Selective Enhancement by an Antiviral Agent of the Radiation-induced Cell Killing of Human Glioma Cells Transduced with HSV-tk Gene

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Abstract

The activation of antiviral drugs as a consequence of thymidine kinase expression has been shown in recent years to have potential as a treatment for malignant tumors. It was hypothesized that the property of the drugs that make them effective against viruses and proliferating cells, namely their ability to interfere with the integrity of the DNA, may be exploited to sensitize cells to radiation damage. The antiviral drug, BVdUrd, structurally a pyrimidine analogue, was found to enhance selectively the radiation cytotoxicity of human tumor cells transduced with HSV-tk thymidine kinase gene. Human glioma cells from the U-251 lineage transduced with HSV-tk and exposed to 40 μg/ml of BVdUrd for 24 h prior to irradiation were more sensitive to radiation compared with control cells under the same conditions; the sensitization enhancement ratio was 1.9. The results suggest that the addition of radiation will improve the effectiveness of HSV-tk gene therapy for the treatment of brain tumors.

Introduction

Malignant glioma of the brain is one of the most lethal cancers in humans. The median survival time after diagnosis is 50 weeks for glioblastoma multiforme. The majority of patients die of local recurrence despite aggressive medical intervention including surgery, radiotherapy, and nitrosourea-based chemotherapy. Recent attention has been focused on gene therapy of tumors. One particularly appealing approach is to incorporate the viral gene which codes for the enzyme thymidine kinase into the replicating cancer cell so that the viral-derived thymidine kinase can toxify a systemically administered antiviral drug (1). The thymidine kinase enzyme is able to convert purine and pyrimidine derivatives, widely used antiviral drugs such as acyclovir, ganciclovir, and BVdUrd, into nucleoside intermediates, thereby disrupting the integrity of cellular DNA. An advantage of incorporating the HSV-tk gene to effect cell death, compared to other toxic gene products, is that thymidine kinase is not toxic by itself. Hence, a retroviral vector which codes for thymidine kinase, such as herpes simplex virus type 1 thymidine kinase, HSV-tk, offers a conditional killing mechanism for proliferating cells.

The effectiveness of the antiviral agent ganciclovir in the treatment of sarcoma and lymphoma cells in vitro and in vivo transduced with a retroviral vector containing HSV-tk gene was demonstrated by Moolten et al. (2). Ezzeddine et al. also showed selective killing of C6 glioma cells in culture and in vivo by retrovirus transfer of the HSV-tk gene in combination with ganciclovir (3). Both groups noted the toxicity of the direct injection of the HSV-tk vector producer cells i.v. or i.p. has demonstrated no evidence of illness with or without the drug therapy (5). Based on the experimental data, "suicide" gene therapy for the treatment of malignant glioma of the brain has been approved by the NIH Recombinant DNA Advisory Committee. A Phase I clinical trial is under way (1). Early results suggest that the approach is beneficial to some patients.

Our conjecture was that thymidine kinase-activated antiviral drugs, nucleoside analogues derived from either purine or pyrimidine building blocks, may be potent radiation sensitizers to the virus-infected mammalian cells, such that the addition of radiation therapy may provide additional benefit. The techniques of gene therapy have been shown to be clinically feasible. Moreover, the techniques involved in pinpointing radiation delivery to a small region in the brain, so-called radiosurgery, are routinely applied in many cancer centers (6, 7). What was lacking in the literature was experimental evidence demonstrating that cancer cells transduced with the virus-derived thymidine kinase were selectively vulnerable to radiation when in the presence of antiviral drugs.

Materials and Methods

The colony-forming ability of human glioma cells growing in cultured media was used to assess cytotoxicity to antiviral drug and radiation in cells containing the viral-derived thymidine kinase gene. Experiments were performed using the U-251 cell line which included the HSV-tk gene (U-251-tk cells). The control cell lines used were the original parental U-251 glioma cell line (U-251 cells) and the parental cell line infected with the same retrovirus but lacking the HSV-tk gene. The cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum. No antifungal agent was used and a test for Mycoplasma infection was carried out routinely.

U-251 parent cells were transduced using the HSV-tk vector, G1TkSvNa.53 obtained from Genetic Therapy, Inc. (Gaithersburg, MD). The G1 backbone of the vectors was derived from the Moloney murine leukemia virus. The G1TkSvNa.53 vector contains the herpes simplex thymidine kinase gene downstream of the 5' long terminal repeat sequence and uses the long terminal repeat sequence as its promoter. In addition, the plasmid contains the neomycin phosphotransferase gene, NeoR, which confers resistance to the neomycin analogue, G418. The SV40 early promoter serves as an internal promoter for NeoR. PA317 was used as the retroviral packaging line following transfection with plasmids containing the retrovirus-like sequence. PA317 derived from NIH 3T3 cells contains a stably integrated replication-incompetent retroviral genome. The resultant G1TkSvNa.53 producer cell line generates a supernatant with a titer of 5.0 × 10^7 colony-forming units/ml.

