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(71) Applicant *(for all designated States except US)*: DANA-FARBER CANCER INSTITUTE [US/US]; 44 Binney Street, Boston, MA 02115 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FINE, Howard, A. [US/US]; 352 Commonwealth Avenue, Unit 4, Boston, MA 02115 (US). KAELIN, William, Jr. [US/US]; 12 Commonwealth Avenue, Boston, MA 02116 (US).

(74) Agents: EISENSTEIN, Ronald, I. et al.; Dike, Bronstein, Roberts & Cushman, LLP, 130 Water Street, Boston, MA 02109 (US).

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(54) Title: METHOD OF TARGETING MALIGNANT CELLS USING AN E2F RESPONSIVE PROMOTER

(57) Abstract

A method of selectively expressing a gene in a malignant as opposed to a non-malignant cell is taught. This permits one to selectively express proteins that would be deletorious to normal cells with minimal harm. The method involves the use of a nucleic acid cassette having an E2F responsive promoter operably linked to a gene of interest, which encodes either a positive or negative potentiator such as antibodies, dominant negative mutants, suicide genes, antisense RNA, ribozymes and cytotoxic agents.

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METHOD OF TARGETING MALIGNANT CELLS USING AN E2F RESPONSIVE PROMOTER

BACKGROUND OF THE INVENTION

1. Field

The present invention is directed to a method of selectively targeting malignant cells. More specifically it involves the use of an E2F responsive promoter operably linked to a gene whose expression is desired in a tumor cell, but not non-malignant cells.

2. Background

One of the difficulties in treating individuals with malignant cells exhibiting uncontrolled growth, such as typically associated with tumors including solid tumors, leukemia, etc., is that antineoplastic agents are not highly selective. For example, many agents (e.g. radiation, chemotherapy) do not distinguish between malignant and non-malignant cells, but are directed to dividing versus non-dividing cells. Thus, such agents do not effect quiescent malignant cells while adversely effecting normal dividing cells.

There have been proposals to use gene therapy techniques in treating malignant cells. For example, targeting a cell with a positive potentiator such as a cytokine or costimulatory molecule to enhance an immune reaction to the tumor or a suicide gene or cytotoxin to kill the malignant cells. Many proposals have focused on using retroviruses that preferentially infect actively dividing cells. However, not all the cells in a tumor are actively dividing at any one point in time. And it is well

known that one of the problems in cancer treatment is from the cells not killed in the initial treatment. Moreover, not all actively dividing cells are tumor cells.

Alternatively strategies that focus on using a vector that will infect both resting and actively dividing cells such as adenoviral vectors also have problems. While some degree of selectively can be obtained by means of administration, the vector wil still infect the normal tissue surrounding the tumor as well as the tumor. Consequently, the treatment will also cross-over to the normal tissue. For example, delivery of a cytotoxin or suicide gene will lead to some degree of host toxicity due to delivery of the gene to the surrounding normal cells. Thus, methods for increasing selectivity between malignant and non-malignant (normal) cells would be desirable.

The E2F family (E2F1-E2F5) are capable of activating transcription when bound to DNA. For example, E2F-1 is a ubiquitously expressed growth regulated, gene exhibiting peak transcriptional activity in S-phase [Tevosian, S.G., et al., Cell Growth and Diff. 7:43-52 (1996), Kaelin, W.G. et al., Cell 70: 351-364 (1992)] Transcription of the gene is cell cycle dependent as a result of E2F DNA-binding sites within its promoter [Neuman, E., et al, Mol. Cell Biol. 14:6607-6615 (1994)]. E2F activity is regulated, in part, by complex formation with cell cycle

regulatory proteins such as cyclin A, cyclin K, cdk2, and members at the retinoblastoma protein (pRB) family (pRB, p107 and p130) [Weinberg, R.A., Cell 81:323-330 (1995); Adams P.D. and Kaelin, W.G., Seminars in Cancer Biology 6 : 99-108 (1995)] Members of the pRB family actively repress transcription when bound to DNA via E2F [Sellers, W.R., et al. Pro. Natl. Acad. Sci. USA 92: 11544-11548 (1995); Weintraub, S.J. et al. Nature 358: 259-261 (1992)]. Overproduction of E2F can override a pRB-induced growth arrest [Qin X.-Q, Mol.Biol. Cell. 15: 742-755 (1995); Zhu. L, et al. Genes Dev. 7: 1111-1125 (1993)] Many malignant cells for examle solid tumors such as malignant gliomas, have disrupted pRB function; either due to RB-1 gene mutations or due to mutations affecting upstream regulators of pRB such as cyclin D1 or p16/1NK 4aIMTSI [Weinberg RA, Cell, supra, He J. et al, Cancer Research 54:5804-5807 (1994)]; Schmidt, EE, et al. Cancer Research 54:6321-6324 (1994). Although this might suggest that greater levels of E2F are expressed in malignant cells, E2F is still expressed in non-malignant cells.

SUMMARY OF INVENTION

We have now discovered a system that is substantially more tumor-specific than current antineoplastic agents such as radiation and

chemotherapeutic agents. This system uses a "vector system" containing an E2F responsive promoter operably linked to a heterologous gene of interest, preferably encoding a negative or postive potentiator, more preferably a negative potentiator such as a suicide protein or a cytotoxic protein. This sytem results in high selectivity *in vivo* between malignant and non-malignant cells. Preferably, the malignant cell is from a solid tumor.

The E2F transcription factor (sometimes referred to as E2F protein or E2F) can regulate expression of numerous genes effecting cellular proliferation including proto-oncogenes and genes regulating cell cycle progression. For example, the retinoblastoma tumor suppressor gene product (Rb) interacts with E2F to form a complex where E2F expression is inhibited.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a schematic of the Adenoviral vectors. The backbone adenoviral sequences are derived from an E3-deleted adenovirus serotype 5, with a bacterial plasmid insert in the E1a region. Following recombination, E1a is replaced by the early CMV promotor (Ad.CMV-βgal) or from the upstream region of the E2F-1 gene (basepairs -218 to +51), a segment containing 4 intact E2F, 1 NF-ķB, and 4 Sp1 consensus sequences. The

β-galactosidase gene was derived from a Lac Z-containing adenoviral shuttle vector, previously described [25].

Figures 2A and b show in vitro cell cycle vector induction. Ad.E2F1-βgal cell cycle selective gene expression is shown. Fig. 2A shows the percentage of cells in S-phase in serum refed (▲) versus serum starved C6 (Δ) cells. Figure 2B shows the relative β-galactosidase expression in C6 cells: (●) Ad.CMVβgal, serum fed; (0) Ad.CMV-βgal, serum starved; (■) Ad.E2F1-βgal serum fed; (□) Ad.E2F1-βgal serum starved.

Figures 3A-F Ad.E2F1-βgal mediated tumor selective transgene expression in vivo:

Fig. 3A shows Ad.CMV-βgal injected into normal brain; Fig 3B shows Ad.CMV-βgal injected into tumor-bearing brain; Fig. 3C shows Ad.E2F1-βgal injected into normal brain; Fig. 3D shows Ad.E2F1-βgal injected into tumor bearing brain; Fig. 3E shows Ad.AE2F1-βgal injected into normal brain; Fig. 3F shows Ad.AE2F1-βgal injected into tumor bearing brain.

Figures 4A-4F show Ad.E2F1-βgal transduction of normal regenerating liver

whole mount liver sections stained for β-galactosidase; Figures 4C and 4D show thin sections of liver tissue stained for β-galactosidase activity and PCNA expression; Figures 4E and 4F show immunofluorescence of thin sections of the liver for expression of the adenoviral fiber protein. Figures 4A, C and E show transduction by Ad.CMV-βgal; Figures 4B, D and F show transduction by Ad.E2F1-βgal.

