In Vitro Evaluation of in Vivo Brain Tumor Chemotherapy with 1,3-Bis(2-chloroethyl)-1-nitrosourea

Mark L. Rosenblum, Kenneth T. Wheeler, Charles B. Wilson, Marvin Barker, and Kathy D. Knebel

The Howard C. Naffziger Laboratories for Neurosurgical Research, Department of Neurological Surgery, University of California Medical Center, San Francisco, California 94143 [M. L. R., K. T. W., C. B. W., M. B., K. D. K.], and the Laboratory of Radiobiology, University of California Medical Center, San Francisco, California 94143 [K. T. W.]

SUMMARY

An in vitro colony formation assay was used to determine the efficacy of in vivo therapy with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) on a rat brain tumor. The fraction of clonogenic cells surviving in vivo therapy was determined by a comparison between the in vitro colony-forming capacity of cells derived from previously treated and untreated tumors. With this intracerebral solid tumor a direct correlation was found between the surviving fraction of cells and animal survival, implying that the in vitro assay system is a reliable test of therapeutic effect. The BCNU dose-response curve was exponential up to a dose of 0.75 times the LD_{10} dose with little additional cell kill noted at higher drug levels. This plateau does not appear to represent a resistant subpopulation of cells, since retreatment of tumors derived from cells surviving an LD_{10} dose were as sensitive to BCNU as those with no prior drug exposure. Instead, it may represent, at least in part, failure of the drug to reach and/or enter cells in all parts of solid tumors. On the average BCNU doses of 0.75 times the LD_{10} dose or greater resulted in slightly more than a 3-log cell kill and doubled the life-span for our tumor-bearing animals. The finding that an increase in animal life-span requires at least a 1-log tumor cell kill indicates that survival studies with intracranial tumor models may be insensitive to single courses of many chemotherapeutic agents with modest but significant antitumor activity.

INTRODUCTION

Precise and reproducible systems for detecting small numbers of surviving cells after anticancer therapy will be useful in the rational planning of the most effective therapeutic regimens. The analysis of chemotherapeutic effect on solid-tumor systems has, until recently, relied primarily on gross tumor measurements. Studies based on tumor size measurements run the risk of underestimating drug effect because of interfering factors such as the swelling of damaged cells, retarded dead cell removal (9, 19), and tumor repopulation by cells previously in the nonproliferating as well as proliferating pools (8). In addition, host factors such as effects of the immunological system may further complicate the interpretation of results. Intracerebral solid-tumor models that rely on single end points, such as the time until the appearance of symptoms or death, are subject to the same criticism, since these systems predictably reflect the volume of the tumor.

We have developed an in vitro colony formation assay that directly measures the cellular effects of in vivo therapy on our brain tumor model (12). A comparison between the in vitro CFE of control and treated tumors gives an estimate of the surviving fraction of cells capable of subsequent proliferation (clonogenic tumor cells). We have used this assay to determine the efficacy of in vivo treatment on an intracerebral solid tumor with BCNU, an agent that has shown significant antitumor activity in both animals and man (2, 20). In addition, we have attempted to demonstrate the inherent limitations in determining chemotherapeutic effect from survival studies on brain tumor models.

MATERIALS AND METHODS

Animal Tumor Model. A rat brain tumor, originally induced by N-methylnitrosourea and described as a malignant astrocytoma (4), was transplanted into the brains of 150- to 200-g adult male Fischer 344 rats by a previously reported technique (2). This tumor has been reclassified as a gliosarcoma or sarcoma because of changes occurring during serial animal and cell culture passage (2).

