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Comparisons of Tumor Suppressor p53, p21, and p16 Gene Therapy Effects on Glioblastoma Tumorigenicity *in Situ*

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The mutation and/or deletion of tumor suppressor genes have been postulated to play a major role in the genesis and the progression of gliomas. In this study, the functional expression and efficacy in tumor suppression of 3 tumor suppressor genes (p53, p21, and p16) were tested and compared in a rat GBM cell line (RT-2) after retrovirus mediated gene delivery *in vitro* and *in vivo*. Significant reductions in tumor cell growth rate were found in p16 and p21 infected cells (60 ± 12% vs 66 ± 15%) compared to p53 (35 ± 9%). *In vitro* colony formation assay also showed significant reductions after p16 and p21 gene delivery (98 ± 5% vs 91 ± 10%) compared to p53 (50 ± 18%). In addition, the tumor suppression efficacy were investigated and compared *in vivo*. Retroviral mediated p16 and p21 gene deliveries in glioblastomas resulted in more than 90% reductions in tumor growth (92 ± 26% vs 90 ± 22%) compared to p53 (62 ± 18%). Tumor suppressor gene insertions *in situ* further prolonged animal survival. Overall p16 and p21 genes showed more powerful tumor suppressor effects than p53. The results were not surprising, as p16 and p21 are more downstream in the cell cycle regulatory pathway compared to p53. Moreover, the mechanism involved in each of their suppressor effects is different. This study demonstrates the feasibility of using tumor suppressor genes in regulating the growth of glioma *in vitro* and in situ. © 2001 Academic Press

Key Words: **p53; p21; p16; glioblastoma; tumor suppression.**

Mutational inactivation of the tumor suppressor genes is a frequent feature of many human neoplasms. The molecular mechanisms by which the tumor suppressor protein may contribute to deregulation of cellular growth and malignant progression are not clearly understood. Many studies have suggested that the protein products of these wild-type tumor suppressor genes play a negative regulatory role in cellular proliferation and tumor formation. Among these tumor suppressor genes, p53, and p16 which involved regulation of pRb protein is thought to be one of the important groups and been widely investigated (1–5). Tumor suppressor gene p16 has been known as cyclin-dependent kinase inhibitors, whereas, p53 involved in several critical cellular pathways supporting cellular differentiation and suppressing abnormal cell proliferation (6).

p16, known to bind cyclin-dependent kinase (CDK4) and preventing its forming an active complex with cyclin D proteins, demonstrate the ability of hypophosphation of retinoblastoma protein (pRb, a substrate of activated cdk4) (7). It has been proposed that by compete with relative abundance of D-type cyclins intracellularly will prevent the accumulation of cdk4 kinase to promote transit through the G1 phase of the cell cycle which suggested that the pl6 protein may be a negative regulator of cell proliferation (8, 9).

Data from molecular biological and gene mapping studies of p53 and pl6 strongly suggest that disruption of one or both of their associated growth-regulatory systems is important to the development of most cancers (10), whereas, p53 transactivates p21 in response to DNA damage which may control DNA replication, promote differentiation (11, 12). Although p53, p21, and p16 genes affect the cell cycle and cell senescence, their roles in astrocyte differentiation are not clear.

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They are, however, one of the most commonly mutated groups of genes found in glioblastomas. Hence, it is of interest to explore the applications of these tumor suppressor genes in the treatment of glioma.

Previously, adenovirus mediated p53 gene delivery had been shown to induce apoptosis and *in vivo* tumor suppression in malignant tumors such as lung, cervical, ovarian, head and neck carcinomas and gliomas (13–17). p16 or p21 gene insertions were also found to induce significant cell cycle arrest and tumor growth suppression *in vivo* in various cancers (18–24). However, adenoviral vectors, known to induce significant immune response and cytotoxicity, were predominantly used in these studies to deliver tumor suppressor genes *in vivo*. Very few approaches applied retroviral or nonviral vectors to deliver these tumor suppressor genes. The purpose of this study is to investigate the effect of these genes in glioblastomas. Emphasis was made to compare the tumor suppression efficiency of p53, p16, and p21 genes *in vitro* and *in situ* after retrovirus mediated gene delivery. Intuitively, by replacement of a missing gene or over expression of genes that affect the cell cycle at a specific check-point should have a more profound inference on tumor suppression therapy.