Figures 5A-D show in vitro and in vivo anti-tumor activity of Ad.E2F1-tk.

In Fig. 5A

C6 glioma cells were infected with viral vectors at an MOI of 50 for 1 h, then washed, and replated in tissue culture plates containing GCV at various concentrations. The cells were fixed 72 h later, stained with methylene blue, and absorbance at 600 nm (A_{600}) was determined on a microplate reader. (Δ) no virus; (○) Ad.CMV-βgal; (●) Ad.CMV-tk; (□)] Ad.E2F1-βgal; (■) Ad.E2F1-tk.

Figure 5B shows Kaplan-Meier survival curves of animals treated by stereotactic injection of viral vectors into 6 d old established intracerebral C6 gliomas, followed by twice a day treatment with intraperitoneal GCV (15 mg/kg) or saline. (●) Ad.CMV-tk, GCV; (0) Ad.CMV-tk, saline; (■) Ad.E2F1-tk, GCV; (□) Ad.E2F1-tk, saline; (▲) Ad.AE2F1-βgal, GCV; (Δ) Ad.E2F1-βgal, saline. Figures 5C and 5D show

Hematoxylin-eosin stained thin section of brain from animals intracerebrally injected with Ad.CMV-tk (Fig. 5C) or Ad.E2F1-tk (Fig. 5D) and treated with CCV (15 mg/kg) twice daily for 7 d.

DETAILED DESCRIPTION OF THE INVENTION

Vector systems containing a E2F responsive promoter operably linked to a gene of interest can be used to selectively express that gene in significantly higher levels in a malignant cell in contrast to a non-malignant cell. The gene of interest is a gene whose expression is desired in the malignant cell but not the non-malignant cell. Preferably, the gene would be express a cytotoxic or therapeutic protein.

We have found that similar levels of expression of a suicide gene such as the herpes thymidine kinase (tk) gene are obtained in malignant cells whether the gene is operably leaked to a E2F responsive promoter or another promoter such as the cytomegalovirus early promoter (CMV). This observation is based upon cell death based upon subsequent treatment with ganciclovir (GVC). Cells expressing tk are sensitive to GVC and are killed (See Fig. 5A and B). Animals treated with tk and GCV live significantly longer than untreated animals. However, whereas extensive tissue damage was seen in normal tissues injected with tk under the control of a CMV promoter after GVC treatment, no obvious

normal tissue toxicitiy was seen in animals injected with tk operably linked to a E2F responsive promoter, except from that local trauma resulting from the injection, which was indistinguishable from sham injected animals (See Fig. 5C). Figure 4 further shows that the expression of a gene operably linked to an E2F responsive promoter is selective for malignant versus normal tissues. This selectivity is all the more remarkable because E2F is normally expressed in cells in a cycle dependent manner.

Although not wishing to be bound by theory we believe that there is an excess of "free" E2F, as well as loss of pRb/E2F repressor complexes in such cells, which results in the selective expression of for example the cytotoxic or therapeutic gene in the malignant cell.

Preferably, the E2F responsive promoter (sometimes referred to as the E2F promoter) is a mammalian E2F promoter, still more preferably it is a human E2F (i.e. has at least one E2F binding site) promoter. The E2F responsive promoter does not have to be the full length wild type promoter, but it must respond to a factor we believe to be E2F as determined by expression in a malignant cell having disruption of pRb function of a heterologous gene (sometimes referred to as transgene) under the promoters control as opposed to a lack of expression in the presence of pRb/E2F complexes. As explained in Sellers, EW.R., et al,

Proc. Natl. Acad. Sci USA 92:11544-11548 (1995). E2F responsive promters typically share common features such as Sp1 and/or ATF sites in proximity to their E2F site(s), which are frequently located near the transcription start site, and lack of a recognizable TATA box. E2F responsive promoters include E2F promoters such as the E2F1 promoter, dihydrofolate reductase (DHFR) promoter, DNA polymerase α (DPA) promoter, c-myc promoter and the B-myb promoter. The E2F1 promoter contains four E2F sites that act as transcriptional repressor elements in serum-starved cells. Preferably, an E2F responsive promoter has at least two E2F sites. However, additional E2F sites are not necessary.

As used herein "responsive" means a sequence having at least 70% wild-type E2F promoter activity in expressing a heterologous gene. This activity can be determined by a number of assays using known techniques based upon the present disclosure. For example, using a marker gene such as *E. coli* β-galactosidase (βgal) in an expression vector. For example, one can use a retroviral vector (e.g. a murine moloney leukemia virus such as pMV7), an adenoviral vector, a herpes simplex viral vector, etc. to transduce a glioma cell line (e.g. the 9L gliosarcoma cell line) operably linked to the E2F promoter. As a control,

a vector with a different promoter can be used such as a CMV promoter or even a reference standard can be used. The transduced cells can then be screened for expression of βgal by known techniques. See Figures 1-5.

In normal tissues E2F responsive promoters are typically repressed by pRB/E2F complexes. The ability of pRB to act as a growth suppresser is linked to this property [Sellers, W.R. et al. Proc. Natl. Acad. Sci. U.S.A. 92:11544-11548 (1995)]. We demonstrated that the selectivity of the E2F responsive promoter for tumor cells as opposed to normal cells can be removed by introducing mutations into an E2F responsive promoter to remove the E2F responsive promoter to remove the E2F binding sites, ΔE2F responsive. See Figure 3E which shows β-galactosidase expression in normal cells, which is in contrast to a relative lack of expression in such cells transduced by an isogenic vector except for an intact E2F consensus sequence (Figure 3C). Moreover, expression in a tumor cell such as a glioma was less with the Δ E2F promoter than the isogenic E2F promoter except for intact E2F consensus sequences. (Fig. 3F). Although not wishing to be bound by theory we believe this is due to induction from "free" E2F.

The target cells include any malignant cells. For example,

cancers, or leukemia such as solid tumors, preferably breast, kidney, liver, brains and colon cancers or leukemias. More preferably, a brain tumor such as glioma, or a liver tumor. Still more preferably, a brain tumor such as glioma.

One can readily determine if a malignant cell is an appropriate target tissue for a molecule by introducing a E2F vector with a marker gene into that cell and determining if the marker is expressed. If the marker is not expressed, it indicates the E2F responsive promoter is being repressed.

One can insert a cassette containing just the E2F responsive promoter operably linked to the heterologous gene into a cell. The cassette is a nucleic acid segment containing the E2F promoter operably linked to a nucleic acid segment encoding the heterologous gene of interest. Preferably the cassette is inserted in a vector. The cassette is preferably DNA. The cassette can be introduced into the target cell by any method which will result in the uptake and expression of the E2F cassette by the target cells. These can include vectors, liposomes, naked DNA, gene gun, adjuvant-assisted DNA, catheters, etc. Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or

RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses and HIV-based viruses. One preferred HIV-based viral vector comprises at least two vectors wherein the *gag* and *pol* genes are from an HIV genome and the *env* gene is from another virus. DNA viral vectors are preferred. These vectors include herpes virus vectors such as a herpes simplex I virus (HSV) vector [Geller, *A.I.* et al. *J. Neurochem* 64: 487 (1995); *Lim, F. et al., in DNA Cloning: Mammalian Systems, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995)*; Geller, A.I. et al., *Proc Natl. Acad. Sci. U.S.A. 90*: 7603 (1993); Geller, A.I., et al., *Proc Natl. Acad. Sci USA 87*: 1149 (1990)], adenovirus vectors [LeGal LaSalle et al., *Science 259*: 988 (1993); Davidson, et al., *Nat. Genet 3*: 219 (1993); Yang, et al., *J. Virol. 69*: 2004 (1995)] and adeno-associated virus vectors [Kaplitt, M.G., et al., *Nat. Genet. 8*:148 (1994)].

Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the E2F

cassette into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include naked DNA, $CaPO_4$ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection,viral vectors, etc.

For example, one can use the vector to target any desired target cell such as a glioma. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location in the brain (e.g. a glioma). Stereotaxic surgery is performed using standard neurosurgical procedures (Pellegrino and Cushman, (1971)). Additionally, the vechicle containing the cassettes (or even "naked" cassettes) can be delivered by intracerebroventricular ("icv") infusion using a minipump infusion system, such as a SynchroMed Infusion System. A method based on bulk flow, termed convection, has also proven effective at delivering large molecules to extended areas of the brain and may be useful in delivering the vector to the target cell (Bobo et al., *Proc. Natl. Acad. Sci. USA 91:2076-2080 (1994) Am. J. Physiol. 266:*

292-305 (1994); Morrison et al.,). Other methods that can be used including catheters, intravenous, parenteral, intraperitoneal and subcutaneous injection, oral or other known routes of administration.

One would inject a sufficient amount of the vector to obtain a concentration in the tissue containing the E2F cassette of the encoded protein ranging between about 1 pg/ml to 20 μg/ml. More preferably between 0.1 μg/ml to 10 μg/ml. Still more preferably, between about 0.5 μg/ml to 10 μg/ml.

The E2F responsive promoter can be combined with a desired nucleic acid sequence encoding a heterologous gene such as one for a positive potentiator (such as a gene for a cytokine or a costimulating molecules, a gene for a missing or defective protein, (many cancers are believed to result in part from missing or defective proteins such as tumor suppressor genes e.g. retinoblastoma, p53, others from changes from a proto-oncogene to an oncogene such as with ras, etc.) or a sequence for a negative potentiator (such as a toxin, an anti-sense RNA, a suicide gene such as HSV thymidiac kinase (tk), a ribozyme, a dominant-negative mutant, an antibody such as an antibody with an intracellular localization signal etc.). Negative potentiators are preferred. Toxins and suicide genes are more preferred. For example, when the

nucleic acid encodes a toxin, one preferably takes care to alter the toxin gene to minimize its potential to affect nontargeted cells. This can be done by standard techniques such as deleting those sequences encoding recognition domains. Toxins are well known and include diphtheria toxin and truncated versions thereof, pseudomonas exotoxin, and truncated versions thereof, Ricin/abrin, Blocked ricin/abrin, Ricin ToxinA-chain, ribosome inactivating protein, etc. All these proteins have different domains. For example, the gene encoding PEA has several domains: Domain I is responsible for cell recognition, Domain II for translocation of the toxin cross-membrane and Domain III for adenosine diphosphate (ADP)-ribosylation of elongation factor 2, which is the step actually responsible for cell death. [Gary, G.L., et al., Proc. Natl. Acad. Sci. USA 81:2645-2649 (1984); Allured, V.S., et al., Proc. Natl. Acad. Sci. USA 83:13220-1324 (1986); Siegall, C.B., et al., J. Biol. Chem. 264:14256-14261 1989)]. Accordingly, by alterations in Domain I or Domain II, that render those domains incapable of expression, for example, by a frameshift mutation, insertion of termination sequences, or deletions one can minimize the ability of the toxin to affect neighboring cells. Thereafter, the skilled artisan can use standard techniques to insure that the other domains, or portions of domains where expression is desired, are used.

For example, as indicated above, with PEA only Domain III is absolutely required. However, partial sequences from other domains makes the toxin more effective. For example, one can prepare PEA mammalian expression vectors in which Domain III (mature PEA amino acid residues 405 to 613) only, is expressed and one which encodes Domain III and partial Domain IB, a sequence of amino acids 385 to 613 is expressed. These sequences should be operably linked to the E2F responsive promoter which will permit expression in the target cell. For example, the human E2F-1 promoter. The toxin proteins encoded by these gene fragments lack a recognition domain. They are non-toxic to surrounding cells and are only toxic when expressed inside a cell. These expression vectors can readily be tested to determine how well they express a product intracellularly by a simple *in vitro* assay. For example, the expression of those DNA sequences encoding PEA toxin fragments can be tested by transforming an E2F high expressing malignant cell with the cassette and observing the cytotoxicity of the cell. Suicide genes such as tk work by sensitizing the cell to a compound which the cell would otherwise not be affected by, e.g. ganciclovir. One preferred type of antibodies works by binding to a target intracellularlly (e.g. an intrabody). For example, by targetting a gene product that is overexpressed in malignant cells such as a gene erbB-2.

In some instances, even with immunotoxins, resistant mutants can develop. In such instances, one can readily insert a different toxin gene or different types of nucleic acid segments into the E2F nucleic acid cassette. Thus, the present system permits the production and use of a wide range of DNA and RNA segments, DNA segments are preferred.

In some preferred embodiments one would administer a cocktail of different nucleic acid cassettes where the heterologous nucleic acid segment that is delivered is changed to widen the spectrum of products delivered to the target cell.

Combination therapy is particularly preferred. For example, the use of both a toxin and a suicide gene. Alternatively, one can use a cytokine or costimulating molecule to enhance an alternative strategy such as immune targetting of tumors. Thereafter, one can administer a toxin to the remaining cells. This can also be done with other anti-neoplastic agents such as radiation, chemotherapy, etc.

The E2F cassette may be administered alone, or as part of a pharmaceutical composition, together with one or more acceptable carriers thereof and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The compositions include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes, and may be prepared by any methods well known in the art of pharmacy.

Such methods include the step of bringing into association with the molecule to be administered ingredients with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid, or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion, or packed in liposomes and as a bolus, etc.

A tablet may be made by compression or molding, optionally with

one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture tablets may be made molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored any may be formulated so as to provide slow or controlled release of the active ingredient therein.

Compositions suitable for topical administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia, and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Compositions suitable for topical administration to the skin may be presented as ointments, creams, gels and pastes comprising one or more compounds of the present invention and a pharmaceutically acceptable carrier. A suitable topical delivery system is a transdermal patch containing the ingredient to be administered.

Compositions suitable for rectal administration may be presented

as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Compositions suitable for nasal administration wherein the carrier is a solid include a coarse powder having a particle size, for example, in the range 20 to 500 microns which is administered in the manner in which a compound is inhaled, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid, for administration, as for example, a nasal sprain or as nasal drops, include aqueous or oily solutions of the active ingredient.

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed

ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

For example, solid dose forms that can be used for oral administration include capsules, tablets, pills, powders and granules. In such solid dose forms, the active ingredient, i.e., the cassettes with the E2F responsive promoter and nucleic acid segment encoding a heterologous protein is mixed with at least one insert carrier such as sucrose, lactose or starch. Such dose forms can also comprise additional substances other than inert diluents, e.g., lubricating agents, such as magnesium stearate. Furthermore, the does forms in the case of capsules, tablets and pills may also comprise buffering agents. The tablets, capsules and pills can also contain time-release coatings to release the particles over a predetermined time period.

