct if it were injected systemically. With the HSVTk/GCV combination, toxicities in normal tissue in the liver were considerable (253). Restricting Tk expression to tumor cells with specific promoters alleviates this problem. This was recently demonstrated with the CEA promoter in a subcutaneous tumor model where systemic injection did not cause any liver toxicity compared to the CMV promoter at the same viral dose (300). However systemic injections were not sufficient to cause tumor reduction and that effect was demonstrated via direct intratumoral injections.

Dependence of Tk suicide gene therapy efficacy on delivery method

Our results on the use of adenoviral or retroviral vectors for transgene expression have shown that viral gene delivery systems can interfere with treatment outcome. Adenoviral vectors that were used in our study are from the so-called first generation of recombinant adenoviruses. A common observation with these E1/E3-deleted, replication defective vectors is leakiness of viral protein expression in host cells. This was reported in several studies done with these vectors in vivo. Short-lived transgene expression as well as strong inflammatory responses in injected organs support the idea of the immune system recognizing and reacting to certain viral proteins or transgene-derived ones expressed in host cells. While immune responses to viral proteins may not be problematic in the context of in vitro studies we were doing, it is conceivable that interactions between cellular and viral proteins are affecting the final sensitivity of a cell population. Interactions of these proteins with cellular factors can affect sensitivity to activated drug or interfere with cell death pathways. The adenoviral vectors we used were deleted

between 339 and 3533 bp, corresponding roughly to map units 1 to 9.5, and between 27865 and 30995 bp, or map units 76 to 85. This region encompasses the E1A and E1B regions, as well as a nearly complete E3 deletion. The E1A region encodes several proteins that were demonstrated to have a role in inducing cellular DNA synthesis and promoting apoptosis by overactivating p53 (136; 301; 302). This apoptosis inducing action is inhibited by E1B genes, which interact with p53 to prevent premature cell death. Therefore both E1A and E1B are needed for viral replication. E1B genes also interact with proteins other than p53, such CED-5 to inhibit caspase activation and ensuing apoptosis (136; 303). E3 genes are implicated in control of host cells activation of inflammatory responses that lead to cell death and that help the virus evade immune surveillance in vivo. It was shown to interact with cellular proteins to inhibit NF-kappa B activity, thereby inducing apoptosis, and prevent TNFα induced cytolysis (136; 304). These actions may seem contradictory, but not all functions of E3 proteins have been characterized yet to give a global idea on the outcome of such interactions with host defense mechanisms. The E4 region of adenoviral vectors has also been shown to produce proteins that interact with cellular proteins to inhibit apoptosis. Seven proteins are made from E4 genes, but E4orf6 effects are the most documented. E4orf6 or E4 34 kd was first thought to inhibit apoptosis through interactions with E1B 55 kd, but it has also recently been shown to interact independently with p73 (305). p73 is a p53-related protein that has similar actions on cell death. E4 34 kd inhibits p73 transcriptional activation and cell killing. Moreover, E4 34 kd has been shown to interact with several other cellular proteins whose normal function hasn't been elucidated yet (306). This leads us to speculate about interactions of adenoviral proteins with cellular ones in ZR75, the

AdRSVTk/GCV resistant cell line we characterized. It is conceivable that these interactions are inhibitory to kinases directly involved in GCV metabolism. Some E1 viral proteins have been documented to affect the synthesis and activity of several enzymes involved in DNA precursors metabolism, such as dihydrofolate reductase, thymidylate synthase, thymidine kinase, and cell cycle-regulatory enzymes such as cdc-2 (132). Although the vectors we are using are E1-deleted, other viral regions that are less well characterized can be producing proteins with similar activities. Another possibility is interference of viral proteins from the remaining E4 region with cell death signals. However this hypothesis has to be verified first by confirming that apoptosis is the mechanism for Tk/GCV mediated toxicity.

