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MOLECULAR NEUROSURGERY USING GENE THERAPY TO TREAT MALIGNANT GLIOMA

JUN YOSHIDA

Department of Neurosurgery, Nagoya University School of Medicine Nagoya, Japan

ABSTRACT

In the last decade, the prognosis of brain tumor patients has dramatically improved due to recent advances in microsurgical techniques and the development of functioning neuroimaging, computer-assisted neuronavigation, endoscopic surgery, intravascular surgery and radiosurgery. According to a report by the Committee of Brain Tumor Registry of Japan, the ten year survival rate of patients with benign brain tumors (meningioma, neurinoma and pituitary adenoma) is more than 95%. In contrast, patients with glioma (which constitute 33% of primary brain tumor cases) still have a poor prognosis, especially in the case of malignant (anaplastic astrocytoma and glioblastoma). This poor prognosis is related to the fact that malignant glioma cells aggressively infiltrate into normal brain tissues, making total removal of the tumor impossible. The median survival time of glioblastoma patients is less than 2 years, despite multimodality treatment with extensive surgical resection and adjuvant therapies using radiation and immunochemotherapy. In order to overcome this formidable neoplasm, the effectiveness of molecular neurosurgery using gene therapy has been investigated since 1992. In this paper, molecular genetic studies and the current state of gene therapy for malignant brain tumors are described, and the future direction of this fascinating approach is discussed.

Key Words: gene therapy, brain tumor, liposome, adeno-associated virus, cytokine gene

HUMAN GENE THERAPY

The first authorized human gene therapy was started in the United States on September 14, 1990 on 4 year old girl who had been born with a defective adenosine deaminase (ADA) gene which caused severe combined immunodeficiencies. For 2 years, she had been on enzyme replacement therapy, polyethylene glycol (PEG)-conjugated ADA, but after an initial improvement, her T cell number again decreased with frequent infection. At this point, she received an intravenons infusion of her own gene-corrected T lymphocytes. The therapy has continued at 2 to 3 month intervals through the present. With a combined treatment consisting of gene transfer of the ADA gene and administration of PEG-ADA, she is apparently in good health without any side effects.

Since this successful treatment, a variety of human gene therapy protocols have occurred world wide.[1] According to the worldwide gene therapy report by TMC development, more than 1500 patients are enrolled in these protocols as of June 1996. Most trials are performed in the US and European countries, with a few in Asian countries including Japan (Table 1). Target diseases for this therapy are expanding from congenital metabolic disorders to encompass acquired life-threatening diseases such as cancer and AIDS. Chronic benign diseases are also expected to be a focus of this therapy. At present, cancer is by far the most popular protocol, involving 848 patients. AIDS (372 patients), cystic fibrosis (152 patients), leukemia/myeloma (89 patients) are second, followed by arterial disease (16 patients) and ADA deficiency (12 patients).

Table 1. Patients enrolled in gene therapy protocols

1. USA	1229	8. Egypt	15	15. Japan	1
2. UK	61	9. Italy	14		
3. Netherlands	55	10. Spain	9		
4. Germany	47	11. Austria	9		
5. France	44	12. Sweden	5		
6. Canada	22	13. China	4		
7. Switzerland	19	14. Finland	3		

METHODS OF GENE THERAPY

Three different approaches are proposed for human gene therapy. 1) Genes of disease interest are delivered into patient cells in order to produce a therapeutic protein; 2) Amplified genes or abnormal genes are suppressed by antisense RNA or ribozyme and 3) Abnormal gene is replaced to normal genes by homologous recombination and repaired genomic DNA. Most protocols approved by the recombinant DNA Advisory Committee (RAC) involve the first approach (Fig. 1), which contains three key components; a) the vector, b) the gene cassette (the gene containing the therapeutic protein and control elements), and c) the target cells. The vectors for gene transfer are classified into viral and non-viral. In the former, retrovirus vectors have been studied most extensively and hence are most commonly used for clinical application. However,