For parenteral administration, one typically includes sterile aqueous or non-aqueous solutions, suspensions or emulsions in association with a pharmaceutically acceptable parenteral vehicle. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils such as olive oil and corn oil, gelatin and injectable organic esters, such as ethyl oleate. These dose forms may also contain adjuvants such as preserving, wetting, emulsifying and dispersing agents. They may be sterilized by, for example, filtration through a bacterial-retaining filter, by incorporating sterilizing agents into the composition, by irradiating the compositions, etc., so long as care is taken not to inactivate the active ingredient (e.g. a vector). They can also be manufactured in a medium of sterile water or some other sterile injectable medium before use. Further examples of these vehicles include saline, Ringer's solution, dextrose solution and 5% human serum albumin. Liposomes may also be used as carriers. Additives, such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives, may also be used.

The preferred range of active ingredient in such vehicles is in concentrations of about 1 mg/ml to about 10 mg/ml. More preferably, about 3 mg/ml to about 10 mg/ml.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

Tumor Cell Line and Animals.

The rat C6 astrocytoma cell line was maintained in Dulbeccos MEM (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin. Adult female Sprague Dawley rats (150-175g) were purchased from Charles River Laboratories (Wilmington, MA).

Recombinant Adenovirus.

The recombinant adenoviruses Ad.E2F1-βgal and Ad.CMV-βgal were constructed by homologous recombination between pJM17, a plasmid containing the genome of adenovirus type 5 (Ad5) with deletions in the El and E3 regions, and a shuttle plasmid that includes Ad5 sequences (map units 0.0 to 1.3 and 9.2 to 17.3) and the *E. coli* lacZ gene driven by either the cytomegalovirus (CMV) early gene enhancer/promoter or the E2F-1 promotor. The E2F-1 promotor utilized

in Ad.E2F1-βgal contains the upstream region of the E2F-1 gene (basepairs -218 to +51), a segment containing 4 intact E2F (two imperfect palindromes) and 4 Sp1 consensus sequences. Construction of the ΔE2F-1 promoter and the Ad.CMV-tk vector has been previously described 13, 25. The recombinant viruses were plaque purified and propagated in 293 cells as previously described 26. The structure of the resulting recombinant vectors were confirmed by restriction enzyme digestion and sequencing. The virus stocks were purified by two cesium chloride ultracentrifugations, dialyzed against 10% glycerol, 10 mM Tris (pH 8.0) and 1 mM $MgCl_2$ (viral vehicle) and stored at -80°C. Virus titers were determined as plaque forming units (pfu) assayed in semisolid cultures of 293 cells.

In Vitro Studies

Cell Cycle Inducibility

C6 cells were serum starved (0.5 % fetal bovine serum) for 48 h and then transduced by either Ad.E2F1-βgal, Ad.CMV-βgal or Ad.dE2F1-βgal at a multiplicity of infection (MOI) of 100. Serum starved cells were split into two groups 48 h later, one refed with 10% FBS and the other maintained in low serum. Cells were harvested at the indicated time

points, fixed with ethanol, stained with propidium iodide (P.1.) or with an anti.-β-galactosidase antibody (Boehlinger-Manheim) and analyzed by ELISA.

Cytotoxicity Assays

C6 glioma cells were seeded in 75 cm^2 tissue culture plates, washed, and infected with viral vectors at various MOIs for 1 h in 0.5% media. Cells were then washed, and cultured in normal media for additional 12 h followed by replating at a density of 500 cells/well in 96-well tissue culture plates containing GCU (Cytovene, Hoffman-La Roche, N.J.) at various concentrations. The cells were fixed 72 h later with the addition of formaldehyde (3.7% final), washed extensively with water, and stained with 200 ml of 0.05% methylene blue for 15 min. Unbound dye was washed away, and bound dye was released for quantitation by addition of 200 Mg/Ml 0.33 N HCl for 15 min. Absorbance at 600 nm (A600) was determined on a microplate reader after mixing for 5 sec.

Animal Studies

Implantation of intracerebral tumors/Vector injections.

C6 glioma cells were stereotactically implanted into the right caudate nucleus of Sprague Dawley rats using a modification of the method of Kobayashi 27. In brief, rats (150-175 g) were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and placed in a small animal stereotaxic frame Kopf Instruments). A sagittal incision was made through the scalp to expose the skull and a small burr hole was made 1.3 mm posterior and 4 mm to the right of the bregma. Twenty thousand tumor cells, suspended in 10 Ml of HBSS, was injected with a 701 Hamilton syringe over 30 seconds to a depth of 4.5 mm. The needle was left in place for one minute and then withdrawn slowly. The hole in the skull was plugged with bone wax and the incision was closed with surgical clips (Ethicon Plus). This method resulted in a 100% yield of intracerebral tumors with relatively little leptomeningeal or intraventricular spread. For the tk studies, Ad.CMV-tk (5×10^8 pfu), Ad.E2F1-tk (5×10^8 pfu), or Ad.E2F1-βgal (5×10^8 pfu) was injected into 6 d old established gliomas followed by intraperitoneal injections of GCV (15 mg/kg) or saline twice a day for 7 d.

Ad.E2F1-βgal (10^9 pfu), Ad.CMV-βgal (10^9 pfu), or Ad.E2F1-βgal (10^9 pfu), was stereotactically injected into normal brain or 14 day old intracerebral C6 gliomas utilizing the same coordinates and methods

described above. Animals were sacrificed 3 days later and brains were harvested for further studies.

Hepatectomy and Intrahepatic Vector Injections

Sprague Dawley rats (150-175 g) were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). The hepatic artery, portal vein and bile ducts leading to the middle and left lobes of the liver were identified and ligated, allowing removal of these two lobes amounting to a 70% hepatectomy. Forty eight h following hepatectomy or sham operation (controls), Ad.E2F1-βgal (10^9 pfu) or Ad.CMV-βgal (10^9) were injected into the right femoral vein. Animals were sacrificed 4 d later and livers were harvested for further studies.

Tissue Preparation

Animals were sacrificed by pentobarbital overdose followed by cardiac perfusion, first with ice cold PBS, followed by 4% paraformaldehyde/PBS. Brain or liver tissue was harvested whole and further fixed in 4% paraformaldehyde/PBS for a further 2 h at 4C, and

then sequentially washed over 3 nights in 10%, 20%, and 30% sucrose in PBS/2 mM MgCl. Brain was then cut coronally along the needle track and liver lobes cut lengthwise and slices used directly for whole mounts or embedded in O.C.T. compound (Tissue Tek) and frozen for thin sections.

For X-gal straining of whole mounts, the fixed samples were rinsed once with cold PBS and incubated in PBS solution containing 2 mM $CaCl_2$, 0.01% sodium deoxycholate and 0.02% NP-40 for 10 min at 4°C. Samples were then stained for 6 h at 37°C in the same solution containing 5 mM $K_3(Fe(CN)_6$, 5 mM K4(Fe(CN)$_6$ and 1 mg/ml X-gal (5-βromo-4-chloro--5-indolyl-β-D-galatopyranoside), mounted and photographed.

For thin sections, 6 μm sections were cut using an IEC Minotome cryostat and placed on poly-lysine coated slides. Slides were allowed to air dry and stored at -20°C. For staining of liver sections, slides were briefly washed in PBS, fixed with cold acetone and allowed to air dry. X-gal staining was then carried out overnight (18h) with the X-gal staining solution as described above. Anti PCNA primary antibody (PC10, Dako

Co.) or polyclonal anti-adenovirus anti-serum was diluted 200-fold in PBS/BSA/Tween-20 and applied for 30 min followed by vigorous washing (Chemicon Int. Inc, Temecula, CA.). A biotinylated horse anti-mouse IgG secondary antibody was used for visualization utilizing a peroxidase ABC system (Vectastain, Vector Laboratories) and DAB with nickel enhancement as the substrate as previously described. Stained sections were counterstained with nuclear fast red (Vector Laboratories) to highlight cell nuclei. The anti-adenovirus monoclonal was detected using a goat-antimouse IgG-FITC-conjugated antibody (Chemicon Int. Inc, Temecula, CA.), and visualized under fluorescent microscopy. Standard hematoxylin and eosin (H+E) staining of brain sections was also carried out after fixing the slides in cold methanol.

