

Antisense Gene Therapy of Brain Cancer with an Artificial Virus Gene Delivery System

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Therapeutic genes are delivered to the nuclear compartment of cancer cells following intravenous administration with a non-immunogenic "artificial virus" gene delivery system that uses receptor-specific monoclonal antibodies (MAb) to navigate the biological barriers between the blood and the nucleus of the cancer cell. Mice implanted with intracranial U87 human glial brain tumors are treated with a nonviral expression plasmid encoding antisense mRNA against the human epidermal growth factor receptor gene (*EGFR*). The plasmid DNA is packaged within the interior of polyethylene glycol-modified (PEGylated) immunoliposomes, and delivered to the brain tumor with MAbs that target the mouse transferrin receptor (TRFR) and the human insulin receptor (INSR). The mouse TRFR MAb enables transport across the tumor vasculature, which is of mouse brain origin, and the INSR MAb causes transport across the plasma membrane and the nuclear membrane of the human brain cancer cell. The lifespan of the mice treated weekly with an intravenous administration of the EGFR antisense gene therapy packaged within the artificial virus is increased 100% relative to mice treated either with a luciferase gene or with saline.

Key Words: gene therapy, liposomes, blood-brain barrier, brain cancer, insulin receptor, transferrin receptor, epidermal growth factor receptor

INTRODUCTION

Most solid cancers, including brain cancers, are dependent on the epidermal growth factor receptor (EGFR) [1,2], and antisense gene therapy directed at the gene *EGFR* is a potential treatment strategy. The delivery of therapeutic genes to solid cancers is hindered by the tumor microvascular barrier, which in brain forms the blood-brain barrier (BBB). Viral vectors for gene therapy do not cross the BBB; therefore, it is necessary to administer the vector by means of intracerebral injection. However, this has limited success owing to poor diffusion of the vector from the injection site, which prevents distribution of the therapeutic gene to most cells within the brain cancer [3]. The preexisting immunity to viral vectors is another difficulty, as a single injection of the virus into the brain causes inflammation and demyelination in humans and primates [4,5]. The principal nonviral gene transfer technology uses complexes of plasmid DNA and cationic lipids or cationic proteins. However, these complexes aggregate in physiological saline and are not suitable for gene transfer to the brain following intravenous administration. The problems in gene transfer presented by either viral vectors or DNA/cationic complexes are eliminated with the use of a polyethylene glycol-modified (PEGylated) immunoliposome (PIL) gene delivery system that is capable of transport across the BBB

and can be administered by intravenous injection [6–8]. This targeted gene delivery system uses an "artificial virus" wherein the therapeutic gene encoded in a nonviral expression plasmid is encapsulated within the interior of an 85 nm PIL (Fig. 1A). The PIL construct is unrelated to cationic liposomes, as the plasmid DNA is encapsulated in the interior of a spherical liposome with a net anionic charge [7]. The surface of the PIL gene delivery system contains one or more peptidomimetic monoclonal antibodies (MAb). These targeting antibodies bind to specific receptor systems to facilitate transport of the PIL across the multiple biological barriers separating the blood from the nuclear compartment of the cancer cell. With this approach, a therapeutic gene can be delivered to the nucleus of a cancer cell following an intravenous injection.

In this investigation, experimental human brain cancer develops in severe combined immunodeficient (SCID) mice following the intracerebral injection of U87 human glioma cells. The brain cancer is treated weekly with an intravenous injection of a plasmid encoding antisense mRNA to human *EGFR*. This plasmid DNA encoding the *EGFR* antisense mRNA is designated clone 882. When the plasmid is encapsulated within the interior of the PIL and delivered to human U87 glioma cells in tissue culture, there is a 70% reduction in thymidine incorporation into