Only tumors that had attained a size (usually 5 mg and larger) allowing excision and separation from adjacent, uninvolved brain tissue were used in this study. This tumor grows intracerebrally, compresses microscopically uninvolved adjacent brain tissue, and forms an easily discernable

---

1 This work was supported by NIH Grant CA 13525, The National Phi Beta Psi Sorority, The Joe Gheen Medical Foundation, and the Association for Brain Tumor Research.
2 Recipient of NIH Individual Postdoctoral Research Fellowship Award 1 F22 CA 02196-01. To whom requests for reprints should be addressed.
3 Supported by NIH Career Development Award 70739.
4 The abbreviations used are: CFE, colony-forming efficiency; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; LD_{10}, dose toxic to 10% of animals; ILS, increase in life-span; CCS, cell-cycle-specific; CCPS, cell-cycle-phase-specific.
colonies containing more than 25 cells were determined and counted in a hemocytometer. A single-cell suspension was aliquoted into five 60-mm plastic Petri dishes containing complete medium in a total volume of 5 ml. The complete medium consisted of Eagle's basal medium supplemented with 10% fetal calf serum, L-glutamine, nonessential amino acids, vitamins, and antibiotics. Feeder cells (10⁴ heavily irradiated rat tumor cells) were added to dishes containing dilutions of less than 10⁴ cells. All cultures were incubated in 5% CO₂ for 12 days at 37°C. The resultant cell suspension was filtered through stainless steel mesh and centrifuged at 1500 rpm for 10 min at 4°C. The supernatant was decanted, 10 ml of fresh medium were added, and the cells were resuspended and counted in a hemocytometer. A single-cell suspension was obtained with a yield of 1.5 to 3.0 × 10⁶ cells/ml of solid tumor tissue. Each of at least 2 dilutions of the cell suspension was aliquoted into five 60-mm plastic Petri dishes containing complete medium in a total volume of 5 ml. The complete medium consisted of Eagle’s basal medium supplemented with 10% fetal calf serum, L-glutamine, nonessential amino acids, vitamins, and antibiotics. Feeder cells (10⁴ heavily irradiated rat tumor cells) were added to dishes containing dilutions of less than 10⁴ cells. All cultures were incubated in 5% CO₂ for 12 days at 37°C. The plates were then fixed and stained, and the number of microscopically.

The CFE was calculated by dividing the number of colonies scored by the number of cells plated, averaged for all cell dilutions, and expressed as a percentage. The standard error, obtained from 5 to 15 plates for each tumor, was usually less than 10% of the mean CFE. In all experiments control tumors were assayed along with treated groups. For avoidance of tumor cell changes induced by long-term tissue culture passage, our tumor line was rejuvenated every 3 months from a frozen tumor cell stock. The CFE for each treated tumor was compared to the mean CFE obtained from all control tumors assayed during each 3-month period. In this manner, the best representation of the untreated tumor was used for the evaluation of therapy. Over the 9 months of this study, the mean control CFE’s were 7.9, 16.9, and 21.1% and represented 19, 29, and 18 untreated tumors, respectively. Similar biological variation has been noted with other tumor systems analyzed by CFE assay (1, 11, 18). The fraction of clonogenic tumor cells surviving treatment was calculated by dividing the treated CFE by the control CFE. The log tumor cell kill was determined as the negative log of the surviving fraction or:

\[ \text{Log cell kill} = -\log \left( \frac{\text{CFE treated}}{\text{CFE control}} \right) \]  

where each unit of log kill represents a 10-fold decrease in the number of clonogenic tumor cells.

**BCNU Treatment.** BCNU was obtained from the Drug Development Branch of the National Cancer Institute, NIH, reconstituted in a solution containing 10% ethanol and 0.9% NaCl, and then immediately injected i.p. All doses of BCNU were administered as fractions of the LD₁₀ dose of 13.3 mg/kg (2). Colony formation analysis was performed 20 to 24 hr after BCNU administration, since no significant difference in tumor cell kill was observed during the 1st 24-hr posttreatment interval (unpublished data).

