Research Report

Conditional Gene Expression in Human Intracranial Xenograft Tumors

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INTRODUCTION

Advances in the treatment of brain tumors remain rather limited. Current methods involve surgery followed by radiation treatment or chemotherapy, which, in most instances, only modestly delay tumor recurrence. More recently, gene therapy techniques have been employed that attempt to introduce vectors that encode gene products involved in cell homeostasis (1,6,7,9,12,13). However, existing protocols are hampered by two primary drawbacks: the introduction or modulation of genes whose function has not been completely established compounded by inefficient delivery of the potential therapeutic gene.

Xenografts of human glioma cell lines placed intracranially into immunocompromised mice are often used to model brain tumors. Properties of the established tumors, such as invasive capacity or vascularization, can be determined by immunohistochemistry following sacrificing the animal and removal of the brain. Similarly, the effect of treatment regimen, such as chemotherapy or ionizing radiation on the growth properties of the tumors, can also be assessed using these mouse models.

A number of systems have been described in which the expression of a gene product can be made conditional in mammalian cells grown in culture (4,10). In one such method, the Tet-On/Off system, the expression of a candidate gene can be controlled by treating the cells with tetracycline (or analogs) (4). In this system, the candidate gene is under control of tetracycline responsive elements (tetO). The cells also express a tetracycline repressor protein (tetR) that has been fused to

the potent transcriptional activation domain of the herpes simplex virus protein 16 (VP16) protein. There are two derivatives of this fusion protein: one is unable to bind DNA in the presence of tetracycline (tTA, Tet-Off), while the other one binds to tetO sequences in the presence of tetracycline (rtTA, Tet-On).

Whereas the tet-inducible system has been used successfully for inducible gene expression rat model systems (11) and in human glioma cells (2,14,15) *in vitro*, it has not yet been tested in human xenograft tumors growing in the brain of a living animal. We embarked on these studies to determine the feasibility of conditionally controlling the expression of a transgene in human xenograft tumors growing intracranially. In this report, we describe the generation of glioma-derived cell lines that express β -galactosidase (β -gal) following treatment with the tetracycline analog doxycyclin (Dox). The cells retain the properties of their parental cells, including the ability to form tumors when grown as xenografts. Tumors formed by these cells express functional β -gal when mice harboring them are fed a diet containing Dox in their drinking water. Based on the success of this model system, the effect of potential therapeutic genes on the growth properties of tumors growing in the intracranial milieu can now be evaluated, circumventing the issue of inefficient delivery of the candidate gene.

MATERIALS AND METHODS

Cell Culture

The glioma cell lines SNB-19 and

ABSTRACT

In an initial effort to determine the effect of expressing potentially therapeutic gene products on the growth properties of glioma tumor xenografts, we describe the development of cell lines that can conditionally express β -galactosidase (β -gal). To achieve this, we generated stable cell lines that express the modified tetracycline repressor molecule (rtTA) and the β -gal gene under control of tetracycline-responsive cis-elements. The resulting cell lines express functional β -gal following treatment with the tetracycline analog doxycyclin (Dox). These cells were then used to form intracranial tumors after injection into the brain using an implantable guide-screw system. The xenografts were found to express β -gal when the animals were fed drinking water containing Dox. From these studies, we conclude that the expression of a target gene in a human xenograft growing in the brain of a living mouse can be conditionally regulated.

U87-MG were obtained from the ATCC (Manassas, VA, USA). These cells were maintained in DMEM plus 10% fetal bovine serum (FBS). Stable cell lines were selected with G-418 (500 $\mu\text{g/mL}$). Double-selected cell lines were selected in G-418 (250 $\mu\text{g/mL}$) and hygromycin (100 $\mu\text{g/mL}$).

Construction of Plasmids

The plasmid encoding β -gal under control of tetracycline-responsive elements was made by excising a *Bam*HI-*Bgl*II fragment containing the β -gal gene from pcDEB:lacZ (kindly provided by Yusaku Nakkabeppu, Kyushu

University, Fukuoka, Japan). Following purification, this fragment was ligated to *Bam*HI-linearized pTRE (Clontech Laboratories, Palo Alto, CA, USA). A plasmid with the desired orientation was determined by restriction analysis (pTRE- β -gal). Expression of β -gal from the resulting plasmid was confirmed by co-transfecting COS-7 cells with pTet-On. The transfected cells were then treated with Dox for 48 h, and cell extracts were used to test for β -gal activity using O-nitrophenyl β -D galactopyranoside (ONPG) (8).

