Effects of Gamma Knife surgery on C6 glioma in combination with adenoviral p53 in vitro and in vivo

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Object. The authors sought to study the combined potential of wild-type p53 gene transfer and Gamma Knife surgery (GKS) for the treatment of glioblastomas multiforme. Modification of the radiation response in C6 glioma cells in vitro and in vivo by the wild-type p53 gene was investigated.

Methods. Stable expression of wild-type p53 in C6 cells was achieved by transduction of the cells with adenoviral p53. Two days later, some cells were treated with GKS. Forty-eight hours after irradiation, the comparative survival rate was assessed by monote-trazolium (MTT) assays. Treated and control C6 glioma cells (4 × 10³ per well) were plated into a 96-well plate in octuplicate and tested every 24 hours. Meanwhile, immunohistopathological examination of proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase—mediated deoxyuridine triphosphate (TUNEL) assays were performed. The MTT assays indicated the p53, GKS, and combined treated cells proliferated at a significantly lower rate than those of the control group (p < 0.01, Days 2–6) and the positive fraction of PCNA in p53-treated group and GKS-treated group was 70.18 ± 3.61 and 50.71 ± 2.61, respectively, whereas the percentage in the combined group was 30.68 ± 1.49 (p < 0.01).

Fifty-six male Sprague-Dawley rats were anesthetized and inoculated with 10⁶ cultured C6 glioma cells into the cerebrum. Forty-eight hours after transduction with adenoviral p53, some rats underwent GKS. A margin dose of 15 Gy was delivered to the 50% isodose line. Two days later, six rats in each group were killed. Their brains were removed and paraffin-embedded section were prepared for immunohistopathological examination and TUNEL assays. The remaining rats were observed for the duration of the survival period. The survival curve indicated that a modest but significant enhancement of survival duration was seen in the p53-treated or GKS alone groups, whereas a more marked and highly significant enhancement of survival duration was achieved when these two treatment modalities were combined. When PCNA expression was downregulated, apoptotic cells become obvious after TUNEL staining.

Conclusions. The findings of this study suggest that p53-based gene therapy in combination with GKS may be superior to single-modality treatment of C6 glioma.

KEY WORDS • adenoviral p53 • Gamma Knife surgery • glioma • radiosensitivity • radiosurgery

It is well known that gliomas are the most common primary central nervous tumors, accounting for 40 to 60% of intracranial tumors. Extensive research devoted to improving treatment of malignant gliomas over the past three decades has yielded little progress; the prognosis for patients who harbored malignant gliomas remains grim. Conventional radiotherapy has been one of the most important adjuvant modalities in the management of malignant gliomas and it has achieved better results than surgery alone; however, its long-term efficacy is poor. How to improve radiosensitivity of these tumors is the hot spot in the radiotherapy research.

It has been reported that enhancing the antitumor effects by combining radiation with other agents often allows lower doses to be used, thereby minimizing radiation side effects. Gene therapy in combination with radiation is one such promising strategy. Evidence indicates that p53 gene may play a role in improving the radiosensitivity of tumors by inducing cell cycle arrest and apoptosis. Meanwhile, many authors have reported that fractionated radiotherapy in combination with adenoviral p53 can improve antitumor efficacy. Gamma Knife surgery is not equivalent to radiotherapy; there are differences between them in terms of radiation dose and radiobiological effects. Radiosurgery is a minimally invasive technique designed to produce a destructive radiobiological response within an imaging-defined target volume in a single session. The use of Gamma Knife surgery allows the delivery of a high dose of radiation to the tumor site with a sharp dose falloff to normal surrounding tissue. We are aware of only a few reports of the use...
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of GKS in combination with p53 transfer to treat gliomas, which prompted us to perform the following experiments.

Materials and Methods

Cell Culture

Rat glioma cell line C6 and packaging cell line 293 were obtained from Tianjin Medical University General Hospital and Laboratory of Neuro-oncology. The cells were cultured in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin, which were grown at 37°C in 5% CO2.