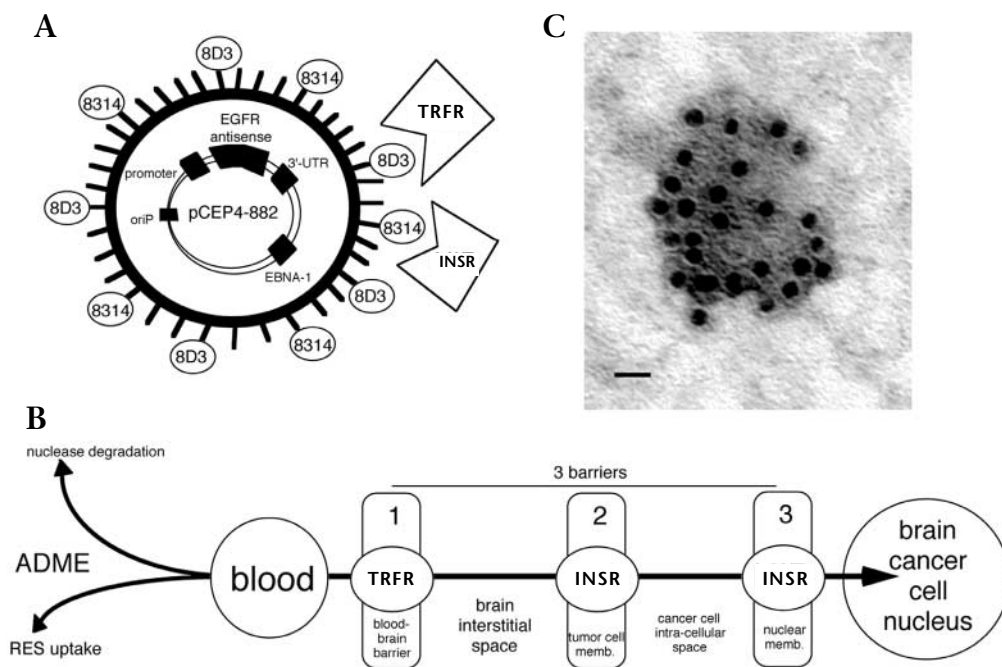


FIG. 1. Gene delivery across biological barriers. (A) The 85 nm PEGylated immunoliposome (PIL) includes approximately 2000 strands of 2000 Da PEG conjugated to the surface of the liposome to minimize rapid uptake by the RES. Approximately 2% of the PEG strands are tethered at the tips of the strands with either the 8D3 or the 83-14 MAB to cause binding of the PIL to either the mouse TRFR or the human INSR, respectively. The expression plasmid (clone 882) is derived from the pCEP4 plasmid, encodes *EGFR* antisense mRNA, and is driven by the SV40 promoter [9]. (B) Multiple barriers must be circumvented before a therapeutic gene injected in the bloodstream can distribute to the nuclear compartment of the cancer cell. The 8D3 MAB to the TRFR causes receptor-mediated transcytosis of the PIL across the tumor microvascular barrier, or BBB. The 83-14 MAB to the INSR mediates transport of the PIL across the tumor cell plasma membrane and the nuclear membrane of the tumor cell [9]. (C) Electron microscopy of the PIL conjugated with the INSR MAB, and complexed with a conjugate of 10 nm gold and an anti-mouse secondary antibody. Magnification bar, 20 nm.

the cells and a 79% reduction in the level of immunoreactive EGFR protein [9]. In this study, clone 882 plasmid DNA is encapsulated within the interior of the PIL targeted with both the rat 8D3 MAB to the mouse transferrin receptor (TRFR) and the mouse 83-14 MAB to the human insulin receptor (INSR; Fig. 1A). The brain tumor, composed of human U87 glioma cells, is perfused by a microvasculature of mouse brain origin. The 8D3 MAB causes receptor-mediated transcytosis of the PIL across the tumor BBB by targeting the endogenous mouse TRFR on the tumor microvascular endothelium (Fig. 1B) [8]. The 83-14 MAB causes receptor-mediated endocytosis of the PIL into the U87 tumor cell [9]. In addition, confocal microscopy studies show that the INSR MAB also targets the PIL across the nuclear barrier (Fig. 1B) [9]. The PIL that is conjugated with both the 8D3 MAB and the 83-14 MAB is designated the 8D3/83-14 PIL (Fig. 1A).