Generation of Tet-On Cell Lines

SNB-19 cells were transfected with the plasmid pTet-On (Clontech Laboratories) by calcium phosphate (3), and neomycin-resistant colonies were selected with G-418. To test for the expression of the Tet repressor/activator, individual clones were expanded and used in transient transfections with a plasmid encoding luciferase under control of tetracycline-responsive elements (pTRE-luc; Clontech Laboratories). The ability of the derived cell lines to activate luciferase activity in a Dox-dependent means was tested by treatment with Dox (1 $\mu\text{g/mL}$) for 48 h. A plasmid encoding β -gal was also included to correct for transfection efficiency. The cells were collected and lysed using a kit from Promega (Madison, WI, USA), and luciferase assays were performed as described by the manufacturer. β -gal assays were also performed, and the relative luciferase activity was determined with respect to β -gal activity. The cell lines with low endogenous and highly inducible luciferase activity were chosen for further study. These resulting cell lines (five of each line) were tested for their ability to form tumors in the flank of nude mice, a property of the parental cells. These cells were then transfected with pTRE- β -gal and pTK-Hyg (Clontech Laboratories), and hygromycin-resistant colonies were then selected while maintaining G-418 selection. Double-resistant colonies were expanded and were tested for Dox-dependent expression of β -gal. The cells were washed with ice-cold PBS, fixed with 0.5% glutaraldehyde in PBS, and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-gal) (de-

Figure 1. Screening pTet-On stable cell lines for Dox-induced pTRE-luciferase activity. (A) U87-MG cell lines. Nine independently derived neomycin-resistant cell lines were transiently transfected with the plasmids encoding pTRE-luciferase and β -gal. Two days following transfection, the cells were treated with Dox for an additional 48 h, at which time cell extracts were prepared and analyzed for luciferase and β -gal activity. Duplicate samples were untreated and served as uninduced controls. The bar graphs represent relative luciferase units (RLU), indicating the fold induction following Dox treatment. Background luciferase values are indicated below the bar graph for each cell line. (B) Performed identically to panel A but using 13 SNB-19-derived cell lines.

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scribed in detail below). Double-selected cells that consistently had the lowest background with the highest level of β -gal expression were used for subsequent animal studies.

Conditional Expression of β -Gal in Cultured Cells

SNB-19 Tet-On/ β -gal cells were plated in 6-well microplates (1×10^5 /dish) or 10-cm dishes (1×10^6 /dish) in DMEM plus 10% FBS. All experiments in which the cells were induced with Dox were performed in medium containing FBS (Clontech Laboratories) that is tested for optimal induction of Tet-On vectors (most commercially available serum contains low levels of tetracycline that can interfere with the Dox induction). To induce β -gal, the cells were either left untreated or induced with Dox at the concentrations indicated in the figure legends for 48 h. The cells were then washed with ice-cold PBS, fixed with 0.5% glutaraldehyde in PBS, and stained with X-gal. A stock solution of X-gal (20 mg/mL) in dimethyl formamide (DMF) was diluted into a buffer containing 5 mM $K_3Fe[CN]_6$, 5 mM $K_4Fe[CN]_6$, and 2 mM $MgCl_2$ in 100 mM sodium phosphate (pH 7.3). In the experiments, to determine time dependence of induction of SNB-19 Tet-On/ β -gal cells, the cells were plated in 6-cm dishes (5×10^5 /dish). The following day, they were treated with Dox (1 μ g/mL) for the times indicated in the Figure 3 legend. The cells were washed twice with ice-cold PBS and resuspended in lysis buffer (Promega). Cell lysates were assayed for β -gal using ONPG. The absorbance of the reactions was determined at 420 nm, and the values of the readings were averaged and are presented as fold induction compared to the uninduced samples.

Conditional Expression of β -Gal in Intracranial Tumor Xenografts

Steel canula were fitted into the skulls of nude mice as described previously (5). Briefly, an implantable guide-screw system was used which consists of a 2.6-mm guide screw with a central 0.5-mm diameter hole that accepts the 26-gauge needle of a Hamil-

ton syringe. The screw is implanted into a small drilled hole made 2.5 mm lateral and 1 mm anterior to bregma. Tumor cells are injected in a freehand fashion by using a Hamilton syringe. The syringes are fitted with a block at 3.5 mm from the opening that insures placement of the tumor cells at a consistent depth in the brain (5). Using this model, 2×10^6 cells (in 10 μ L) were injected in the caudate nucleus of nude mice. Tumors were allowed to form for 10–14 days, after which time the animals were divided into two groups. One of the groups was left untreated, while the other group had Dox added to their drinking water (2 mg/mL, plus 5% sucrose). After 48 h, the animal hearts were perfused with 3% formaldehyde in PBS, were then sacrificed, and their brains were removed. The brains were fixed with 4% formaldehyde for 30 min

and transferred to 30% sucrose for three days at 4°C. Frozen sections were then prepared using a cryostat and stained with X-gal (as described above) and/or hematoxylin and eosin (H&E).