Adenoviral Vectors and Gene Delivery In Vitro

The replication-defective adenoviruses carrying wild-type p53 (ad-p53) were also obtained from Tianjin Medical University General Hospital and Laboratory of Neuro-oncology. Adenovirus p53, which contains a basepair sequence encoding the wild-type p53 protein, driven by the immediate early cytomegalovirus promoter upstream of a polyadenylation signal, were used as vectors in this experiment and had been amplified in 293 cells, amounting to 1.8 x 10⁹ pfu/ml. Rat C6 cells in monolayer culture were incubated with Dulbecco modified Eagle medium containing 2% FCS in six wells with or without the vectors at a multiplicity of infection of 100. After this incubation, the culture medium containing 10% calf serum was added to each well. Some cells were collected by trypsinization and subjected to GKS. Other mock-irradiated cells were treated in a similar manner except they were not irradiated.

Western Blot Analysis

Forty-eight hours after adenovirus transfer, some cells were trypsinized and washed with ice-cold phosphate-buffered saline three times. Then the cells were solubilized in 1% Nonidet P-40 lysis buffer (20 mM Tris, pH 8, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 mM sodium orthovanadate), and a protease inhibitor mixture. Homogenates were clarified by centrifugation at 20,000 G for 30 minutes at 4°C, and protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Bioscience). Samples were adjusted to equal protein concentration and volume and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 8% sodium dodecyl sulfate–acrylamide gel. Separate proteins were transferred to polyvinylidene difluoride membranes (Millipore), followed by blocking. The membranes were incubated with primary antibody against p53 (1:1000 dilution; Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase–conjugated secondary antibody (1:1000 dilution; Zymed). The specific protein was detected using a Superfinial protein detection kit (Pierce Bioscience). After washing with siring buffer, the membrane was reprobed with an antibody against β-actin (1:500 dilution; Santa Cruz Biotechnology) by using the same procedures described previously.

The MTT Assay

Growth inhibition of cells after GKS was evaluated using an MTT assay (Sigma). Briefly, 4 x 10⁴ cells were plated in 96-well plates and grown overnight. In all 20 µL MTT (5 g/L) was added into each well for 4 hours. After the medium containing MTT was aspirated, the formazan crystals were dissolved in 200 µl dimethyl sulfoxide. The absorbance was recorded using a Teanagan 96-well spectrophotometer at a wavelength of 570 nm, with a wavelength of 630 nm as the reference. The data are presented as the mean ± SD, derived from triplicate samples of at least three independent experiments.

Tumor Inoculation and Viral Vector Injection

Fifty-six male Sprague–Dawley rats, each weighting 225 ± 25 g, were anesthetized with intraperitoneal 10% chloral hydrate (300 mg/kg) and placed in a stereotactic device. Each rat was then inoculated, using a Hamilton microliter syringe, with 10⁶ cultured C6 glioma cells into the cerebrum in the anterior fontanel region 3 mm to the right of the midline and 5 mm deep from the surface of cranium (Fig. 1).

The rats bearing well-established cerebral gliomas, demonstrated by MR imaging at 6 days postimplantation, were divided into four groups of 14: Group 1, a control group in which each rat was only inoculated with C6 glioma cells; Group 2, the ad-p53–treated group in which each rat was injected with a 20-µl solution containing ad-p53 on Days 6 and 8 after inoculation; Group 3, Gamma Knife surgery group in which rats were inoculated 10 days prior to GKS, which was performed with a 4-mm collimator and a 201-source 60Co gamma unit (model C; Elekta Instruments AB, Stockholm, Sweden). The 50% isodose line (15 Gy) matched the approximate tumor size; Group 4, the ad-p53 and GKS–treated group, in which rats were treated with ad-p53 2 days after implantation and underwent GKS on Days 6 and 8 postimplantation.