The expression plasmid encoding the *EGFR* antisense gene is encapsulated within the interior of the 85 nm PIL (Fig. 1A). The PIL acts as a nanocontainer or artificial virus and shields the therapeutic DNA from the ubiquitous endonucleases present *in vivo*. The surface of the liposome is conjugated with several thousand strands of 2000 Da polyethylene glycol (PEG²⁰⁰⁰). The PEG polymers extend

from the surface of the liposome [6]. About 2% of the PEG strands are tethered at the tip with the targeting MAB, and there are typically 35–50 MAB molecules per individual liposome [6–8]. The PEG strands prevent the absorption of serum proteins to the surface of the liposome [10], which reduces the uptake of the PIL by cells lining the reticulo-endothelial system (RES; Fig. 1B). PEGylation of the liposome gives it prolonged blood residence time and optimized properties of absorption, distribution, and metabolism (ADME) [10], similar to other drugs (Fig. 1B). An effective *in vivo* gene transfer vehicle must have the dual properties of stability in the bloodstream and ability to navigate the biological barriers separating the blood from the nucleus of the target cell (Fig. 1B).

RESULTS AND DISCUSSION

The PEG-extended MAB molecules projecting from the surface of the PIL were revealed by electron microscopy (Fig. 1C). The PIL has a diameter of nearly 100 nm, which approximates the size of many viruses. The average number of 83-14 or 8D3 MAB molecules conjugated per liposome is computed from trace amounts of [³H]8D3 MAB or [¹²⁵I]-83-14 MAB (37 ± 2 and 35 ± 3 , respectively). The