Pharmacological modulation of the bystander effect

Delivery vehicles have been limited by the inefficiency of present technology to deliver therapeutic genes effectively to a large proportion of the tumor cell population, even with intratumoral injection. Recent progress in vector technology may solve some of these problems (307). One characteristic of suicide gene therapy with various combinations is the bystander effect. It offers a way to access a greater cancer cell population, after infection, through the transport of the activated anticancer agent to adjacent cells that were not originally targeted. Efforts to enhance this effect may have a significant impact on the efficacy of this therapeutic approach.

Studies on the bystander effect using the vTK/gancyclovir system were carried out using Cx43 stable transfectants (271), Cx43 delivery systems, or cells with naturally higher levels of Cx43 (261). Furthermore, there is evidence for a dose effect of the Cx43 expression, in that the enhanced bystander effect observed in the Cx-transfection studies

was most obvious in the clones expressing the highest concentration of Cx43 (271). While these studies established the role of GJIC as its main mediator, they didn't offer any insight into a therapeutically applicable method of enhancing it. An alternative approach would be to pharmacologically induce Cx43 and GJIC, and subsequently the bystander effect. A chemical inducer can potentially access a greater number of the cancer population as compared to a virus delivery system. The first attempt to induce the bystander effect pharmacologically was recently demonstrated utilizing retinoic acid (308). In this study, although retinoids enhanced GJIC, they also had GJIC-unrelated effects on cell killing, since they affected cell survival even when all cells were infected with a vTK-expressing virus. Also, there was no examination of the bystander effect on infectivity levels below 10%, which is likely to be a clinically relevant level of cell targeting when adenoviral delivery is used *in vivo*.

We demonstrated that 8-Bromo-cAMP clearly leads to increases in GJIC and Cx43-containing gap junctions in a breast cancer cell line, as previously reported for other cancer cell models (273). This is associated with enhanced cell killing in a mixed population of breast cancer cells, at different levels of Tk expressing cells present in the population, and more significantly at levels between 2% and 50% of the total population. We have shown this effect for cell lines both stably transduced with a retroviral vector and infected with an adenoviral vector. Subsequently, we have shown that cAMP does not affect virus infectivity, DNA stability, or gancyclovir cytotoxicity. Chemically enhancing the bystander effect appears adequate to decrease by half the number of cells that need to be targeted in order to attain 50% killing. These observations add strength to the rationale of developing clinically-tolerable potent gap junction inducers (309), to

enhance the efficacy of suicide gene therapy. However, testing of this hypothesis in vivo should be done in immunocompetent animals to evaluate efficacy in the context of a contribution from the immune system.

Efficacy of a CYP-based suicide gene therapy

Three different groups have published on the use of the CYP2B1 gene to sensitize tumor cells to cyclophosphamide (110-113; 310; 311). From all of these studies the lowest IC_{50} achieved, even with co-expression of a P450 reductase, is 250 μM. This IC_{50} is quite high, considering that the liver can metabolize cyclophosphamide at least as well. Although it is a prodrug, it doesn't fit in the ideal description of a prodrug, which is pharmacologically inert, until it is activated by the tumor-directed suicide gene. Therefore the application of this strategy in a clinical setting may not lead to an improvement in therapeutic index. A more recent study revealed a strategy to improve on the use of CYP2B1 to activate cyclophosphamide by tumor cells (312). In this case, an oncolytic viral mutant, which selectively replicates in tumor cells that express the complementing protein ribonucleotide reductase, was used for gene delivery. Growth of tumors treated with this replicating virus, which expresses the CYP2B1 gene, was completely inhibited in vivo, whereas tumors treated with replication defective adenoviruses or retroviruses for CYP2B1 delivery continued to grow even in the presence of cyclophosphamide. This shows a much higher level of CYP2B1 is required to be expressed in tumor cells than can be achieved with traditional viral gene delivery.