For comparison of the results of the colony formation assay and animal survival studies, various doses of BCNU (0.25, 0.50, 0.75, and 1.00 × LD₁₀ dose) were administered to groups of 10 to 24 animals harboring intracerebral tumors transplanted 17 to 19 days previously. The assay was performed on 2 to 3 animals at each dose level and 7 to 21 similarly treated animals were followed for survival. Each experiment also contained similar-sized groups of comparably analyzed, untreated, tumor-bearing controls. Therapeutic results were expressed as the percentage of ILS, determined from the median posttransplant survival of treated and untreated groups:

\[ \% \text{ILS} = \left( \frac{\text{median treatment survival (days)}}{\text{median control survival (days)}} \right) - 1 \times 100 \]  

These results were plotted against the mean log tumor cell kill from comparable animals as determined by the colony formation assay in the usual manner. The line resulting from all such points was determined by a least-squares regression analysis.

In order to determine a dose-response curve, BCNU doses of 0.25, 0.50, 0.75, 1.00, and 2.00 × LD₁₀ were administered to animals 12 to 19 days after tumor cell inoculation in experiments that also included untreated tumor controls. Colony formation analysis was performed in the usual manner and the surviving fraction of clonogenic tumor cells was determined for each tumor.

In order to determine whether tumor cells that proliferate after exposure to large doses of BCNU (>0.75 × LD₁₀) have an inherent resistance to the effects of the drug, cells were harvested from plates containing tumor cell colonies grown from primary explants after in vivo exposure to an LD₁₀ dose of BCNU. Cells were analyzed simultaneously from plates of control tumors. These 2 groups of cells were then implanted intracerebrally using the same techniques as tumors transplanted in the routine fashion (2). Both of these groups contained 15 animals and were followed for subsequent tumor development. The histological characteristics, untreated CFE, and results of treatment with an LD₁₀ dose of BCNU were determined for 3 tumors in both groups in the usual manner.

**RESULTS**

A direct correlation exists between the results of CFE assays and simultaneous survival studies in animals harbor-
In Vitro Evaluation of BCNU Brain Tumor Therapy

A change in tumor size after chemotherapy will depend on the balance between cell kill, dead cell removal, and the induction of a delay in proliferation kinetics on one hand and swelling of damaged cells, extracellular edema, and proliferation of surviving clonogenic cells on the other hand. As a result, tumor volume measurements will often underestimate the percentage of tumor cells killed by a chemotherapeutic agent (19). Furthermore, since dead cell removal seems markedly retarded from intracerebral locations (9), survival studies with brain tumor models may not even show an increase in animal life-span when low levels of cytotoxicity are obtained. This was, in fact, confirmed in our system with the observation that no significant ILS was noted for doses of BCNU resulting in less than 1 log tumor cell kill. This implies that survival studies with intracranial tumor models may be insensitive to single

**DISCUSSION**

A change in tumor size after chemotherapy will depend on the balance between cell kill, dead cell removal, and the induction of a delay in proliferation kinetics on one hand and swelling of damaged cells, extracellular edema, and proliferation of surviving clonogenic cells on the other hand. As a result, tumor volume measurements will often underestimate the percentage of tumor cells killed by a chemotherapeutic agent (19). Furthermore, since dead cell removal seems markedly retarded from intracerebral locations (9), survival studies with brain tumor models may not even show an increase in animal life-span when low levels of cytotoxicity are obtained. This was, in fact, confirmed in our system with the observation that no significant ILS was noted for doses of BCNU resulting in less than 1 log tumor cell kill. This implies that survival studies with intracranial tumor models may be insensitive to single
courses of many chemotherapeutic agents with modest but significant antitumor activity. Most CCS and CCPS agents would fall into this category (3, 5, 7, 15). Since the growth fraction for most solid tumors is often 0.5 or less (14), a single, totally effective short-term course of treatment with a CCS or CCPS agent would be able to kill, at the most, 50% of all tumor cells. This 2-fold decrease in the total number of viable cells is significantly less than the 10-fold (1-log) cell kill necessary to demonstrate antitumor activity in the present model. Nevertheless, the results of this investigation do not imply that all survival studies with intracranial tumor models inaccurately reflect BCNU effect. On the contrary, the correlation noted with tumor cell kill confirms that life-span studies do measure antitumor activity with this agent. However, because of the 1-log-kill threshold, investigators should be cautious in labeling a chemotherapeutic agent as inactive when no increase in survival is noted from single, short-term courses of therapy in intracranial tumor systems. Agents that show modest antitumor activity (<1 log kill) may be useful in long-term courses or in combination with other effective drugs.