MATERIALS AND METHODS

Cell culture. The RT-2 cells were grown in Dulbecco's modified Eagle medium (DMEM, high glucose) with 10% bovine calf serum (Gibco, MD). The cell line has a 95% plating efficiency with doubling time about 8–12 h. At the log phase, it has spindle morphology. RT-2 cells are capable of transactivating a wtp53 specific pg13CAT promoter construct. By using the TA cloning system (Invitrogen) and with a pair of rat p53 cDNA terminal sequence primers, a full length cDNA was cloned from the RT-2 cell line and was found to be identical to wtp53.

Retrovirus constructions and propagations. Gene transfer into RT-2 cells was accomplished by infection with helper-free pCL retrovirus encoding human p53, p21 and p16 cDNA and the neo-resistance gene. pCLp53, p21 and p16 retroviral constructs were generated by insertion of a full length human p53, p21 and p16 cDNA digested with its specific restriction enzyme and redeposited into the multiple cloning site of the pCL vector. Virus was produced by transfecting the 293 packaging cell line and the resulting viral titer was assayed on BALB/3T3 cells. A titer of 1–5 $\times$ 10^6 infected particles/ml was routinely obtained. A titer of 5 $\times$ 10^6 I.U./ml was used for these experiments. The vector pCL without the p53, p21 and p16 construct was designated as the viral vector control.

Colony formation assay and growth curves. Forty-eight hours after infection, the cells were replated in 10 cm^2 plates and maintained in selection medium containing 800 μg/ml of G418 (Geneticin, GIBCO). All cells in the mock-infected plates died within 7 days under G418 selection. Infected cultures were replated in the densities of 1 $\times$ 10^3, 5 $\times$ 10^2, or 2.5 $\times$ 10^2 in 10 cm^2 plates in triplicates and maintained for 2 weeks and the neo-resistant colonies were fixed with methanol and stained with Giemsa for counting. The number of colonies on the control dishes (infected with vector only) was used as the 100% in this assay. Cell growth was determined by plating 10^4 cells in the DMEM medium without G418 in quadruples. The cells were harvested with trypsin and counted at 24 h intervals.

Western blot analysis. Total cell lysates were prepared by incubating the cells 48 h after p53 lipofection in lysis buffer [20 mM Imidazole–HCl (pH 6.8), 100 mM KCl, 2 mM $MgCl_2$ 20 mM EGTA (pH 7.0), 300 mM sucrose, 1 mM NaF, 1 mM Na-vanadate, 1 mM Na molybetadate, 0.2% Triton X-100] for 1 h at 4°C. Proteins (10 μg/sample assessed by BCA protein assay, Pierce Chemical Co., IL) were subjected to 12% SDS–Tris glycine gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., NH). The membrane was blocked with 5% nonfat milk in TBST buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 0.1% Tween 20] and incubated with the primary antibody (DO-1, 1:1000, Santa Cruz Biotech, CA; p21WAF1 [Ab-1], 1:500, Oncogene Science, MA; p16 [M156], 1:500, Santa Cruz Biotech) for 1 h at room temperature. Peroxidase conjugated anti-mouse antibody (1:3000) was used as a secondary antibody. The membrane was developed using a Renaissance protein detection kit (DuPont NEN, MA). The membrane was further stripped in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl (pH 6.7). For the detection of rat endogenous p53 protein, pAb421 (Santa Cruz Biotech) at 1:100 was used.