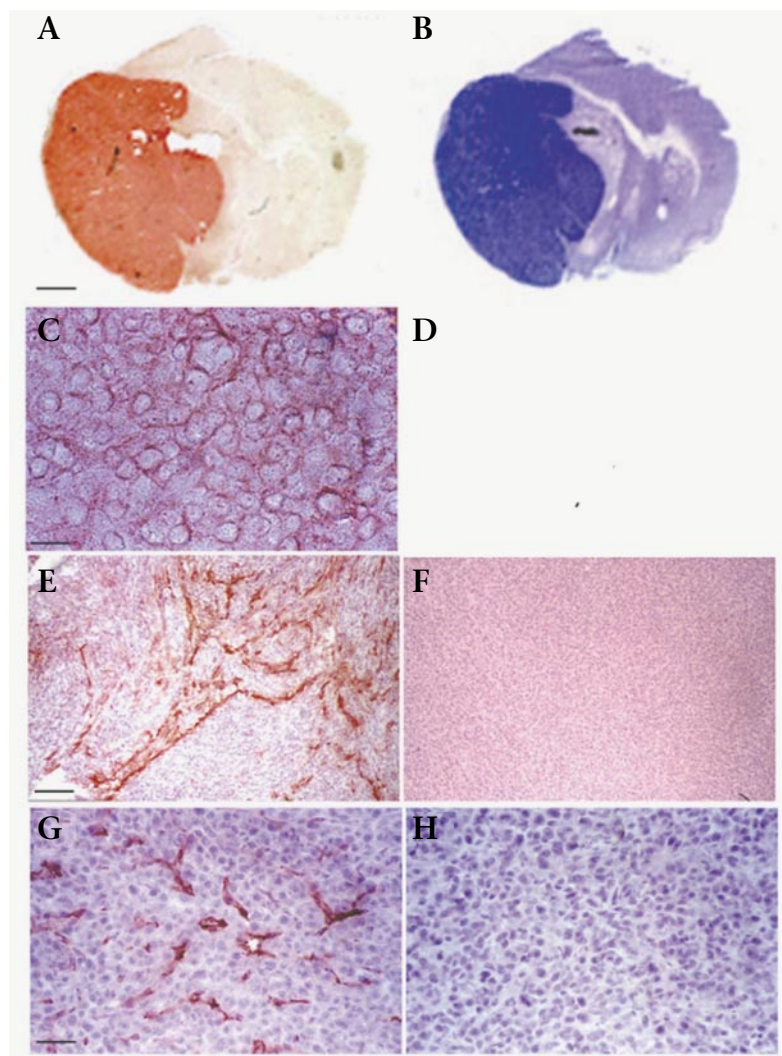


FIG. 2. Immunocytochemistry of frozen sections of mouse brain using the 528 MAb to the human EGFR (A), the 83-14 MAb to the INSR (C), the rat 8D3 MAb to the mouse TRFR (E, G), mouse IgG_{2a} (D), and rat IgG (F, H). (A) Abundant expression of the immunoreactive EGFR in the tumor with minimal expression in normal mouse brain. (B) Frozen section of mouse brain corresponding to (A) counterstained with hematoxylin. The sections in (A) and (B) show the size of the brain tumor at death. (C) Expression of the INSR on the plasma membrane of the tumor cells, whereas the mouse IgG_{2a} isotype control (D) shows no staining of the tumor cells. Abundant expression of the mouse TRFR on the microvasculature perfusing the tumor (E, G), whereas the rat IgG control antibody (F, H) shows no staining of the tumor or tumor vessels. Panels (B) and (E–H) are counter-stained, and (A), (C), and (D) are not counter-stained. The magnification is the same in (A) and (B), (C) and (D), (E) and (F), and (G) and (H), respectively; the magnification bars in (A), (C), (E), and (G) are 1 mm, 11 μ m, 114 μ m, and 29 μ m, respectively.

plasma membrane of the brain tumor cells *in situ* express INSR, as shown by immunocytochemistry of tumor sections with the 83-14 MAb (Fig. 2C). The microvessels perfusing the tumors are strongly immunoreactive for the mouse TRFR, as shown by immunocytochemistry with the 8D3 MAb (Fig. 2E), although the 8D3 MAb does not react with the human TRFR on the U87 cell plasma membrane (Fig. 2G). Therefore, the mouse TRFR and the human INSR, which mediate uptake of the PIL into the tumor, and the target EGFR, are all expressed in these experimental brain tumors *in vivo* (Fig. 2).

The expression of an exogenous gene within the experimental tumor is demonstrated with a luciferase expression plasmid, designated clone 790 [9]. This pCEP4-derived plasmid is identical to the clone 882 plasmid

average DNA encapsulation per liposome preparation is computed from trace amounts of [³²P]DNA ($21 \pm 1\%$ (mean \pm SE), $n = 12$). The doses administered by tail vein per mouse (in a final volume of 400 μ L saline) are as follows: plasmid DNA, $5.2 \pm 0.4 \mu$ g; lipid, $2.4 \pm 0.2 \mu$ mol; 8314 MAb, $125 \pm 13 \mu$ g; 8D3 MAb, $139 \pm 17 \mu$ g; PEG, $200 \pm 20 \mu$ g. The PIL administered is 80% lipid, 11% protein, 9% carbohydrate, and 0.2% DNA. This dose of an 11.0-kb plasmid is equivalent to 9.6×10^{11} plasmid molecules per mouse. Assuming a tumor uptake of 0.1% injected dose per gram [7,11], and 1.8×10^8 tumor cells per gram, then about five to six plasmid molecules are targeted to each tumor cell. Each plasmid may produce multiple copies of the EGFR antisense mRNA within the tumor cell.

The implanted U87 cells grow into large tumors and approximately 50% of the cranium is occupied by tumor at the time of death (Figs. 2A and 2B). These brain tumors express EGFR, as demonstrated by immunocytochemistry with the 528 MAb to the human EGFR (Fig. 2A). The

expressing the EGFR antisense mRNA, except for the expressed gene [9]. The clone 882 plasmid DNA contains the EBNA-1/oriP replication system, which promotes persistence of gene expression and enables extrachromosomal replication of the plasmid DNA at the time of cell division [12]. Gene expression in human U87 glioma cells persists for up to 21 days after a single addition of the PIL gene delivery system to the cells [9]. The clone 790 plasmid is encapsulated within the 8D3/83-14 conjugated PIL and is injected intravenously to tumor-bearing mice at 5 and 12 days after tumor implantation. On days 16–19 after tumor implantation, the luciferase enzyme activity is measured in both the brain tumor and the contralateral normal mouse brain (Fig. 3). Luciferase enzyme activity decreases with a $t_{1/2}$ of 1.02 ± 0.02 days and 1.31 ± 0.52 days in tumor brain and normal mouse brain, respectively. The peak luciferase enzyme activity in brain is observed 2 days after injection [6,8], and the extrapolated luciferase enzyme activity in the brain tumor at 2 days after injection