Gamma Knife Surgery

Radiosurgery was performed 10 days post–C6 cell implantation. The rats were anesthetized and placed on a modified stereotactic frame. Axial and coronal MR images were obtained, and the imaging data were uploaded to Gamma Knife planning computer running GammaPlan software. We first outlined the tumor volume and then designed a conformal treatment plan in which we attempted to cover the entire tumor volume by using a 4-mm radiation isocenter (Fig. 2). A margin dose of 15 Gy was...
FIG. 2. GammaPlan software was used to design a conformal treatment plan to cover the entire tumor volume.

delivered to the 50\% isodose line. Two days later, six rats in each group were killed and the brains were removed, sliced, and embedded in paraffin for immunohistopathological examination and TUNEL assays. The remaining rats were observed during the survival period. After 8 weeks, all the rats were killed, and we performed a survival period analysis.

Immunohistochemical Analysis

The second day after radiosurgery, six rats in each group were killed, and paraffin-embedded tissue sections were used for examination of PCNA expression. The sections were dewaxed, treated with 3\% H$_2$O$_2$ for 10 minutes, and incubated with appropriate antibody (1:100 dilution) overnight at 4°C. Biotinylated secondary antibody (1:100 dilution) was added at room temperature for 1 hour, followed by the incubation with avidin-biotin-peroxidase complex for an additional 1 hour. After washing with Tris-buffer, the sections were incubated with 30 mg DAB dissolved in 100 ml Tris-buffer containing 0.03\% H$_2$O$_2$ for 5 minutes, rinsed in water, and counterstained with hematoxylin.

Detection of Apoptosis

Apoptosis was detected using the TUNEL method. Briefly, sections were dewaxed, incubated with blocking solution (0.3\% H$_2$O$_2$ in double-diluted water) for 30 minutes, and permeabilized with 0.1\% Triton X-100 in phosphate-buffered saline for another 2 minutes on ice. Apoptosis was detected using an in situ cell death kit (Boehringer Mannheim). Positive cells were visualized by fluorescent microscopy. The reaction mixture was incubated without enzyme in a control cover slide to detect nonspecific staining.

Statistical Analysis

A commercially available software package SPSS, version 10.0, was used for statistical analysis. One-way analysis of variance was used to analyze the significance between groups. Statistical significance was determined at a probability level of less than 0.01.

Results

Expressions of Glioma Cells Transduced With ad-p53

We demonstrated transfer of wild-type p53 into rat C6 cells in vitro by using the ad-p53 vector. Wild-type p53 expression was demonstrated by means of Western blot (Fig. 3). Western blot analysis showed that p53 expression was dramatically upregulated in cells transduced with ad-p53.

Effect of p53 Overexpression and GKS on C6 Glioma Cell Proliferation and Apoptosis

To determine the influence of the transduced p53 gene on the sensitivity of glioma cells to radiation, we performed GKS 2 days after exposure to ad-p53; 48 hours later, we examined the comparative survival rate by using MTT assays. The cells in the p53-, GKS-, and combination-treated groups proliferated at a significantly lower rate than those of the control group (Fig. 4a; p < 0.01, Days 2–6). Meanwhile, proliferation of the transduced cells and GKS-treated cells was analyzed by immunohistochemistry for PCNA. As shown in Fig. 4b, the positive fraction of the p53-treated group and GKS-treated group was 70.18 \pm 3.61 and 50.71 \pm 2.61, respectively. The percentage in the combined group was 30.68 \pm 1.49 (p < 0.01). These results suggest that overexpression of p53 can sensitize C6 glioma cells to radiation.

Next, we performed in vitro TUNEL assays to evaluate apoptosis of C6 glioma cells after GKS. The percentage of cells undergoing apoptosis after treatment was determined by calculating the fraction of TUNEL-positive cells in each low-power field examined (Fig. 4c). These results demonstrate that overexpression of p53 in combination with GKS results in more of an increase in apoptosis than in other groups (p < 0.01).

FIG. 3. Western blot analysis of p53 protein expression by using total protein extracted from the control cells and the transduction cells.
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Fig. 4. a: In the MTT assays, treated and control C6 glioma cells ($4 \times 10^3$ per well) were plated into a 96-well plate in octuplicate and tested every 24 hours. The x axis indicates comparative survival rate of glioma cells in parental C6, p53-, GKS- and combined-treated groups. The y axis represents the number of days after cell implantation in the 96-well plate. The survival rate in parental C6 glioma cells was presented as 100%. b: The expression of PCNA is downregulated. c: The apoptotic cells become obvious after TUNEL staining.