E2F-1 is a ubiquitously expressed, growth regulated, gene which exhibits peak transcriptional activity in S-phase[11, 12]. The E2F-1 promoter contains 4 E2F binding sites which act as repressor elements in quiescent cells[5, 13-16]. We constructed an adenoviral vector that utilizes the E2F-1 promotor (basepair -218 to +51; a segment containing 4 intact E2F consensus sequences) to drive expression of an E coli β-galactosidase gene, placed in the E1 region of an E1 /E3 deleted adenoviral vector (Fig. 1) as previously described[17, 18]. Transduction of

proliferating C6 glioma cells by Ad.E2F1-βgal resulted in high level expression of β-galactosidase activity in a titer-dependent manner (data not shown). We next examined whether Ad.E2F1-βgal could mediate cell cycle-dependent transgene expression in vitro. C6 cells were serum starved, transduced by either Ad.E2F1-βgal or Ad.CMV-βgal (an identical vector except for the presence of a constitutively active cytomegalovirus early promotor rather than the E2F-1 promotor; Fig. 1), and then re-exposed to serum. As demonstrated in Fig. 2A and B, serum starvation resulted in a relatively constant number of cells in S phase (~10%), without significant cytotoxicity, whereas re-exposure to serum resulted in an increased S phase fraction to 25%, 46%, and 60% at 16, 20, and 28 h, respectively. β-galactosidase activity was approximately 20 fold above background in the Ad.CMV-βgal transduced, serum starved cells, and remained constant at this level following re-exposure to serum. In contrast, Ad.E2F1-βgal transduced, serum starved cells expressed relatively low levels of β-galactosidase activity. When these cells were re-exposed to serum, however, β-galactosidase activity was rapidly induced 8, 14, and 20 fold over baseline at 16, 20, and 28 hours, respectively (Fig. 2B; $p < 0.001$). These data are consistent with the suggestion that Ad.E2F1-βgal mediated transgene expression is induced following entry into and/or progression through the cell cycle. The fact that the level of β-galactosidase expression in Ad.E2F1-βgal transduced cells 24 h

following serum re-feeding was similar at equivalent MOIs to that seen in cells transduced by Ad.CMV-βgal, suggests that the induced/de-repressed E2F-1 promotor can mediate high level transgene expression. Similar results were obtained with a panel of human malignant glioma cell lines, suggesting that the observations made with the C6 cells were not cell line specific (data not shown).

Malignant gliomas represent a excellent model for therapeutic gene transfer given the ability to inject viral vectors directly into these relatively localized tumors[19-21]. To test the tumor selective properties of the Ad.E2F1-βgal vector in vivo, we stereotactically injected Ad.E2F1-βgal or the control Ad.CMV-βgal into normal rat brains or brains with established C6 gliomas, and sacrificed the animals 3 d later. As can be seen in Fig. 3A, injection of Ad.CMV-βgal into normal brain resulted in high levels of β-galactosidase activity in brain adjacent to the needle tract, and along ependymal surfaces. Injection of Ad.CMV-βgal into a large established C6 glioma resulted in widely dispersed β-galactosidase activity predominantly in the tumor and in edematous brain infiltrated by tumor, but also in normal adjacent brain and along ependymal surfaces (Fig. 3B) as previously described[22]. In marked contrast, normal rat brains injected with Ad.E2F1-βgal resulted in virtually no β-galactosidase staining, whereas injection into an established C6 glioma

resulted in extensive staining of the tumor itself, with little or no staining in the adjacent normal brain (Fig. 3C, 3D, respectively).

Two, non-mutually exclusive, possibilities could be invoked to account for the enhanced β-galactosidase activity in tumor tissue relative to normal tissue following injection with Ad.E2F1-βgal. One explanation, given the cell cycle data described above, would be that a higher fraction of glioblastoma cells are cycling than are normal cells found in the surrounding, mitotically quiescent brain. A second explanation might be that glioma cells, by virtue of pRB inactivation, contain high levels of 'free' E2F and lack pRB/E2F transcriptional repressor complexes, in which case even mitotically active normal cells might not achieve the high level of transgene expression observed in tumor cells. To address these possibilities, we performed two different sets of experiments. In the first set of experiments, rats underwent partial hepatectomy followed by injection of either Ad.CMV-βgal (10^9 pfu) (Fig. 4 A,C,E) or Ad.E2F1-βgal (10^9 pfu) (Fig. 4B, D, F) into the femoral vein 48 h later. Four days later, livers were harvested and stained for β-galactosidase activity, proliferating nuclear antigen (PCNA), and for the adenovirus fiber protein (Fig. 4). Approximately 30% of the hepatocytes were positive for PCNA under these conditions, confirming the presence of a large number of normal proliferating cells in the regenerating livers

(Fig. 4C,D). Ad.CMV-βgal transduced livers demonstrated diffuse β-galactosidase staining with no apparent difference between PCNA positive versus negative cells (Fig. 4A). In contrast, Ad.E2F1-βgal transduced hepatocytes, including those which were PCNA positive, did not express detectable levels of β-galactosidase (Fig. 4B). Detection of adenovirus fiber protein in the liver sections demonstrated similar levels of hepatic transduction by both the Ad.CMV-βgal and Ad.E2F1-βgal vectors (Fig. 4E,F). Similarly, Ad.CMV-βgal, but not Ad.E2F1-βgal, produced high levels of β-galactosidase activity following transduction of non-hepatectomized livers (data not shown). These data suggest that the high level E2F-1 promotor-mediated transgene expression in vivo is not merely a function of active cell cycling.

In the second set of experiments, we constructed a virus identical to Ad.E2F1-βgal, except that mutations were introduced into the two E2F-binding site within the E2F-1 promotor (Ad.ΔE2F1-βgal). These mutations have previously been shown to render the mutated E2F-1 promotor unresponsive to E2F in vitro[13] In experiments similar to those outlined in Fig. 2, transduction of serum starved and refed C6 cells in vitro by Ad.ΔE2F1-βgal resulted in constitutive transgene expression independent of cell cycle (data not shown). When Ad.AE2F1-βgal was stereotactically injected into non-tumor-bearing rat brain, β-

galactosidase activity was readily apparent in normal cells (Fig. 3E). This is in marked contrast to the lack of β-galactosidase expression in normal cells when the vector contained the intact E2F-consensus sequence (Ad.E2F1-βgal; Fig. 3C). Interestingly, Ad.ΔE2F1-βgalmediated β-galactosidase expression in established gliomas in vivo was less than that seen with Ad.E2F1-β-gal Fig. 3F). These data suggest that the E2F-responsive sequences within the E2F-1 promoter are essential for the tumor selective properties of the vector. Furthermore, they strongly suggest that the tumor selectivity of the E2F responsive vector is due to a combination of factors, namely, repression in normal tissue by pRB/E2F complexes and activation in tumor tissue due to loss of pRB repressor complexes and an excess of free E2F.