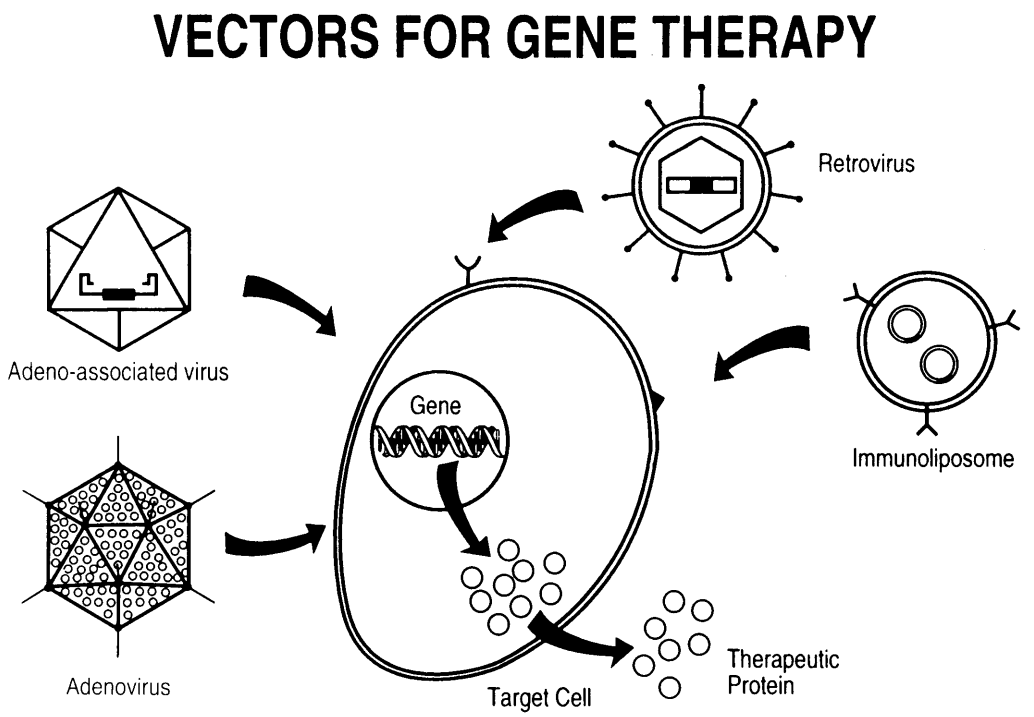

Fig. 1.

the retrovirus vector has a limitation because it requires that target cells be dividing to achieve gene delivery and is rapidly inactivated in the blood. Thus most clinical applications of the retrovirus vector involve a complex ex vivo procedure whereby patient cells are removed and the gene is delivered in vitro.

Another type of viral vector is derived from the adenovirus and adeno-associated virus (AAV). The adenovirus vector is capable of efficiently delivering a gene to several dividing and non-dividing cells, gene therapy for cystic fibrosis now relies primarily on a vector based on a crippled adenovirus. However, adenovirus genes express proteins that trigger an immune response. This immune response is believed to limit the length of time that gene expression can be maintained in the target cell. AAV vectors are derived from AAV, a common non pathogenic human parvovirus. They may offer several potential advantages over other vectors. These advantages include efficient delivery of genes to both dividing and non-dividing target cells, potential site-specific integration of chromosome 19 and the absence of viral genes that may be responsible for causing an undesirable immune response.

A major limitation in the development of clinical applications for AAV vectors has been the lack of an efficient production method. On the other hand, non-viral vectors, especially DNA/liposomes are known to be much safer because they are non-infectious and non-immunogenic. Liposomes, artificially generated lipid vesicles that can entrap genes within their aqueous compartment or in the lipid bilayer, have been regarded as a useful gene delivery system. In 1987, Felgner et al. developed a cationic liposome with N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium (DOTMA). The authors reported that DOTMA interacts spontaneously with DNA to form a DNA-lipid complex and facilitates fusion of the complex with the cell membrane, resulting in both uptake and expression of the DNA.[2] Since this pioneer work, cationic liposome-mediated gene transfer has been widely used in the field of basic molecular biology and for gene therapy studies. Several groups have explored more efficient and less toxic cationic liposome compositions using different cationic lipids.[3] We have also developed novel cationic liposomes with high transfection efficiency and low cytotoxicity which permit their use for in vivo gene transfer. Our liposomes are multilamellar vesicles (MLV) prepared by a simple procedure with N-(α-trimethyl ammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroyl phosphatidylcholine (DLPC) and dioleoyl phosphatidylethanolamine (DOPE) in a molar ratio of 1:2:2.[4] To transfer the gene selectively and efficiently into target cells, we coupled a monoclonal antibody (MCA) with the liposomes (immunoliposomes).[5] For this purpose, we developed two monoclonal antibodies (G-22 and 3C10) against cell surface molecules expressed on human glioma cells. G-22 MCA operates against the standard or hematopoietic form of CD44, which is known to be overexpressed in the cell adhesion molecules on most glioma cells.[6] 3C10 MCA operates against the truncated epidermal growth factor receptor (EGFR), encoded by the type III in-frame deletion mutant.[7] It has been identified in about 10–20% at glioblastoma patients. In our experiments, the proportion of glioma cells that expressed the transferred DNA sequences and the absolute levels of expression obtained were markedly higher when the DNA/immunoliposomes were used, compared to the levels, achieved with the DNA/liposomes.[5]