The colony formation assay does not attempt to determine the absolute number of clonogenic cells present in a tumor, since the cells must divide in an artificial environment. However, it is probably valid to assume that the comparison of treated and control tumors permits a true estimation of the surviving fraction of clonogenic cells. The direct correlation demonstrated between tumor cell kill and animal survival (ILS) appears to confirm this hypothesis. Other investigators have shown correlations between in vitro colony formation assays and studies of the clonogenic population required to produce a 50% tumor "take" after s.c. administration of a single-cell suspension used in the evaluation of in vivo tumor radiotherapy (1, 11). These observations support our view that the colony formation assay is a reliable test of therapeutic effect.

The cells that form colonies in vitro must come from both the proliferative and nonproliferative tumor cell pool since the 3-log kill required to double the life-span of the animal could not be produced by killing cells in the proliferative compartment alone.

To our knowledge, the only other direct quantitation of cell survival following BCNU treatment of a solid tumor was reported by Wharam et al. (18). Using techniques similar to ours, they observed a 1-log kill from approximately a 0.25 LD₁₀ dose on a subdermally grown EMT-6 murine mammary tumor. This result closely conforms with ours at that drug dose. Among nonsolid tumors, the effects of BCNU have been reported using the spleen colony assay for a transplanted AK lymphoma (6, 17) and a life-span bioassay for L1210 leukemia (13, 14). With both tumors, a 0.5 LD₁₀ dose of BCNU resulted in a 4 to 5-log cell kill, a much greater effect than we observed in our system. Differences in tumor type and growth characteristics probably account for the markedly heightened tumor sensitivity of nonsolid tumors.

The most likely explanation for the observed limit in the effectiveness of BCNU is a problem with drug delivery based on pharmacokinetic parameters. Although BCNU, like its analog 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (10), crosses the blood-brain barrier and accumulates in brain tumors, the entry of the drug [or its active metabolite(s)] into tumor cells may be limited by other factors. This phenomenon may be peculiar to solid tumors. A drug might bind to or react with substances in the tumor extracellular spaces, or, because the distance between capillaries and tumor cells appears to increase as the tumor grows (16), some tumor cells may not receive a cytotoxic drug concentration. The results of previous studies with BCNU on L1210 murine leukemia (14) may be interpreted as supporting this hypothesis. Leukemic cells injected i.v. or intracranially tend to grow in "solid" form at their sites of implantation, and the dose-response curves for BCNU treatment of both appeared to show a plateau at LD₁₀ doses, similar to the results of our study. In contrast, L1210 cells growing i.p. are suspended in an ascitic transudate, and the BCNU dose-response curve was exponential up to an LD₁₀ dose. Similarly, other investigators studying the in vitro effects of BCNU in other systems, have failed to demonstrate a resistant subpopulation of tumor cells (3, 7).

Obviously, further studies are necessary to elucidate and modify any drug distribution problems that may exist. An understanding of these limitations should lead to improved results in the treatment of solid tumors.

ACKNOWLEDGMENTS

The tumor used in this study was provided by William H. Sweet, Paul L. Kornblith, Jannette L. Messer, and Beverly O. Whitman of Massachusetts General Hospital, Boston, Mass.

We thank Carol Maroten, Alfonso Usog, Kathleen Smith, and Rhonda Borg for their technical assistance.

REFERENCES

In Vitro Evaluation of BCNU Brain Tumor Therapy


In Vitro Evaluation of in Vivo Brain Tumor Chemotherapy with 1,3-Bis(2-chloroethyl)-1-nitrosourea


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/35/6/1387

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.