RESULTS

Generation of U87-MG and SNB-19 Glioma Cell Lines Expressing pTet-On

The glioma-derived cell lines were transfected with the plasmid pTet-On, encoding a reverse tetracycline-controlled transactivator (rtTA), and neomycin-resistant colonies were selected using G-418. To determine clones that expressed rtTA, numerous individual clones were expanded and tested for the ability to induce the ex-

Figure 2. Induction of β -gal activity in SNB-19 (pTet-On)(pTRE- β -gal) cell lines. Three independently derived double-resistant cell lines from SNB-19 (pTet-On) isolate no. 16 (Figure 1, panel B) were obtained following transfection with the pTRE- β -gal plasmid and pTK-Hyg. The cells were grown to near confluence and left untreated (panels A–C) or treated with 0.1 μ g/mL (panels D–F) or 1 μ g/mL Dox (panels G–I) for 48 h, at which time the cells were fixed and stained with X-gal as described in Materials and Methods.

pression of a luciferase gene under control of tetracycline-responsive elements in a Dox-dependent manner. The induction profiles of nine representative U87-MG clones are depicted in Figure 1, panel A, while the induction profiles of 13 representative SNB-19 clones are shown in Figure 1, panel B. To test that these cell lines were capable of forming tumors in nude mice, five of each of the cell lines that regulated the expression of luciferase tightly (i.e., low uninduced yet highly inducible levels) were injected into the flank of nude mice. In all instances, palpable tumors were detected within two weeks, indicating the expression rtTA did not affect the tumorigenic capacity of the G-418-resistant cell lines (data not shown).

Generation of SNB-19 Glioma Cell Lines that Express β -Gal Conditionally

One of the five SNB-19 cell lines was then chosen for further studies (Figure 1, panel B, no. 16). The cells were then co-transfected with pTRE- β -gal and pTK-Hyg, which confers hygromycin resistance, and G-418/hygromycin double-resistant colonies

were selected. The resulting clones were expanded and tested for the induction of β -gal when treated with Dox. This was assessed by detecting functional β -gal enzymatic activity by incubating with X-gal, a chromogenic substrate of the enzyme. The staining of three independent SNB-19-derived cell lines are shown in Figure 2 after treatment with two concentrations of Dox for 48 h. While no staining was observed without Dox treatment (Figure 2, panels A–C), some slight activity was seen at the lower Dox concentration (Figure 2, panels D–F), and a much increased level of β -gal activity was detected at the higher Dox level (Figure 2, panels G–I). We then used another more sensitive technique to assess the expression of β -gal in the population of cells as a function of the concentration of Dox. Cells were induced with Dox (1 μ g/mL) and harvested at various times after treatment. The β -gal activity present in the cell lysate was determined using the chromogenic substrate ONPG. As seen in Figure 3, as early as 4–8 h following treatment, β -gal activity begins to accumulate, although more significant activity is detected after 24 h.

Figure 3. Kinetics of β -gal activity following Dox induction of SNB-19 (pTet-On) (pTRE- β -gal cells) (the cells are those shown in Figure 2, panels A, D, and G). The bar graph represents the fold induction of β -gal activity over a 48-h time course. The mean and standard error of duplicate dishes are shown. The graph is representative of two independent experiments.

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Intratumor Expression of β -Gal in Intracranial Xenografts

Having demonstrated that β -gal expression was dependent on Dox in glioma cells growing *in vitro*, we next tested the feasibility of inducing expression of the transgene in xenograft tumors growing intracranially. To test this, SNB-19 (pTet-On) (β -gal) cells were grown in medium containing serum with low levels of tetracycline (see Materials and Methods). The cells were injected into the brains of nude mice that had been previously fitted with a steel canula in the skull. Following injection, the canula was sealed with a bolt. Tumors were allowed to grow for 10–14 days, at which time half of the mice were changed to a diet of drinking water containing sucrose and Dox. After 48 h, the mice were sacrificed, and their brains removed and processed for histochemical analysis. Serial sections were prepared and stained with H&E (Figure 4, panels A, C, E, and G), and functional β -gal was detected with X-gal and counterstained with H&E (Figure 4, panels B, D, F, and H). Figure 4 depicts four independent brains containing tumors. The results clearly show that β -gal activity is detected in the tumors of animals that had a diet containing Dox in their water supply, while no activity was observed in the untreated animal's tumors (compare Figure 4, panels B and D to panels F and H). These results demonstrate that the Tet-On system can be used to conditionally induce the expression of a transgene in human tumor cells growing in the brain of a mouse.