Our results using a similar combination base on CYP3A4 and R-IFA proved to be not sufficient to activate prodrugs efficiently after retroviral-mediated gene transfer. Moreover, we have shown that the reductase gene is normally present in some human

tumor cell lines, like MCF-7, and that it is further induced by the expression of the CYP3A4 gene, in the case of the MDA-MB-231 cell line. These novel observations show it is unnecessary to use bicistronic vectors to express both a reductase and a CYP gene. A study published recently documented the use of various CYP proteins with either CPA or IFA (295). While this group's results demonstrated activation of CPA and IFA by CYP2B1 and CYP3A4 respectively, and that it is useful to co-express a reductase with these genes, their strategy to do so differed substantially. Stable cell lines, all derived from 9L gliomas, were generated using retroviral constructs. Most importantly, clonal selection was performed using the prodrugs themselves, CPA and IFA to select the best clones. This protocol heavily favors the selection of clones sensitive to prodrugs, and this sensitivity could be due to the expressed gene or to another unknown and unaccounted for factor. Moreover, this strategy is incompatible with the concept of gene therapy, where there can be no selection for gene transfer. The efficacy of a suicide gene strategy has to be tested taking into account the limitations of gene transfer of presently available delivery tools.

Although the Tk/GCV system is the most widely used, a study comparing it to a cytosine deaminase/5-fluorocytosine system showed that the latter was more efficient (313). Criteria used were levels of specific enzyme activity, IC_{50}, and bystander effect. Other studies(100) were done on HSVTk to improve its substrate specificity. These studies could be extended to other enzymes (314) used in suicide gene therapy keeping these three criteria in mind to find a truly ideal combination.

Relevance of in vivo models

Studies characterizing the use of CYP2B1 and CYP3A4 have demonstrated the concept that CYP genes may activate cyclophosphamide when introduced into tumor cells. However, this demonstration was done in an artificial context of in vitro cultures or in vivo tumor models that are very far from the reality of a heterogeneous tumor in a syngeneic patient. A recent evaluation of the C6 glioma tumor model that was used in two of these studies (113; 310) has shown that this syngeneic tumor model, used by many to demonstrated the efficacy of tumor gene therapy, is in fact an allogeneic tumor model, where tumors can regress spontaneously and that protective anti-tumor immunity ensued without any therapeutic intervention (315). Experimental studies including gene therapy ones are generally evaluated with direct comparison to negative controls. However, the results of these comparisons have to be kept in perspective. While they may show a statistically significant difference between the control and the treatment groups, they don't necessarily guarantee a successful outcome in a more natural setting. Models utilizing spontaneous tumor models are more relevant in this case. They have been used in the case of HSVTk/GCV gene therapy (286), and showed an advantage in Tk modified tumors. However the use of such models is not as widespread as subcutaneously implanted tumor models. This criticism may not apply for studies requiring the evaluation of a therapeutic response in human derived cells. In this case the use of xenograft models is inevitable. It is very useful to validate a proof of concept in an in vivo setting, but it doesn't provide direct answers for the validity of a therapeutic approach to treat tumors in human beings.

Contributions to Original Knowledge

1- Identification of a novel use for the rat hexokinase type II promoter in cancer gene therapy. Demonstrated specific promoter activation, relative to normal cells, in a series of human tumor cell lines of different origins.

2- Demonstrated retention of specificity in an adenoviral vector, compatible for in vivo use of this therapeutic approach. This demonstration was done by showing tumor cell specific killing with the HSVTk /GCV combination directed by the HK II promoter.

3- Previous studies of glucose metabolism identified a pattern of regulation for this promoter that is different in normal and tumor cells of rats. This differential regulation was confirmed in human tumor and normal cells.

4- cAMP has been used previously to upregulate gap junction intercellular communication. Contributed to the demonstration of an upregulation of the bystander effect for HSVTk/GCV therapy using cAMP, and demonstrated that this effect was not due to non specific effects on adenoviral infectivity and adenoviral-mediated gene transfer.

5- Demonstrated that the CYP 3A4-based suicide gene system is not efficient to achieve tumor cell killing. Co-expression of the reductase gene is not necessary, since the expression of this protein is upregulated by the expression of CYP3A4.

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