CHARACTERISTICS OF MALIGNANT GLIOMA

Astrocytic gliomas are the most frequent human brain tumors. They are classified into three malignancy grades (astrocytoma, anaplastic astrocytoma and glioblastoma) on the basis of histopathological parameters. Genetic alteration is commonly encountered in astrocytic glioma. Loss

of heterozygosity (LOH) on chromosome 17p occurs in approximately half of all grades of malignancy, and most of these are mutations in the conserved region of the p53 genes.[8,9] Low-grade astrocytomas bear a significant risk of malignant progression.[10] LOH of chromosome 9p21 and chromosome 10 are known for potential involvement in this progression event. A multiple tumor suppressor-1 (MTS-1) gene for a newly identified inhibitor of cell cycle-dependent kinase 4 (CDK-4), a protein that goes by the name p16, has been confirmed to be localized in the areas of 9p21.[11] It was recently reported that deletion of the genes occurs frequently in anaplastic astrocytoma and glioblastoma.[12] Amplification of EGFR gene is the most common genetic alteration in glioblastoma.[13] Malignant glioma (anaplastic astrocytoma and glioblastoma), in general, infiltrates aggressively into the surrounding normal brain tissue.

Several factors including matrix metallo proteinase (MMP)-II,-IX, CD44,[14] Tenascin[15] were reported to be strongly correlated with the invasion of tumor cells. For these reasons, total resection of gliomas by surgery is impossible, although recent advances in microsurgical technique are remarkable. Postoperative radiation is applied to all patients with malignant glioma. The tumors do show a response to the radiation in many cases, but high doses are necessary to achieve control of the tumor. The normal brain around the tumor can generally tolerate no more than 60 Gy, a dose which below the curative level for glioma.

Other approaches to malignant glioma treatment have included chemotherapy using nitrosourea derivatives either as an adjuvant to radiation[16] or at the time of relapse, and immunotherapy with interferon,[17] intra-tumoral LAK cell instillation,[18] intra-arterial TNF-α infusion,[19] and intra-tumoral injection of radiolabeled monoclonal antibodies.[20] These adjuvant therapies help to prolong survival at least for anaplastic astrocytoma. However, none of these methods are curative, and the median survival time for malignant glioma patients which is less than 2 years at present. Nevertheless, this malignant glioma has important features make it an excellent candidate for gene therapy. The brain is a closed cavity separated from the general circulation system by the blood-brain barrier, and it has been noted to be an immunologically privileged site with no lymphatic system. Normal glia and neuron are relatively quiescent compared to tumor cells. Furthermore, the glioma arising from a glia is a localized tumor in the central nervous system (CNS) with no extra-CNS metastasis.

GENE THERAPY FOR BRAIN TUMOR

Two gene therapy approaches were studied for the treatment of malignant glioma; a) suicide gene therapy using the herpes simplex thymidine kinase (HSV-tk) gene and ganciclovir (GCV) and b) immune gene therapy using cytokine genes.

In the case of suicide gene therapy (Fig. 2), a team of molecular biologists and neurosurgeons at the National Institute of Health in the US (NIH) developed a remarkable new form of treatment: molecular neurosurgery based on gene therapy.[21] Mouse cells were genetically modified by inserting a retroviral vector carrying a herpes gene coded for thymidine kinase. When the mouse cells were injected directly into a brain tumor, they start budding out copies of the retroviral vector, which infected nearby tumor cells. The infected tumor cells then produced HSV-tk, laying themselves open to attack by intravenous injection of the antiviral drug GCV. A team from Howard Hughes Medical Institute and the Baylor College of Medicine reported the efficacy of adenovirus (ADV)-mediated gene therapy to treat brain tumor.[22] Tumors were generated in syngeneic rats by stereotaxic implantation of 9L gliosarcoma cells into the caudate nucleus. Eight days later, the tumors were injected and transduced in situ with a replication-defective ADV carrying the HSV-tk gene, and the rats were treated with ganciclovir. No tumors