Cloned vector producer cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mm L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. U-251 cells were exposed to the supernatant added with polybrene to a concentration of 8 μg/ml for about 4 h. To achieve an enriched population of transduced cells, the tumor cells were selected in G418 (800 μg/ml) for 7 days. The surviving colonies were cloned for subsequent radiation and cytotoxic studies of antiviral agents.
The antiviral drug BVdUrd was purchased from Sigma Chemical Company, St. Louis, MO. The cells were irradiated using a 5000-Ci $^{137}$Cs source (Model Mark I; J. L. Shepherd, Inc., San Fernando, CA) at a dose rate of 1.61 Gy/min. Cell survival data were analyzed using a nonlinear least-square algorithm developed by Albright (8). Cells were actively dividing and were asynchronous within the cell cycle.

**Results**

The rates of cell growth and plating efficiency of the transduced cell lines (U-251-tk cells and U-251 cells with the same retrovirus lacking the HSV-tk gene) were similar to that of the parental U-251 cells; the plating efficiency was 45 to 55% and the doubling time was approximately 20 h.

The efficiency of transduction was assessed using a vector similar to the HSV-tk vector but containing the enzyme β-galactosidase instead of thymidine kinase. The presence of β-galactosidase can be detected visually under light microscope because it stains cells blue. Transduction was performed as before including selection by G418. The number of brilliantly stained blue cells as opposed to total cells indicated the efficiency of transduction to be 68%.

Fig. 1 demonstrates the effects on cellular survival of exposure to BVdUrd as a function of exposure time in the absence of radiation. The parental U-251 cells were minimally sensitive to the antiviral agent studied, up to 100 µg/ml for BVdUrd. Survival of the parental U-251 cells incorporating the retrovirus backbone but lacking the HSV-tk gene was also relatively unaffected by drug alone. In sharp contrast, the HSV-tk-transduced U-251-tk cells were somewhat more sensitive to even low concentrations, 10 µg/ml of BVdUrd when exposure was prolonged for 72 h (Fig. 1B). The cytotoxicity of U-251-tk cells exposed to antiviral drug increased with increasing exposure time and generally increased with increasing drug concentration (Fig. 1B). Similar results were found using the purine analogue acyclovir (data not shown). Acyclovir was not toxic to U-251 tumor cells but was increasingly toxic to U-251-tk cells as exposure time and drug concentration increased.

On the basis of the cytotoxicity data of U-251-tk cells to BVdUrd, we investigated the effect of the drug on the radiation response of U-251-tk cells. Transduced cells when exposed to drug prior to irradiation showed increased radiosensitization with increased time of exposure to BVdUrd. Maximum radiosensitization was observed at 24 h drug exposure prior to irradiation. Forty-eight h exposure to BVdUrd produced the same level of radiation enhancement as observed at 24 h drug exposure.

Fig. 2 shows the radiation enhancement of U-251 parental and U-251-tk cells as a function of BVdUrd concentration. Cells were exposed to BVdUrd for 24 h prior to (Fig. 2A) or following (Fig. 2B) a single dose of 8 Gy irradiation. Twenty-four h prior or subsequent drug exposure relative to radiation delivery did not affect cell survival of U-251 parental cells regardless of drug concentration, up to 200 µg/ml.

In contrast, the transduced U-251-tk cells displayed a marked enhancement in radiation sensitivity when exposed to BVdUrd for 24 h prior to or subsequent to irradiation. The enhancement appeared to be dependent on the concentration of drug, requiring a concentration of at least 40 µg/ml for the maximum effect. The selective radiation enhancement at 8 Gy contributed an additional log of cell kill in HSV-tk-transduced cells relative to parental cells. The effect was observed regardless of whether drug exposure preceded or followed irradiation.

The extent of radiosensitization can be assessed from cell survival curves in which the percentage of cells that survive is plotted as a function of radiation dose with and without the drug. The sensitization enhancement ratio is the ratio of the radiation dose necessary to achieve a given level of cell kill in the absence of the drug relative to the radiation dose necessary to produce the same level of cell kill in the presence of the drug.

Cell survival curves were constructed for U-251 and U-251-tk cells exposed to BVdUrd at 40 µg/ml for 24 h before irradiation. No cytotoxic effect of the drug on U-251 and U-251-tk cells were observed at 40 µg BVdUrd/ml. Fig. 3 demonstrates the effect of drug on the radiation response observed in the parental line and the transduced...
resultados de cultivos de células huérfanas de gliomas malignos observados en el laboratorio, se encontraron limitados en estudios clínicos. El uso de genes activados por radiación presenta una perspectiva prometedora debido a la capacidad de inhibir el reparo de daños en DNA, pero debe ser aplicado con precaución.

**Discusión**

Se produjo una sensibilización de la radiación por el mecanismo de daño genético y la reparación del ADN. El aumento en la sensibilidad a la radiación se observó tanto luego de la exposición a radiación como después de la exposición. Las consecuencias de los resultados con BVdUrd podrían ser significativas en vista de la exposición de células tumorales a la radiación, independientemente de si el BVdUrd estaba presente antes o después de la irradiación. En resumen, se ha demostrado que la derivada pirimidínica puede sensibilizar los efectos de la radiación en células tumorales transducidas para producir derivados de ADN sensible a la radiación. El resultado sugiere que la adición de BVdUrd a la cura de gen puede mejorar la selección de la radiación y disminuir la toxicidad normal. La comunicación representa un primer paso en la iluminación de la susceptibilidad de genes de resistencia a la radiación y sus efectos en la radioterapia clínica.


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