Antitumor Effect of Overexpression of p53 in Combination With GKS in Vivo

The rats bearing well-established cerebral gliomas at 6 days postimplantation, and all other surviving rats underwent MR imaging to allow for observation of tumor changes at 1, 2, 4, and 8 weeks after implantation (Figs. 5 and 6). Two days after GKS, six rats in each group were killed and their brains were removed and embedded in paraffin for H & E staining (Fig. 7). During the observation period, all the rats in Group 1 died of tumor progression; the mean survival of those rats was $18.7 \pm 0.45$ days. Six rats in Group 2 and four in Group 3 died; the mean survival period of rats that died during the observation period was $24.85 \pm 3.76$ and $24.75 \pm 2.5$ days, respectively. Only two Group 4 rats died in observation period; their mean survival time was $30.5 \pm 2.5$ days ($p < 0.01$) (Table 1). Survival curves demonstrating specific augmentation of animal survival time by combining ad-p53 transduction with in vivo GKS are shown in Fig. 8a. These experiment were performed by implanting C6 glioma cells in groups of animals. Six days after cell implantation, rats in Group 2 and Group 4 underwent transduction with ad-p53; 2 days later this process was repeated. On the 10th day after inoculation, rats in Group 3 and Group 4 underwent GKS. A modest but significant enhancement

Fig. 5. a: All rats underwent MR imaging 6 days after inoculation and tumors had already formed, with volumes of $176.7 \pm 13.2$ mm$^3$. b: Representative MR image obtained in a rat in the control group 14 days postimplantation, demonstrating that the tumor has enlarged and the necrosis has occurred in the center of tumor.

Fig. 6. Dynamic MR imaging performed in a rat in the combination-treated group. The images were obtained 1, 2, 4, and 8 weeks after implantation (a-d, respectively) of C6 glioma cells. Glioma gradually regresses and disappears at Week 8 after implantation.

Fig. 7. Photomicrographs of rat brain sections. a: Control group. b: Ad-p53–treated group. c: GKS-treated group. d: Combination-treated group. H & E. Bars = 10 μm.
FIG. 8. a: Graph showing the results of survival analysis in animals harboring intracerebral C6 gliomas with and without p53 transduction and GKS. b: Expression of PCNA is downregulated. c: Apoptotic cells become obvious after TUNEL staining. H & E. Bars = 10 μm.

of survival duration was seen with p53-treated or GKS-treated animals, whereas a more marked and highly significant enhancement of survival duration was achieved when these two strategies were combined. Thus, the findings of enhanced radiosensitivity in C6 glioma cells after transduction with wild-type p53 are seen in vivo as well.

Two days after rats underwent GKS, six rats in each group were killed, their brains were removed, and paraffin-embedded sections were prepared for immunohistopathological examination and TUNEL assays, the results of which are shown in Fig. 8 b and c. The TUNEL staining method showed that there were nearly no apoptotic cells found in the control group; however, apoptosis was prominently increased in the p53- and GKS-treated groups, but mostly aggregated in the combined group. The expression of PCNA was also greatly reduced in tumor tissues derived from the combined group, compared with that in the control group (p < 0.01).

TABLE 1

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<th>Variable</th>
<th>Group 1</th>
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<td>GKS</td>
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*Median not attained (more than half of rats lived more than 56 days).

Discussion

Radiosurgery is a minimally invasive technique designed to produce a destructive radiobiological response within an imaging-defined target volume in a single session. It is capable of delivering a high dose of radiation to the tumor site with a sharp dose fall off to normal surrounding tissue. Several studies have provided evidence that radiosurgery may be an effective procedure in treating recurrent gliomas or newly diagnosed ones.11,18 Despite better efficacy, radiosurgery fails to prevent recurrence at the margin of the treated tumor mass because of infiltration of tumor cells into the surrounding normal brain tissue. These infiltrates cannot be targeted by radiosurgery for fear of causing additional neurological damage. It is these intractable infiltrates that require targeting by additional forms of therapy.