Stereotactic injection. Sprague–Dawley rats (180–200 g) were anesthetized with an intramuscular injection of a cocktail of Ketamine, 22–44 mg/kg, Xylazine, 2.5 mg/kg, and Acepromazine, 0.75 mg/kg. Animals were then placed in a Kopf stereotactic apparatus. A midline linear skin incision was made, and burr holes were drilled 0.8 mm posterior and 3.5 mm lateral to the bregma. Infected and selected RT-2 cells with varied suppressor genes were harvested and re-suspended in a concentration of 10^4 cells per microliter. Ten microliters of RT-2 cells (1 $\times$ 10^5) were injected over 2 min into the striatum at a depth of 5.0 mm. The control tumor cells (vector only) were injected into the left side, whereas the suppressor gene encoded virus was injected into the right side. Injections through the burr holes were carried out with a 27-gauge syringe needle attached to a Hamilton micro-injector. The needle was then retracted over a 2 min period. The burr holes were subsequently sealed with bone wax prior to closure of the incision. The animals tolerated the procedure without any apparent discomfort. Rats were sacrificed 11 days after operations.

Histopathology and immunohistochemistry analysis. Histological studies were done after fixed the brain in 10% formalin and acetic acid. The whole brain was cut into 2 mm slabs prior to paraffin embedding. Thin sections of 5 μm were stained with hematoxylin and eosin prior to volumetric study. Tumor volumes were measured with an NIH imaging program coupled with frame grabber software. Morphologic changes in the tumors infected with p53, p21 and p16, compared to control tumor cells, were assessed independently on these sections by a neuropathologist in a blinded fashion. Immunohistochemistry was performed as described previously (25). DO-1 antibody (1:100) and p21WAF1 (Ab1 1:100) were used to detect exogenous transgene expressions after antigen retrieval using 0.1 mM citric buffer in microwave at 700 watts for 20 min.

Long-term survival assay. Thirty Sprague–Dawley rats under anesthesia were used for long-term survival assay. Ten microliters of RT-2 cells (1 $\times$ 10^5) were injected intracranially into left hemisphere of the striatum in SD rat to create *in situ* GBM. Three days after RT-2 injections, p53, p21, p16, or vector control (20 μl at the titer of 5 $\times$ 10^6 cfu/ml) were injected over 5 min into the striatum at a depth of 5.0 mm into left striatum of the animal. The animals were then observed for 60 days. Survival fractions and the mean survival time of the animals were determined.

RESULTS

Growth Rate and Colony Formation in pCLp53, p21 and p16 Infected RT-2 Cells

The growth rate and colony formation assay were depicted in Figs. 1 and 2. Cells infected with pCLp53