To confirm that the tumor-selective properties of this vector could be successfully utilized for expression of a therapeutic gene, we constructed vectors containing the herpes thymidine kinase (tk) gene driven by either the E2F-1 promotor (Ad.E2F1-tk) or the CMV promotor (Ad.CMV-tk). In vitro transduction of C6 cells by Ad.CMV-tk or Ad.E2F1-tk, demonstrated that both vectors equally sensitized glioma cells to ganciclovir (GCV) (LD_{50} = 0.05 MM; Fig. 5A). In order to confirm the activity of the vector in vivo, Ad.CMV-tk or Ad.E2F1-tk were stereotactically injected into 7 day old intracerebral C6 gliomas, followed

by systemic GCV treatment for 1 week (Fig. 5B). There was no significant difference in survival in animals treated with either Ad.CMV-tk or Ad.E2F1-tk plus GCV (> 60% of animals without evidence of tumor > 3 months from treatment), although animals in both these groups lived significantly longer than animals from the control groups (Log rank analysis of Kaplan-Meier survival curves, $p < 0.0001$; Fig. 5B).

Since the evaluation of normal brain toxicity in tumor-bearing animals can be difficult as a result of changes induced by necrotic tumor tissue, we elected to evaluate vector-mediated normal tissue toxicity in the brains of non-tumor bearing rats. One week following the injection of either Ad.CMV-tk or Ad.E2F1-tk and systemic GCV therapy, non-tumor bearing brains were harvested and pathologically evaluated. Consistent with reports in nonhuman primates, stereotactic injection of a tk constitutively expressing adenoviral vector (Ad.CMV-tk) plus GCV resulted in extensive areas of local brain necrosis, inflammation and hemorrhage (Fig. 5C)[23]. In marked contrast, treatment with Ad.E2F1-tk and GCV resulted in no obvious normal tissue toxicity, except that from the local trauma of the stereotactic injection (indistinguishable from sham injected animals). These studies confirm that an E2F-responsive adenoviral vector can mediate tumor selective cytotoxic transgene expression with similar anti-tumor efficacy, but with significant less

normal tissue toxicity, than that of constitutively expressing vectors.

These results indicate that the activity of E2F responsive promoters in tumor cells exceeds that achieved in mitotically active normal cells, and this difference can be exploited to target tumor-selective gene expression in vivo. This may be particularly relevant for glioblastomas where the p16-cdk4/cyclin D1-Rb signal pathway is deregulated in the majority of cases[2, 9, 10, 24].

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All the references mentioned herein are incorporated by reference.

We claim:

1. A nucleic acid cassette comprising:

(1) an E2F responsive promoter, wherein said promoter in the presence of "free" E2F, expresses a gene operably linked to said promoter;

(2) a nucleic acid segment containing a nucleic acid sequence of interest operably linked to said E2F responsive promoter, wherein said gene of interest is a positive potentiator or a negative potentiator .

2. The nucleic acid cassette of claim 1, wherein the nucleic acid sequence of interest encodes a negative potentiator selected from the group consisting of an antibody, a suicide protein, a dominant negative mutant, and a cytotoxic agent.

3. The nucleic acid cassette of claim 1, wherein the nucleic acid segment is encodes a cytotoxic protein or cytotoxic fragment thereof.

4. The nucleic acid cassette of claim 3, wherein the nucleic acid segment encodes at least Domain III of *Pseudomonas exotoxin* A.

5. The nucleic acid cassette of claim 1, wherein the E2F responsive promoter is selected from the group of promoters consisting of E2F1 promter, dihrydrofolate reductase promoter, DNA polymerase α promoter, c-myc

promoter and B-myb promoter.

6. The nucleic acid cassette of claim 5, wherein the promoter is the human wild type E2F-1 promoter.

7. A cassette containing the nucleic acid cassette of claim 1.

8. The vector of claim 7, wherein the vector is selected from the group consisting of chemical conjugates, fusion proteins containing a targeting moiety and nucleic acid binding moiety, retroviral vectors and DNA viral vectors.

9. The vector of claim 8, wherein the vector is a DNA viral vector selected from the group consisting of herpes viral vectors, adenoviral vectors and and adeno-associated viral vectors.

10. The vector of claim 8, wherein the vector is an adenoviral vector.

11. The vector of claim 10, wherein the nucleic acid sequences of interest encodes a negative potentiator.

12. The vector of claim 11, wherein the negative potentiator is a sucide protein or a cytotoxin.

13. The vector of claim 12, wherien the negative potentiator is a suicide protein and the suicide protein is HSV thymidine kinase.

14. A method of selectively targetting a malignant cell which comprises adding an effective amount of the nucleic acid cassette of claim 1 to a medium containing the malignant cell under conditions where the nucleic acid cassette can transduce the cell and waiting until the nucleic acid cassette transduces the malignant cell.

15. The method of claim 14, wherein the nucleic acid cassettes is present in a viral vector or nucleic acid delivery system.

16. The method of claim 14, wherein the malignant cell is a solid tumor.

17. The method of claim 11, wherein the solid tumor is a glioma.

18. The method of claim 17, wherein the nucleic acid cassettes is present in a vector, wherein the vector is an adenovirus vector or a herpes virus vector.

19. The method of claim 16, wherein the nucleic acid sequence of interest encodes a negative potentiator.

20. The method of claim 19, wherein the negative potentiator is a suicide gene or a cytotoxin.

21. The method of claim 20, wherein the negative potentiator is a suicide gene.

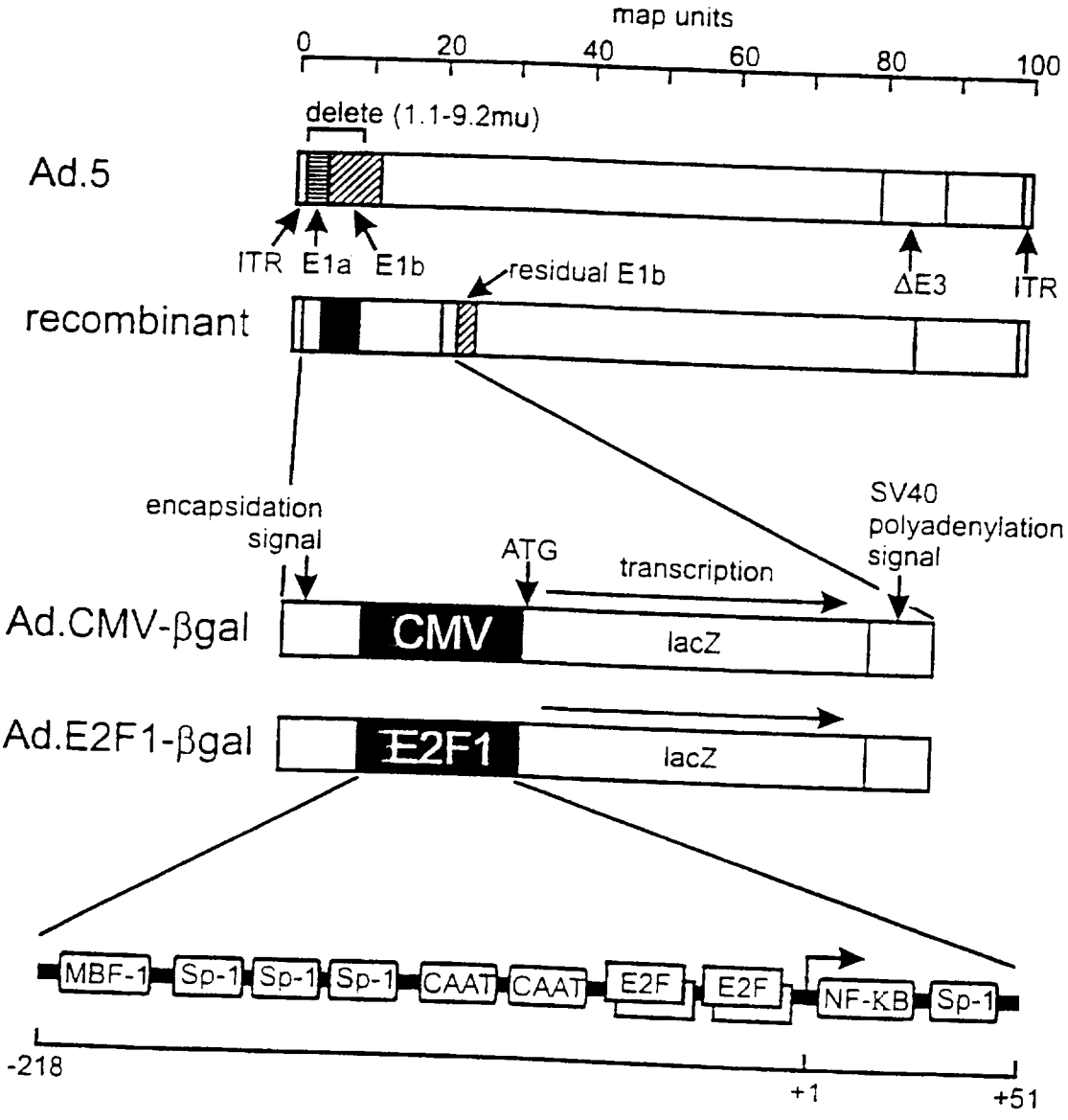
22. The method of claim 21, wherein the suicide gene is HSV thymidine kinase.