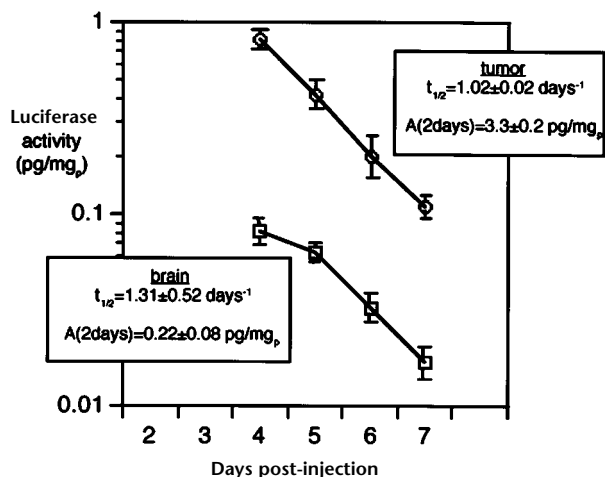


FIG. 3. Luciferase gene expression. Luciferase activity in extracts of either contralateral normal mouse brain or brain tumor is measured at 4, 5, 6, and 7 days after single intravenous injection of clone 790, the luciferase expression plasmid, packaged within the PIL targeted with both the 8D3 and 83-14 MAb. Data are mean \pm SE ($n = 3-4$ animals per group). The data are analyzed by linear regression analysis to yield the slope and y-intercept of the curve. The $t_{1/2}$ of decay in luciferase activity is computed from the slope. The peak luciferase activity at 2 days after injection [A(2days)] is computed from the slope and intercept.

tion is 15-fold greater than luciferase enzyme activity in normal mouse brain (Fig. 3). The INSR MAb, which targets the PIL across both the tumor cell plasma membrane and the tumor cell nuclear membrane [9], is inactive in normal mouse brain as the 83-14 MAb does not cross-react with the mouse insulin receptor. The TRFR MAb and the INSR MAb work in parallel to target the gene to the tumor, whereas the TRFR MAb works alone to target the gene to normal mouse brain. The higher level of gene expression in the tumor, relative to contralateral mouse brain, is attributed to the selective nuclear membrane targeting properties of the INSR MAb [9]. The insulin receptor delivers ligand to the nuclear compartment as demonstrated by either electron microscopic autoradiography or gold labeling [13,14].

The survival of the SCID mice bearing the U87 brain tumors is examined in three groups of mice treated with saline, clone 790 plasmid DNA expressing the luciferase mRNA and packaged within the 8D3/83-14 PIL, and clone 882 plasmid DNA expressing the *EGFR* antisense mRNA and packaged within the 8D3/83-14 PIL. Clone 882, expressing *EGFR* antisense mRNA, induces a 79% reduction in immunoreactive *EGFR* in the U87 glioma cells [9]. In this study, an aggressive tumor model is used in which the initiation of intravenous gene therapy was delayed until large brain tumors were already formed. In this model, 500,000 U87 brain tumor cells are implanted at day 0 and treatment is not initiated until day 5 after tumor implantation. At 5 days after implantation of 500,000 U87

cells in mouse brain, the entire caudate-putamen nucleus is occupied by tumors of up to 3 mm in diameter [15]. At the time of death, the tumor diameter is approximately 6 mm, and tumor tissue occupies half of the intracranial volume (Fig. 2B). The luciferase or *EGFR* antisense gene therapy is administered intravenously at days 5, 12, 19, and 26 after tumor implantation (Fig. 4). Both the mice treated with saline and the mice treated with the luciferase gene expired by 20–21 days and 50% in either group died by 18 days (Fig. 4). In contrast, the mice treated with the *EGFR* antisense gene packaged within the PIL delivery system have a 100% increase in survival with 50% mortality at 36 days (Fig. 4). The last dose of *EGFR* antisense gene therapy is administered at 26 days (Fig. 4).

These results demonstrate that the PIL targeted gene delivery system allows for the enhanced expression of exogenous genes within brain cancer relative to normal brain (Fig. 3). A 100% increase in lifespan is achieved with *EGFR* antisense gene therapy even though the initiation of gene therapy is delayed until large brain tumors had already formed, and therapy is terminated at 26 days (Fig. 4). *EGFR* antisense gene therapy is effective in the human U87 glioma model, although there is no amplification of *EGFR* in this cell line [15]. In contrast, *EGFR* inhibiting MABs have no effect on wild-type U87 tumors that lack *EGFR* mutations [16]. It is possible that further increases in survival can be achieved with dual or triple gene therapy. Many brain cancers, including U87 cells, have a mutation in the tumor suppressor gene *PTEN* [17]. In addition to combining *EGFR* antisense gene therapy with *PTEN* replacement gene therapy, the combined use of gene therapy and radiation therapy may be beneficial in the