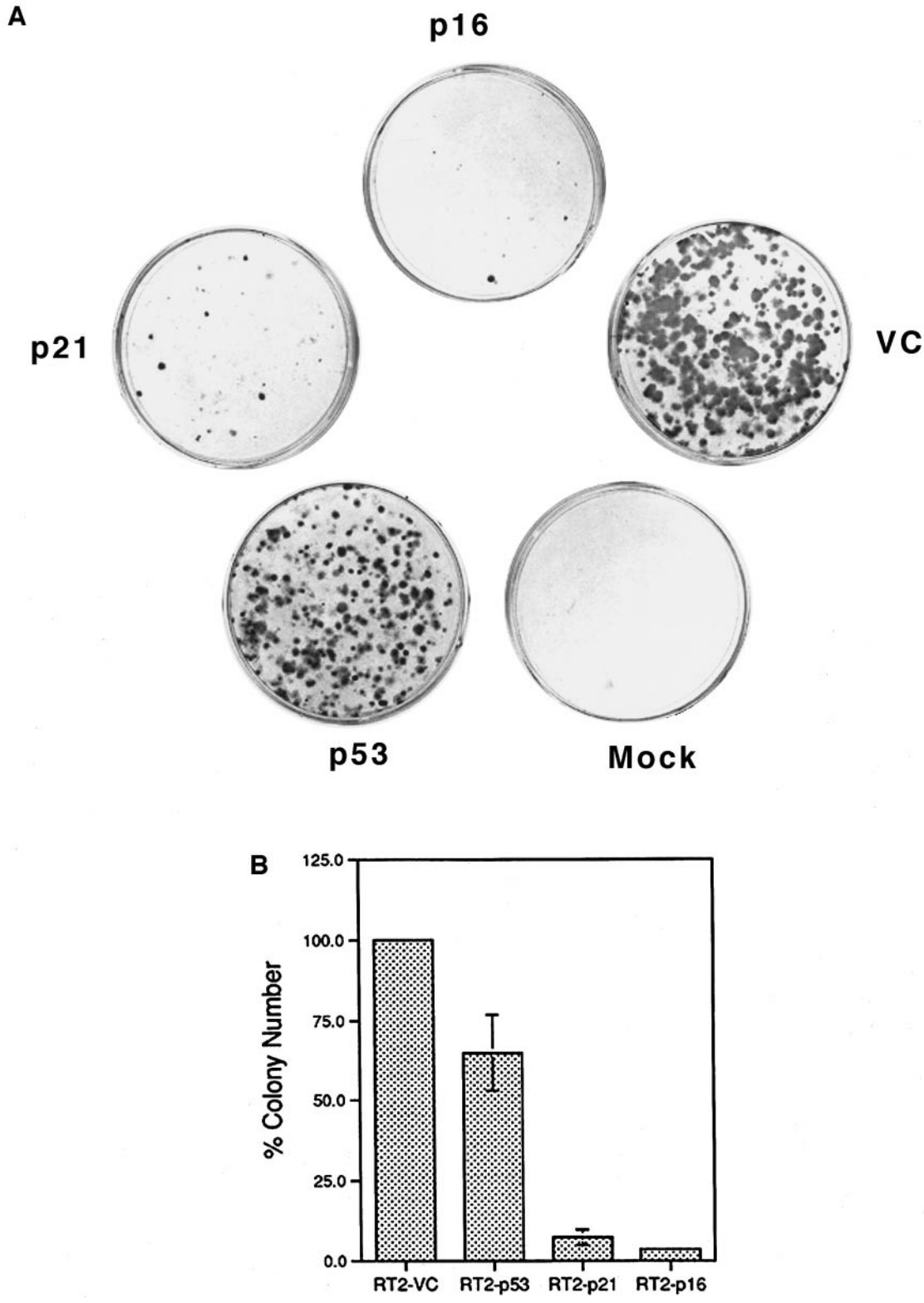

FIG. 1. Comparisons of colony formation potentials of p53, p21, and p16 infected RT-2 cells. (A) Colony formation assay of vector, p53, p21, and p16 infected RT-2 cells. Note significant reductions of colonies formed in the p21, and p16 infected plates. (B) Bar diagram represents the reduction of colony formation by p53, p21, and p16 and is expressed as % of the vector control. Note the number of colonies in the empty viral vector infected dishes were used at 100% control in this assay.

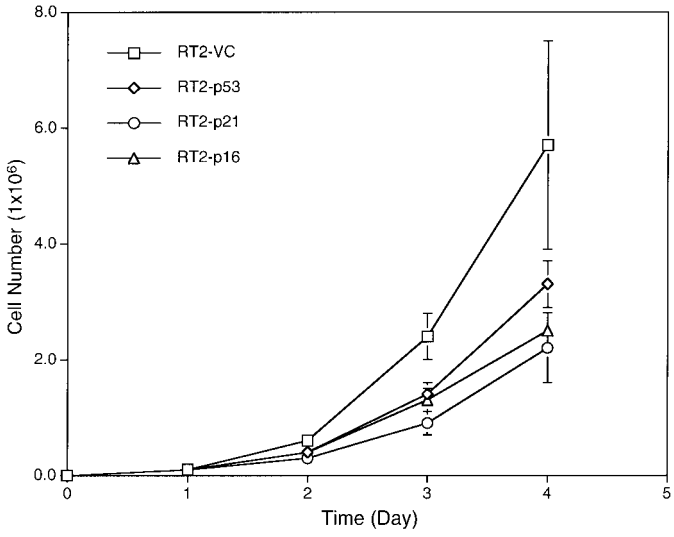

FIG. 2. Comparisons of *in vitro* tumor growth curves in p53, p21, and p16 infected RT-2 cells. A nonreplicating retrovirus pCL encoded with a human full-length cDNA was used to infect glioblastoma RT-2 cells. Exponentially growing cells were plated 24 h before infection in 10 cm^2 dishes High-titered retroviruses were used to infect tumor cells. 2×10^4 infected cells were seeded in quadruplicates to determine the growth curves of the tumor cells after p53, p21, and p16 retrovirus infections.