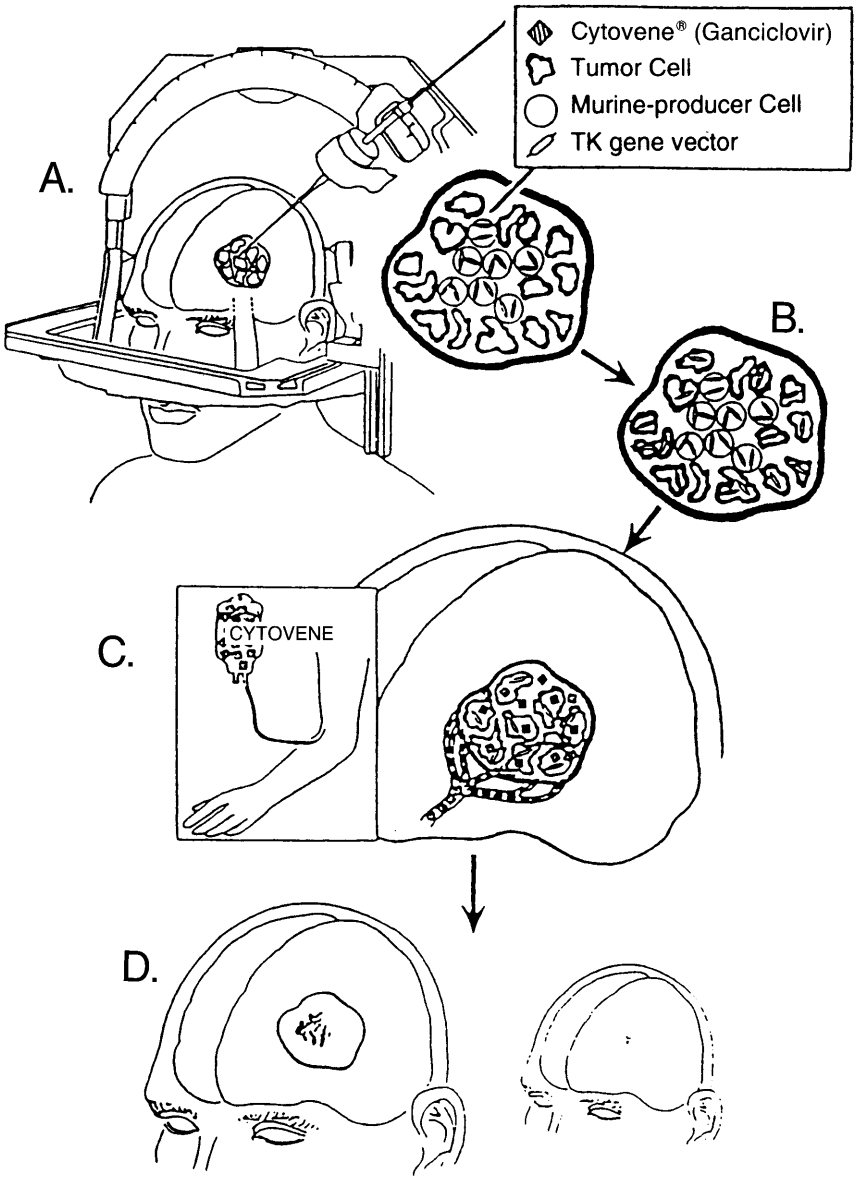

Fig. 2.

were detected in animals treated with ADV-tk and ganciclovir. We are developing a gene therapy using the AAV vector and have demonstrated the efficacy of AAV vector-based gene therapy for malignant glioma in an experimental animal model. We obtained AAV vectors containing the gene for either HSV-tk (AAV-tk) or β-galactosidase (AAV-LacZ) from Avigen Inc. in USA. In several experiments, we confirmed that gene expression was seen in more than 30% of glioma cells by intra-tumor injection of the AAV vector. Following a single injection of an

AAV-tk vector into human glioma transplanted to the brains of nude mice, a significant reduction in tumor size was observed in all animals who also received GCV.[23]

With respect to immune gene therapy, our experimental studies we transfected human glioma cells with a plasmid vector containing the HuIFN-β gene (pSV2IFN-β) by means of our novel TMAG cationic liposome, and found that HuIFN-β produced in the cells had a much stronger inhibitory effect on the growth of the tumor cells than exogenously added HuIFN-β.[24] Our results suggest that the mechanism causing the growth-inhibitory effect of transfection-induced HuIFN-β is different from the exogenous one. The former process is thought to be cytocidal to the transfected glioma cells, which can be ascribed to HuIFN-β production in the cells transfected with its gene by the process of apoptosis.[25] The production of HuIFN-β in the cells and its release from the transfected cells were increased by treating cells with a small dose of TNF-α before transfection.