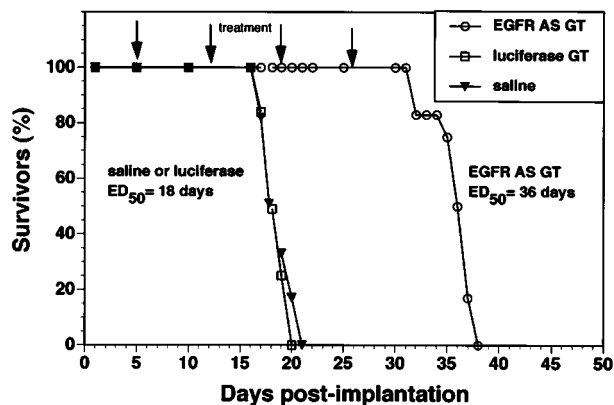


FIG. 4. Survival study. Intravenous gene therapy is initiated at 5 days, and repeated at 12, 19, and 26 days after tumor implantation. There are 12 tumor-bearing mice in each of the three groups. There is no statistical difference between the mortality in the animals treated with either saline or the luciferase expression plasmid (clone 790), although there was a 100% increase in survival of the animals that were treated with the *EGFR* antisense gene therapy (ASGT). The difference in survival in the *EGFR* antisense gene therapy group relative to the saline control, or the luciferase gene therapy group, is significant at the $P < 0.005$ level (Fisher's exact test).

treatment of brain cancer. Radiation induces an overexpression of the EGFR in brain tumors, which has a cytoprotective effect that inhibits further therapeutic effects of radiation therapy [18].

The gene delivery system used in these studies is designed specifically for brain cancer. Peptidomimetic MABs or other targeting ligands of varying tissue specificity may be selected to deliver therapeutic genes to cancers of peripheral origin. However, targeting ligands that cause not only receptor-mediated endocytosis into the tumor cell but also receptor-mediated transcytosis of the therapeutic gene across the tumor microvasculature should be selected. Without dual targeting across both the tumor cell membrane and the tumor microvascular barrier, it is unlikely that therapeutic levels of the gene will be achieved deep within the cancer cell, distal to the microvascular barrier. It may be possible to restrict gene expression to the cancer cell if the exogenous gene is driven by a promoter element taken from a gene specifically expressed in the cancer. Organ-specific gene expression is possible with the combined use of gene targeting technology and tissue-specific gene promoters [8].

This study shows that a liposomal gene delivery system can produce significant therapeutic effects in an experimental brain cancer model when used in conjunction with EGFR antisense gene therapy. The PIL gene targeting system functions as an artificial virus, and has the same size as many viral vectors (Fig. 1C). Similar to viral vectors, the gene is encapsulated in the interior of the nanocontainer [6–8], and the surface of the nanocontainer is decorated with targeting ligands that trigger receptor-mediated transcytosis across microvascular barriers [6–8] and receptor-mediated endocytosis across the tumor cell membrane [9]. The immunogenicity of the targeting MAB ligands is minimized with the use of genetically engineered, or “humanized,” antibodies. The PIL gene transfer technology allows for gene targeting to distant sites following the intravenous administration of a nonviral, non-immunogenic formulation.

MATERIALS AND METHODS

Brain tumor model. We obtained human U87 glioma cells from the American Type Culture Collection (Rockford, MD) and 500,000 cells were implanted in the caudate-putamen nucleus of adult female SCID mice under stereotaxic guidance on day 0. All animals develop large tumors, which are 4–8 mm in diameter at autopsy. The animals are treated with either saline, luciferase gene therapy, or EGFR antisense gene therapy weekly at 5, 12, 19, and 26 days. The use of vertebrate animals is in accordance with approved UCLA Animal Research Committee protocols.