encoded virus grew slower in comparison to those encoded with an "empty" construct. A 35% reduction was noted in this growth paradigm after p53 retroviral infection. Much higher reduction in growth rate was observed in cells infected by pCLp21 (66%) and pCLp16 (60%) (Fig. 2). Significant suppressions in colony forming assays were found in pCLp21 (91%) and pCLp16 (98%) compared to p53 (50%) (Fig. 1).

Retroviral Infections of p53, p21, and p16 Genes Induced Strong Exogenous Transgene Expressions

Figure 3 showed protein expressions of RT-2 cells after infected with retroviral vector (pCL) or tumor suppressor genes (p53, p21 and p16). Figures 3A–3C represented the wtp53, p21, and p16 protein expression patterns after gene delivery. The glioblastoma RT-2 cells showed endogenous expression of wtp53 and a moderate amount of p21 but not p16 expression detected by rat specific antibodies (lane 2 of A–C). In comparisons, pCLp53, p21 and p16 infected clones demonstrated strong exogenous transgene expressions equivalent to human cell line control (lane 3 of A–C vs lane 1 of A–C).

Suppression of in Vivo Tumorigencity by p53, p16, and p21 Gene Delivery

Cells infected by virus encoded an "empty" or full-length cDNA of human wild type p53, p21, and p16 were stereotactically implanted into striatum of rats. Sizable tumors were detectable 10 days postinoculation. In rats harboring p53 infected cells, the tumor volume was significantly reduced compared to controls (3.5 ± 2.5 mm^2 vs 9.3 ± 2.7 mm^2; $62 \pm 26\%$ reduction). Similarly, tumor formation was suppressed in a larger degree in p21 (0.9 ± 0.6 mm^2; $90 \pm 18\%$ reduction) and p16 (0.7 ± 0.3 mm^2; $92 \pm 12\%$ reduction) infected cells. (Fig. 4 right hemisphere of the brain compared to left hemisphere).

p53, p21, and p16 Transgene Expression in Vivo

Expression of human p53 and p21 protein *in vivo* was demonstrated (Figs. 4c–4f) and the transgene expression of these proteins was confirmed by staining with their corresponding antibodies. Immunohistochemical analysis showed that p53 infected in-situ RT-2 tumors had less than 10% of the tumor cells expressing human p53 transgene (Fig. 4c). Vector control infected RT-2 cells did not show expression of human p53 protein (Fig. 4a). In p21 injected RT-2 tumor, however, no human p21 transgene expression was detected (Fig. 4d). p16 injected RT-2 tumor showed