Correspondingly, the antitumor effect of the transfection-induced HuIFN-β was significantly elevated by combination with TNF-α.[26] The intraperitoneal injection of a small amount of TNF-α (1000U) inhibited tumor growth only slightly. On the other hand, prior treatment with TNF-α followed by intratumoral injection of liposomes with entrapped pSV2IFN-β (150 nmol lipids, 2mg DNA) had a remarkable effect. The subcutaneous tumors regressed completely in all nude mice tested; they were tumor free and surviving at the 250th day, the longest time for follow-up.[26] TNF-α was originally discovered in mouse serum by Carswell and Old in 1975. It is a cytokine that possesses a wide variety of biological and immunomodulatory properties, although the problem of dose-limiting toxicity of TNF-α was reported in its clinical trials.[27] Rosenberg et al. began immune gene therapy for cancer patients using gene transfer instead of administration of TNF-α either by adding the TNF-α gene to the tumor-infiltrating lymphocytes (TILs) to make them more effective[28] or by adding a TNF-α gene to the tumor cells to induce a host immune system response.[29]

In our experimental studies of brain tumors, we found that human TNF-α (HuTNF-α) was produced in the glioma cells transfected with liposomes with entrapped pcDVTNF-α and that the growth-inhibitory effect of transfection induced HuTNF-α was much stronger than that of exogenously added HuTNF-α.[30] In our study to analyze this mechanism, we found that it was due to transmembrane-formed TNF-α. TNF-α has a 76-residue-long precursor sequence containing both hydrophobic and hydrophilic regions, and its long precursor sequence serves to anchor the TNF-α precursor polypeptide in the plasma membrane. The transmembrane-formed TNF-α was identified in cytotoxic T lymphocytes (CTLs), macrophages, and activated monocytes, and it is thought to play an important role in the modulation of host immunity. Following transfection of the TNF-α gene into human glioma cells, the transfected cells secreted soluble TNF-α and also expressed transmembrane formed TNF-α. It has also been demonstrated that the transfected cells have the potential to inhibit growth or express cytotoxicity toward adjacent nontransfected glioma cells by means of transmembrane-formed TNF-α through cell-to-cell contact. In vivo experiments using transplanted human glioma growing in the brains of nude mice clearly showed that HuIFN-β was expressed in the solid tumor and that growth of the brain tumor was inhibited by intratumoral injection of liposomes with entrapped pSV2IFN-β, while a high dose of exogenous HuIFN-β or empty liposomes did not significantly inhibit the growth of human glioma.[31]

CLINICAL APPLICATION OF GENE THERAPY

Protocols of suicide gene therapy with the HSV-tk gene, antisense therapy to insulin-like growth factor-1, and immune gene therapy with interleukin-2[32] were approved by an RAC meeting in the US. In 1992, Oldfield et al. started suicide gene therapy in 15 patients with glioblastoma or metastatic brain tumor. Vector producer cells (VPC) that had been genetically engineered into NIH3T3 to continually produce HSV-tk recombinant retroviral vectors, were injected into the brain tumors using an MRI guided stereotaxic approach. On the fifth postoperative day, intravenous injection of GCV started at 5mg/kg/dose twice daily for 14 days. During these suicide gene therapy protocol, some tumors responded to the treatment and the size of the tumors definitely decreased. But prolongation of survival time did not occur, except in one patient whose small tumor went into complete remission. More than 60 patients have been enrolled in a similar protocol in the US and in some European countries, and some of them were treated by direct injection of VPC into the brain tumor or by administration of VPC through an Ommaya reservoir into the residual tumor.

ASSESSMENT OF CURRENT STATUS AND RECOMMENDATION TO FURURE DIRECTION

Since the first human gene therapy started successfully, more than 100 protocols were approved by the RAC, and their clinical applications have been carried out in more than 1000 patients world wide. Gene therapy has been conducted mostly University Hospitals and at other US and European academic centers, supported by the NIH & private companies. The NIH is spending an estimated US$ 200 million a year to develop and test tools and techniques for gene therapy. Academic centers have created gene therapy programs and private companies are sponsoring them in clinical trials by spending hundreds of millions of dollars. They are all expecting that gene therapy will be a formidable tool in treating inherited and acquired disorders. However, an ad hoc NIH committee assessing the current state of gene therapy in 1995 found that its clinical efficacy has not been definitively demonstrated in any gene therapy protocol, because significant problems remain with all basic aspects of gene therapy.[33] In order to confront the major outstanding obstacles to successful gene therapy, the committee recommended a greater focus on basic aspects of gene transfer and gene expression, and an emphasis on research dealing with the pathogenesis of target diseases. In the former, methods for increasing gene transfection efficiency, directing gene transfer to specific cells, and achieving tissue-specific and regulated expression of the transfected genes must be developed.

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