23. The method of claim 22, wherein the negative potentiator is

a cytotoxin.

24. The method of claim 23, wherien the cytotoxic contains at least Domain III of *Pseudomonas extoxin A*.

FIGURE 1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20
40
60
80
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delete (1.1-9.2mu)
Ad.5
ITR
E1a
E1b
residual E1b
ΔE3
ITR
recombinant
encapsidation signal
ATG
transcription
SV40 polyadenylation signal
Ad.CMV-βgal
CMV
lacZ
Ad.E2F1-βgal
E2F1
lacZ
MBF-1
Sp-1
Sp-1
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NF-KB
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-218
+1
+51

FIGURE 2

FIGURE 2A

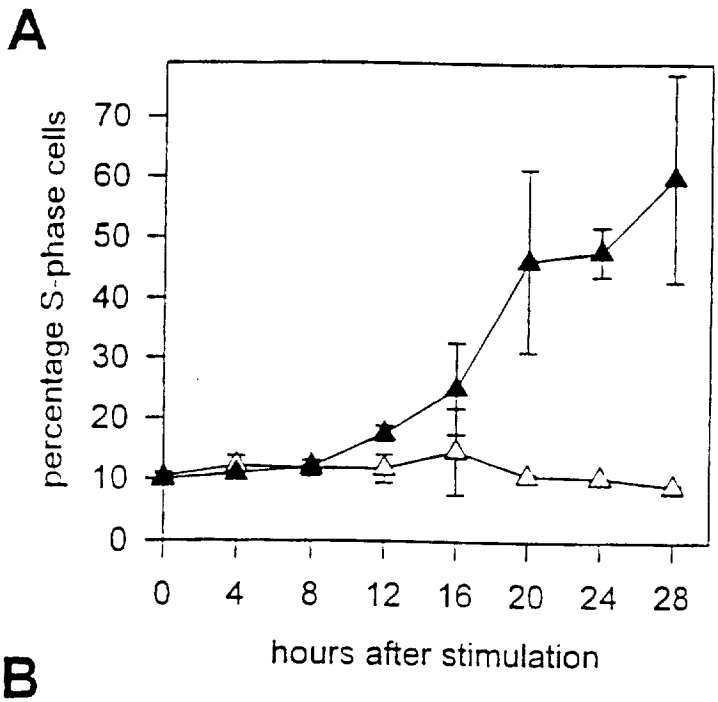


FIGURE 2B

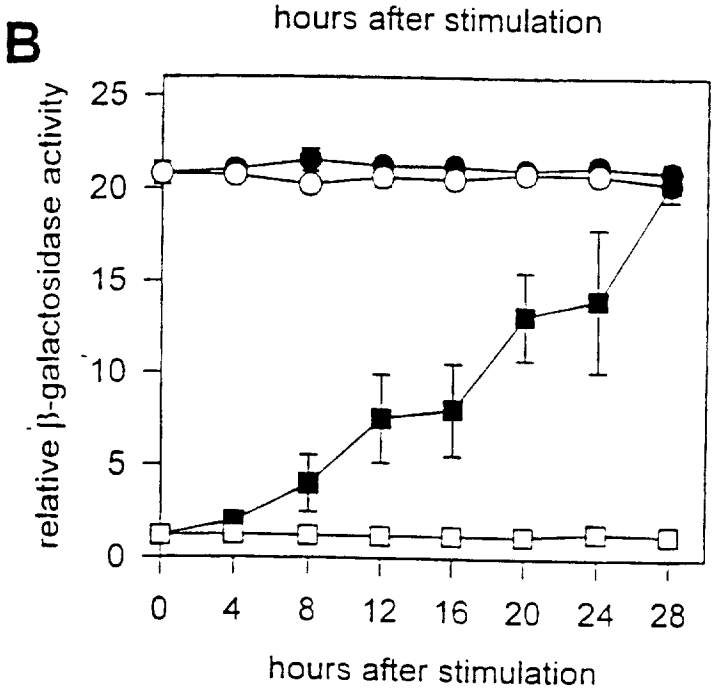


FIGURE 3

FIGURE 3A

A

FIGURE 3B

B

FIGURE 3C

C

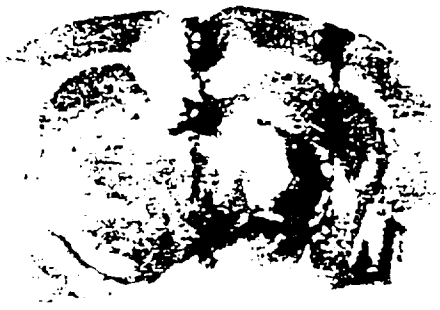


FIGURE 3D

D

FIGURE 3E

E

FIGURE 3F

F

FIGURE 4

FIGURE 4A

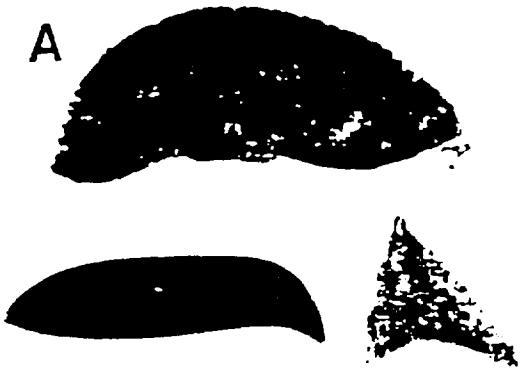


FIGURE 4B

FIGURE 4C

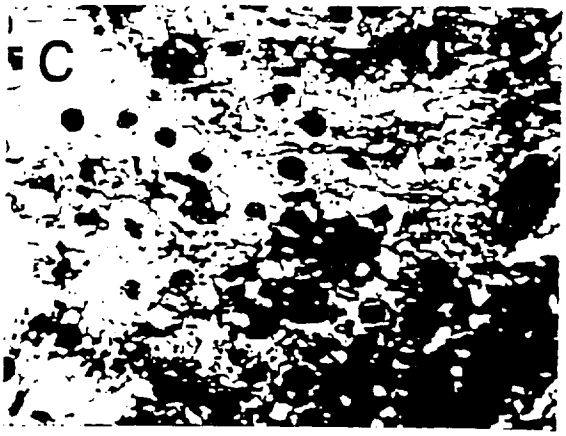


FIGURE 4D

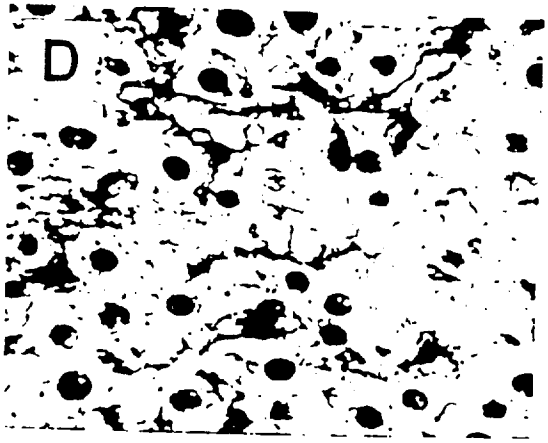


FIGURE 4E

FIGURE 4F

FIGURE 5

FIGURE 5A

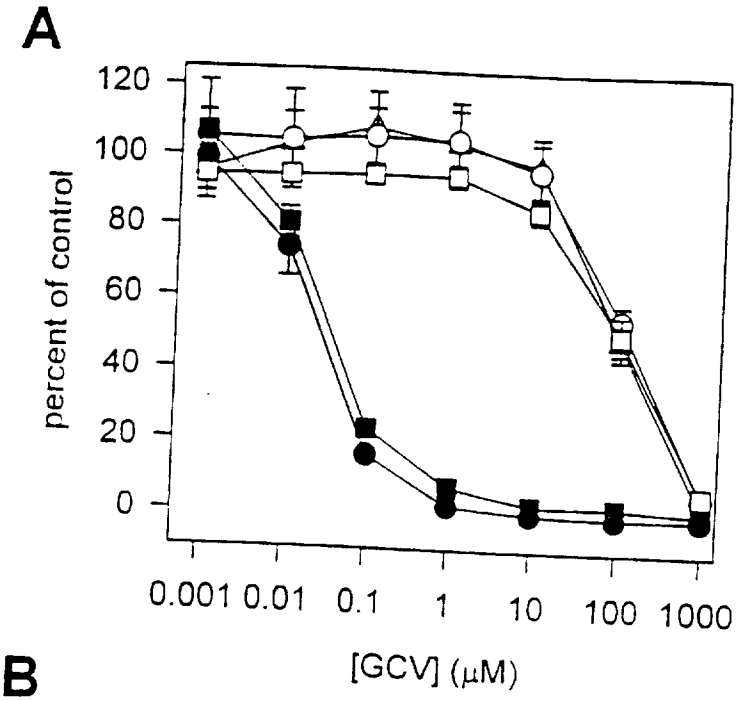


FIGURE 5B

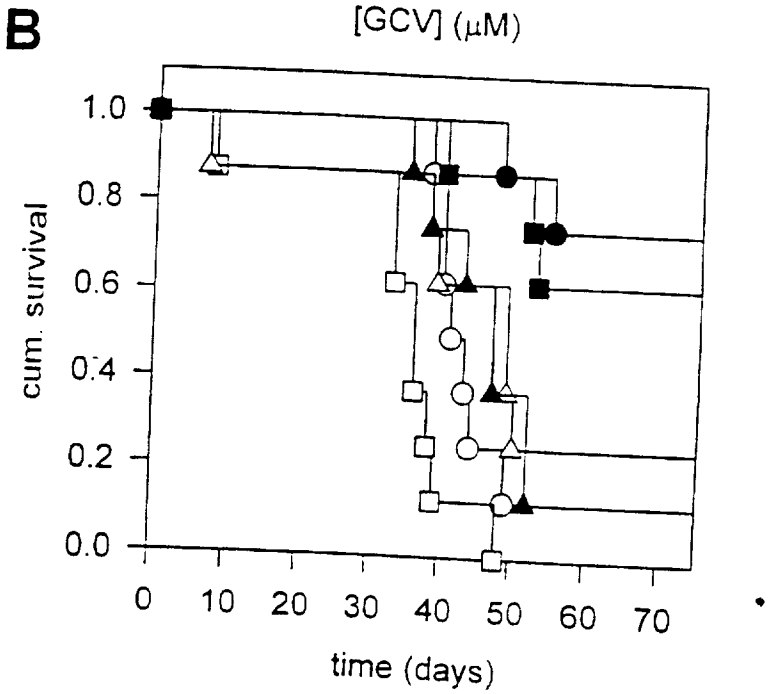


FIGURE 5 (Continued)

FIGURE C

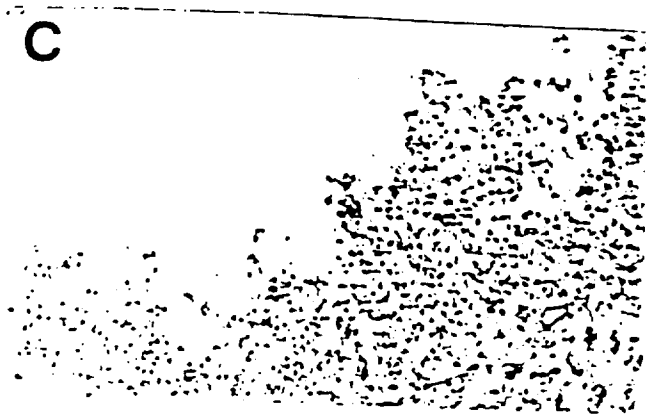
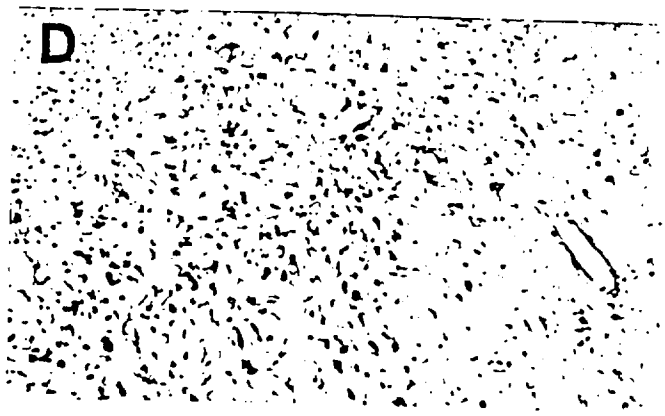


FIGURE D



INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 97/17143