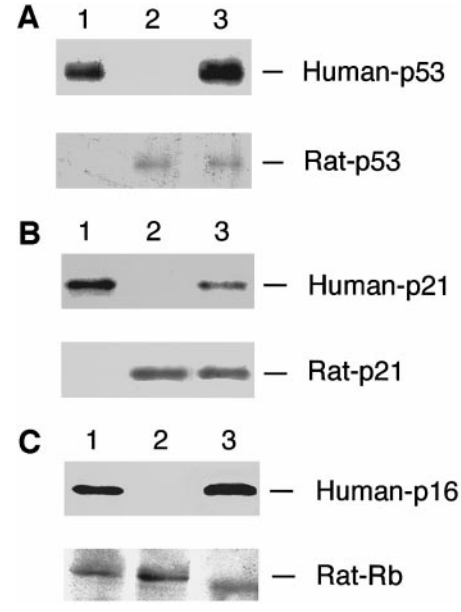

FIG. 3. Western blot analysis of exogenous human p53, p21 and p16 gene expression in retrovirus infected RT-2 cells. (A) p53 protein expression detected by human and rat p53 antibodies. Lane 1, U251 human glioblastoma cell line as human p53 positive control; lane 2, RT-2/VC; lane 3, human p53 infected RT-2 cells. Note strong human exogenous human p53 protein expression compared to weak rat endogenous rat wtp53 expression. (B) p21 protein expression detected by human and rat WAF1 antibodies. Lane 1, U87 human glioblastoma cell line as human p21 positive control; lane 2, RT-2/VC; lane 3, human p21infected RT-2 cells. (C) p16 and Rb protein expression. Lane 1, 293 human embryonic kidney epithelial cell line as human p16 control; lane 2, RT-2/VC; lane 3, human p16 infected RT-2 cells. Note p16 infected RT-2 cells showed hypophosphorylated Rb (lane 3). Also note rat Rb antibody cross react with human Rb protein (lane 1).

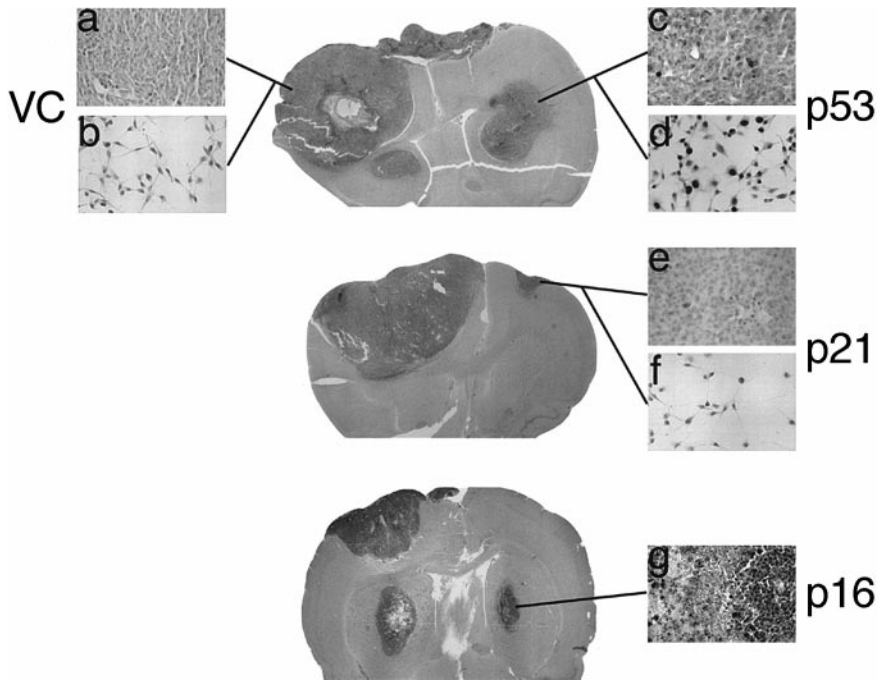

FIG. 4. Transgene expression, tumor volume, and histopathology analyses in *in situ* tumors after p53, p21, and p16 retroviral gene delivery. RT-2 cells were harvested and re-suspended in a concentration of 10^4 per μl. Cells were stereotactically injected into the striatum of a (180–200 gm) Sprague–Dawley rat. Vector Control retroviruses were injected in the left hemisphere and the tumor suppressor gene encoding retroviruses were injected in the right hemisphere at day 8 after implantation. Histopathological and immunohistochemical examinations were performed 3 days after virus injections. The inserts showed the expression of human p53 and p21 in *in situ* tumors. (a, b) Vector control (VC) infected control stained with p53 antibody in-situ (a, 200×) and in explanted cell culture (b, 200×). (c, d) p53 infected cells stained with p53 antibody in-situ (c, 200×) and in explanted cell culture (d, 200×). (e, f) p21 infected cells stained with p21 antibody in-situ (e, 200×) and in explanted cell culture (f, 200×). (g) H&E staining of p16 infected cells showed significant necrosis.