Plasmid DNA. The therapeutic gene is encoded by an expression plasmid derived from the pCEP4 plasmid, that is designated clone 882, with a size of 11.0 kb (Fig. 1A). This plasmid produces a 700 nucleotide human EGFR antisense mRNA that corresponds to nucleotides 2317–3006 of the human EGFR mRNA [9]. The therapeutic gene is driven by the SV40 promoter and the transcript produced from this plasmid contains SV40 3'-untranslated region (UTR) mRNA as well as a 200-nucleotide fragment from SLC2A1 mRNA 3'-UTR, which contains an mRNA stabilizing element [9]. The plasmid also expresses the Epstein Barr nuclear antigen EBNA-1 and oriP (Fig. 1A), which is derived from the pCEP4 plasmid [9], and enables a single round of episomal replication of the plasmid with each cell mitosis [12].

PEGylated immunoliposome production and gene encapsulation. The PIL is prepared by first encapsulating 200 µg of plasmid DNA in 20 µmol of lipid with repetitive freeze/thaw cycles as described [6–8]. The lipid is 94% neutral lipid, POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine), 2% cationic lipid, DDAB (didodecyltrimethylammonium bromide), 3% anionic lipid, DSPE-PEG²⁰⁰⁰ (distearylphosphatidylethanolamine conjugate of 2000 Da polyethylene glycol), and 1% anionic lipid, DSPE-PEG²⁰⁰⁰-MAL (a bi-functional PEG molecule with a DSPE moiety at one terminus of the polymer and a maleimide (MAL) group at the other terminus). Following DNA encapsulation in the large lipid vesicles, small 85–100 nm liposomes encapsulated with DNA are formed by successive extrusions through 400, 200, and 100 nm pore size polycarbonate membranes (Avestin, Ottawa, Canada), as described [6–8]. About half of the DNA is encapsulated within the liposome, and about half is exteriorized. The latter is exhaustively removed by nuclease treatment [6–8]. In parallel with the DNA encapsulation, the targeting antibodies (8D3, 83-14) are individually thiolated with 2-iodoethanol, and the thiolated antibodies are conjugated to the MAL moiety of the PEGylated liposomes overnight [6–8]. The unconjugated antibody and the nuclease digested exteriorized DNA are removed from the PIL by CL-4B gel filtration chromatography [9]. The thiolated antibody and DNA mixture contains a trace amount of [³H]8D3 MAB, [¹²⁵I]-83-14 MAB, and [³²P]DNA, and the radioactivity determinations allow for the calculation of the number of antibody molecules conjugated to the surface of each liposome and the amount of plasmid DNA encapsulated in the interior of the liposome.

Immunocytochemistry. Frozen sections of mouse brain (20 µm) are fixed in either 100% cold acetone for 20 minutes or in 4% paraformaldehyde for 10 minutes. A biotinylated horse anti-mouse IgG or a biotinylated rabbit anti-rat IgG secondary antibody is used with the ABC labeling method from Vector Labs (Burlingame, CA). The concentration of the primary antibody used is 10 µg/ml. The primary antibody used to detect the human EGFR, the human INSR, and the mouse TRFR are the 528 mouse MAB, the 83-14 mouse MAB, and the 8D3 rat MAB, respectively.

Luciferase enzyme measurement. Luciferase enzyme activity is measured in extracts of brain tumor or contralateral mouse brain with a luminometer, and expressed as pg luciferase activity per mg tissue protein [8,9].

Electron microscopy. PIL-conjugated INSR MAB are examined with a conjugate of 10 nm gold and a goat anti-mouse secondary antibody (Sigma G7652). A 4 µL aliquot of the 83-14-PIL (1.8×10^{12} liposomes) is incubated with 200 µL IgG gold conjugate (2.8×10^{12} gold particles) for 1 hour in 0.018 M Tris-buffered saline, pH 8, with 0.9% bovine serum albumin, and 17% glycerol. The mixture is applied to formvar-coated 2000 mesh copper grids, washed once with 0.05 M Tris/0.15 M NaCl, pH 7.4, counter-stained with 2% uranyl acetate, and examined directly by electron microscopy using a Jeol JEM-100CX II electron microscope at 80 kV. Negatives, taken at a $\times 29,000$, are scanned and enlarged in Adobe Photoshop 5.5 on a G4 Power Macintosh.

ACKNOWLEDGMENTS

This work was supported by funds from Accelerate Brain Cancer Cure (ABC²), Inc., and the United States Department of Defense.

RECEIVED FOR PUBLICATION MARCH 15; ACCEPTED MAY 8, 2002.

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