The p53 gene contains 11 exons and encodes a 53-kD nuclear phosphoprotein, which can be divided into three distinct functional domains: an acidic N2-terminal transactivation domain, a central DNA-binding domain, and a basic COOH-terminal oligomerization domain. Each of these domains plays an important role in the regulation of p53 function. The transactivation domain is bound by proteins that regulate the ability of p53 to function as a transcription factor; the central domain of p53 is required for sequence-specific DNA binding. The importance of DNA binding to p53 tumor suppressor function is underscored by the finding that the majority of tumor-derived p53 mutations occur in the central domain and disrupt the ability of p53 to bind DNA; the basic COOH terminus of p53 consists of three functional regions, including the nuclear localization signal, oligomerization domain, and nuclear export signal. The p53 tumor suppressor gene plays a critical role in preventing cancer development by regulating a number of cellular processes that are initiated following genotoxic stress.14 In addition to its ability to initiate the death of damaged cells by apoptosis, it is now understood that p53 is involved in other signaling pathways leading to improving the radiosensitivity.

In this report, we investigated the effect of GKS in combination with ad-p53 against C6 glioma in vitro and in vivo.

Prolongation of Survival Period and Suppression of Proliferation

Animals that received the intracranial injection of cells infected with ad-p53 or underwent GKS lived longer than controls. The rats in the combination-treated group lived longest and this resulted in a statistically significant survival benefit, an observation confirmed in treatments of the C6 glioma. Meanwhile, the evidence of MTT assays
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and immunohistochemistry in our experiment indicate that GKS and p53 gene can suppress the tumor cell proliferation. Two mechanisms have been suggested: First, p53 may interact directly with components of the DNA replication machinery to block initiation of DNA replication. Second, or additionally, p53 may directly or indirectly activate the transcription of a set of genes that negatively regulate key events in cell proliferation.17 Mercer, et al.,16 reported that inhibition of cell cycle after induction of wild-type protein is accompanied by selective down-regulation of PCNA mRNA and protein expression. Shan, et al.,16 studied exposure of a lung epithelial cell line to ionizing radiation arresting cell cycle progression through 48 hours postexposure, they found that PCNA mRNA levels increase transiently at early times and declined as p21/WAF1 mRNA levels increase through 48-hours post-exposure to radiation. So in their opinion, the p21/WAF1, which is the key downstream gene, can inhibit DNA replication by directly binding PCNA. Limited activation of the PCNA promoter by p53 and its modified forms would restrict the amount of PCNA made available for DNA repair and cell proliferation.

Induction of Apoptosis

The induction of apoptosis that is caused by overexpression of the wild-type p53 gene with or without radiation has been examined in a variety of tumor cell lines. Furthermore, the response is observed in vitro or in vivo. The mechanism by which p53 mediates apoptosis can generally be classified into two categories: transcription independent and transcription dependent. Mi-hara, et al.,15 reported that p53 can interact with BCL-XL and BCL-2 to exert its direct apoptogenic function at mitochondria. So far, a large number of p53 target genes with proapoptotic activity have been identified, which included, Puma, Noxa, Apaf-1, KILLER/DR5, and so on14,18. Apoptosis is an important mechanism for cell death after radiation. Preliminary in vitro and in vivo gene transfer studies in which wild-type is used in malignant glioma have established that wild-type p53 gene transfer has dominant effects over endogenous mutant p53 in sensitizing tumor cells to therapy.16 In our experiment, apoptosis was prominently increased in the ad-p53 and GKS-treated groups, compared with the control groups, which coincided with the results previously stated.

Conclusions

The findings of this study suggest that p53-based gene therapy in combination with GKS may be superior to single modality treatment of C6 glioma. Our data support the potential for transduction with p53 to enhance the sensitivity of C6 glioma in vivo and in vitro.

References

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