massive tumor necrosis and no p16 transgene was detected (Fig. 4g). In addition, tumor cells were explanted and plated in cell culture. Immunocytochemical staining also showed similar transgene patterns in p53 and p21 infected cells (Figs. 4d and 4f). These results indicated that p53, p21, and p16 genes exerted strong tumor suppressive effects on in-situ tumors through retrovirus mediated gene delivery. In p53 and p21 injected tumors, however, the formation of the tumors might be due to the escape of retroviral infections by the tumor cells since the remaining tumor did not stain for human p21, and to less extent, human p53 gene (Figs. 4c and 4e).

p53, p21, and p16 Genes Prolonged Animal Survival after Gene Delivery

Survival curve study was performed to determine if retrovirus mediated tumor suppressor gene insertions beneficial to animals. Emphasis was also made to determine which tumor suppressor gene is the most potent gene to prolong the survival of the animal with glioblastomas. Figure 5 showed rats survived longer than controls after p53 retrovirus injections (37.1 ± 12.3 days compared to 15.3 ± 3.5 days). Thirty-three percent cure rate was also noted after p53 gene delivery. p16 and p21 gene deliveries via retrovirus resulted in even better survival and cure rate (53.5 ± 11.2 days with 83% cure rate compared 45.5 ± 15.6 days with 66% cure rate). These results indicated p16 as the most potent tumor suppressor gene in prolonging animal survival and was in agreement with the *in vitro* and *in vivo* tumor suppression results (Figs. 1, 2, and 4).

DISCUSSION

Malignant glioblastomas are the most common primary brain tumors encountered in adults and the most frequent solid neoplasms in children. These tumors are highly resistant to conventional treatment (surgery, radiotherapy, and chemotherapy) (26–29). Researches exploring therapeutic potentials of other approaches such as gene therapy are currently underway. Since most of the primary glioblastomas contained multiple tumor suppressor gene alterations. It is of interest to compare the functional expressions of these genes in glioblastoma. In this study, we investigated the effects of gene therapy in glioblastoma with these seemingly related genes p53, p21 and p16.

In our previous studies, we have shown substantial reduction of tumor growth by p21 or p16 (25, 30). In