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12N15/86 C12N15/62 C12N9/12 C07K14/21
C07K19/00 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 94 18992 A (ONYX PHARMACEUTICALS) 1 September 1994 see page 24, line 31 - page 25, line 19 see page 26, line 5 - page 27, line 22; claims 1-23	1-3,5-7, 9-15, 18-23 4,8,16, 17,24
Y	WO 95 22618 A (DANA FARBER CANCER INST INC ;MARASCO WAYNE A (US); CHEN SI YI (US)) 24 August 1995 see claims 1-12	4,8,24
Y	US 5 529 774 A (BARBA DAVID ET AL) 25 June 1996 see claims 1-8	16,17

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
26 January 1998	11.02.98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/US 97/17143

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 25494 A (MEDICAL RES COUNCIL ;VER NL KANKER INST (NL); THANGUE NICHOLAS BAR) 22 August 1996 see claim 26	1
P,X	DE 196 05 274 A (HOECHST AG) 14 August 1997 see claims 1-18	1-3,5, 9-12
T	MARTIN LA ET AL: "Tumor-selective transgene expression in vivo mediated by an E2F-responsive adenoviral vector." NAT MED;3(10):1145-9 1997, XP002053131 see the whole document	1-7, 10-24

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INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 97/ 17143

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 14-24 (as far as they concern an in vivo method) is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ɔnal Application No
PCT/US 97/17143

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