DISCUSSION

In this report, we describe the generation of glioma-derived cell lines that can conditionally express functional β -gal activity. These cells were also found to conditionally express β -gal when grown as xenograft tumors in nude mice. U87-MG and SNB-19 cells were chosen because they are both commonly used to form xenograft tumors in studies modeling brain tumors. A two-step process was used to make the lines. First, the cells were transfected with a plasmid encoding the modified Tet-activator

gene, and stable cell lines were selected. The resulting cell lines were tested for their ability to activate a transiently transfected plasmid encoding luciferase under the control of tet-responsive elements. Cells that demonstrated tight regulation of the expression of this gene were chosen for further study and were subsequently transfected with the plasmid pTRE- β -gal that encodes β -gal under control of tet-responsive elements. Double-resistant clones were then screened for the ability to express β -gal

following Dox treatment.

In cell culture, we used both tetracycline and its analog Dox to induce the expression of β -gal and found that Dox consistently resulted in higher levels of induction (data not shown). Because of this finding, Dox was used in all of the animal studies. A time course of expression of functional β -gal after induction with Dox indicated that, while there was significant activity by 24 h, the expression levels continued to increase up to 48 h, the last time point as-

Figure 4. In vivo Dox-dependent induction of β -gal in SNB-19 glioma cells growing as intracranial xenografts. SNB-19 cells (pTet-On) (pTRE- β -gal) cells (2×10^6) were injected through canula placed in the skull of nude mice. Tumors were allowed to form for 10–14 days, at which time the animal's drinking water was replaced with water containing Dox (1 mg/mL) and 5% sucrose. The other half of the animals remained on regular water. Following two days of induction, the animals were sacrificed, and the animal's brains were removed and processed as described in Materials and Methods. Frozen sections were then prepared and stained with X-gal. Figure 4 depicts serial sections containing xenograft tumors from four independent mouse brains. The top four panels were from two mice fed a diet containing Dox (panels A–D), and the bottom four were from two mice fed regular drinking water (panels E–H). Panels A and B are sections from mouse no. 1. (A) H&E only. (B) H&E plus X-gal. Panels C and D are sections from mouse no. 2. (C) H&E only. (D) H&E plus X-gal. Panels E and F are sections from mouse no. 3. (E) H&E only. (F) H&E plus X-gal. Panels G and H are sections from mouse no. 4. (G) H&E only. (H) H&E plus X-gal.

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sessed. Whether there are further increases beyond 48 h has not been tested. Based on the induction conditions on the SNB-19-derived cells in culture, a 48-h induction was used in the xenograft experiments.

We found that U87-MG (pTet-On) (pTRE- β -gal) double-resistant cells had relatively high background levels of β -gal expression and, upon induction, did not result in the extent of activity seen with the SNB-19 derived cells (data not shown). We have made stable lines with several independent U87-MG (pTet-On) cell lines and found this to be a consistent property of these cells. For this reason, all animal studies were performed with the SNB-19 derivatives in which the expression of β -gal was not "leaky" and induced more robustly.

Because these studies were designed to test proof of principal, we chose a relatively high level of Dox for the animal studies to increase the likelihood that β -gal would be induced. We do not know precisely how much Dox is necessary to result in the expression of β -gal in the tumor xenografts. Since there was more than one animal in each cage, we could not determine the actual intake of drinking water per animal. Not all of the tumor cells stained positive for β -gal when compared to the H&E staining of serial sections. In most instances, at least 30% to as much as 60% of the tumor stained positive with X-gal. This might reflect the relatively inefficient transfer of the Dox across the blood-brain barrier. It should be noted that a 10-fold increase in Dox concentration resulted in a dramatic increase in β -gal expression (Figure 2) in cultured cells. Also of note in Figure 2 is that, even at the higher level of Dox used, there was not β -gal expression in all of the cells in culture. The kinetics and dose of Dox needed to obtain optimal intracranial induction of the target gene will require further studies in which the water is administered in a more controlled fashion.

The major advantage of this system over others (11) is the use of human tumor cells to form the xenografts. The use of rat cells in xenografts might not accurately mimic all of the properties of human cells grown in the brain. Since the ultimate goal is to develop

therapies that target human tumor cells, our system allows the direct study of such therapies on human tumor cells.

While advances continue to be made in the use of therapeutic gene products to treat tumor cells, the system described here would allow for the systematic testing of the effects of individual or combination of genes alone or in conjunction with other therapies such as radiation or chemotherapy. These studies demonstrated that Dox, administered in the drinking water of the mouse, passed the blood-brain barrier and effectively induced the expression of a gene product in xenograft tumors. While the feasibility of this system was demonstrated by conditionally expressing β -gal, the ultimate goal is to use the system to assess the effect of expression of candidate gene products on the growth properties of intracranial xenograft tumors.

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