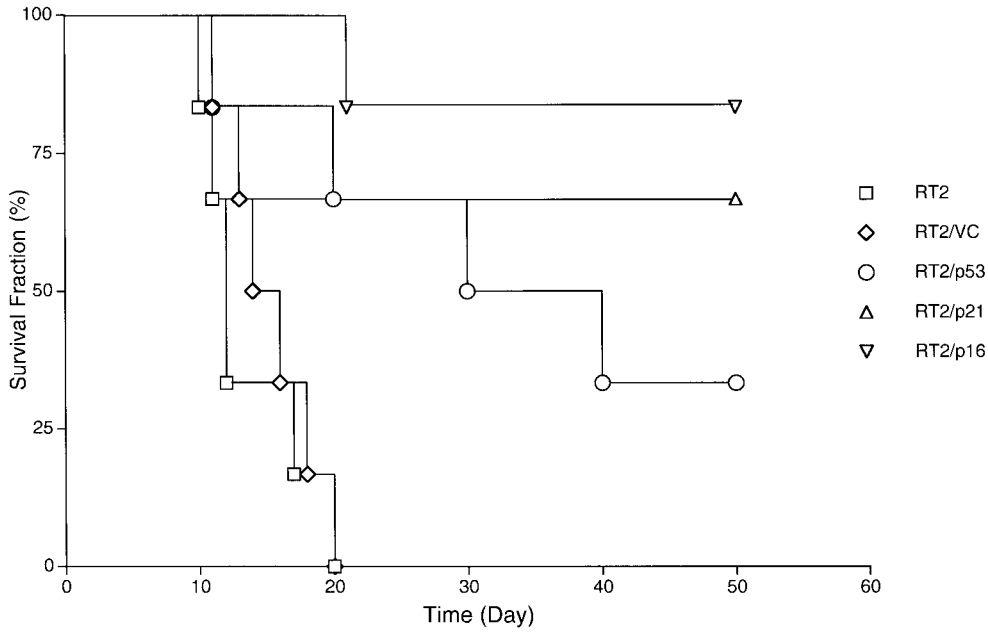

FIG. 5. Comparisons of long-term survival curves in p53, p21, and p16 infected RT-2 tumors. RT-2 tumor cells were injected into 5 groups of 6 rats each into the left striatum. Three days after tumor injections, retroviruses encoding vector control, p53, p21, or p16 was injected into *in situ* tumors through intracranial route. The animals were then kept for the time indicated on the graph. Survival fractions of the animals were determined to plot the survival curves. Mean survival time was determined. Note most of the control animals and the viral control animals died within 20 days.

contrast to the control group, tumor introduced with p21 or p16 gene showed significant reductions in tumor growth. In some cases, complete suppressions of tumor growth were achieved. This study systemically introduced a series of glioblastoma tumor suppressor genes to investigate and to compare the restoration powers of cell-cycle control pathway with long-term survival. Examination of RT-2 cells exhibited normal wild type endogenous p53 expression, little to no expression of p21 or p16 gene. This may coincided with the *in vivo* long term survival data which indicated the restoration of a non-expressed tumor suppressor gene will restore the physiological genetic checking system which will eventually lead the cell through apoptosis. Colony assay in Fig. 1 showed an effective suppression with p16 and p21. Similar results were also found in growth rate (Fig. 2), where p16 and p21 reduced cell proliferations. Although p53 had less inhibitory ability, an average of at least 30% reduction in colonies was seen. Interestingly, growth rate of p53 and p21 infected cells were similar until later time period where p53 decrease its ability as a tumor suppressing mediator. The differences may be due to the fact that cell growth controlled by p53 gene is mediated via p21 gene. Overexpression of p21 might have a more direct effect. Other molecules regulate or regulated by p53, such as MDM2, may play a role in weakening the function of p53 as a tumor suppressor (31–33).

Regulatory network of p16/RB/E2F and p53/p21/RB are the most frequent altered pathways in a variety of cell lines and tumors. The transfer of these genes to correct the genetic defect seems feasible. Our previous results had shown that the transfer of exogenous wild-type p53 cDNA would induce apoptosis in glioblastoma cells expressing endogenous mutated p53 but not in cells expressing endogenous wild-type p53 (34). However, overexpression of p53 in tumors with wild type endogenous p53 had an augmentation effect in tumor suppression. Similar results had also been demonstrated in several studies especially when the exogenous transgene is proved to have the ability to override the "silencing" (6, 34–36) effect of endogenous tumor suppressor genes. The exact interactions among these molecules are still not clear.

Although results in recent studies seemed promising in brain tumor gene therapy through p16, p21 and/or p53 gene transfer, long-term survival analyses of the treated animals have not been evaluated. From our previous results, delivery of endogenous wild-type tumor suppressor gene (such as p53, p21, and p16) into experimental brain tumors with tumor with p53, p21 or p16 mutation would induce tumor regression and prolong animal survival. Therefore, it may be possible to use one of these tumor suppressor gene therapies as augmentation therapy to treat residual brain tumor after surgical resection.

The application of gene therapy in treating brain tumors sheds hope in the future therapeutic treatment of this disease. However, without better understanding of the basic tumor biology and genomic make-up of

individual tumors, the usefulness of these clinical applications remains skeptical. Since most of the tumor suppressor genes were found to have mutations in only portions of the malignant brain tumors. The application of the most potent tumor suppressor gene may only cure certain tumors with defective or mutated genetic background. However, by comparing their *in vivo* behaviors with the genetic alterations, the therapeutic effects of these tumor suppressor genes to treat malignant brain tumors may become predictable. The combination of these different tumor suppressor genes in one gene therapy vector may offer some hopes to cure the devastating glioblastomas regardless of their genetic background.

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