Combined Modality Treatment Using Radiation and/or Chemotherapy in an Athymic Nude Mouse-Human Medulloblastoma and Glioblastoma Xenograft Model

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ABSTRACT

A human medulloblastoma (BN-2) and a glioblastoma (BN-3) which were previously established in nude mice were used to determine the effect of combined modality therapy with γ-radiation, and three chemotherapeutic agents, procarbazine, 1,4-cyclohexadiene-1,4-dicarbamic acid, 2,5-bis(1-aziridinyl)-3,6-dioxo diethylester (AZQ), and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). The tumor cells were grown in tissue culture and implanted intracranially in the right cerebral hemisphere of NIH Swiss nude mice to a depth of 3 mm. The mice were randomized, and treatment was started 3 days after tumor implantation. Procarbazine and AZQ were injected i.p. every 5 days for three treatments. BCNU was injected one time for a single treatment. Radiation was localized to the head. A 60Co unit was used for irradiation at the rate of 125 rads/min 3 days after tumor implantation. Ten experiments were performed using six to nine mice per group and different drug-radiation dose combinations. The drug dose ranged from 400 to 500 mg/kg/injection for procarbazine, 7.5 mg/kg/injection for AZQ, and 10 to 20 mg/kg/injection for BCNU. The radiation dose ranged from 320 to 1050 rads/mouse (whole head). The day of death was recorded for each animal, and the mean of the death times for each group were used to calculate the percentage increase in life span (ILS) compared to the untreated control group. Chemotherapy alone produced a minimal effect, while radiation alone produced minimal effects at 320 to 640 rads and progressively positive effects at 800 and 1050 rads. When the combination treatment of the human medulloblastoma xenograft with procarbazine was used, the ILS was significantly increased in all four experiments, ranging from 25 to 41%, and was superior to single-modality treatment in all but the 1050-rad treatment, where it showed an equal effect. The combination treatment using AZQ and BCNU showed no ILS for the medulloblastoma tumor. Combination treatment of the human glioblastoma xenograft using BCNU produced significant ILSs of 105 and 119% and was superior to single-modality treatment with a drug dose of 10 mg/kg and radiation doses of 540 and 800 rads, respectively. The nude mouse-human tumor xenograft model was found to be useful for combined modality studies and should give valuable information for the experimental design of pilot Phase III clinical studies against a variety of brain tumors.

INTRODUCTION

The objective of combined-modality treatment is improved therapeutic results in terms of local or disseminated cancer disease control. If a positive interaction between 2 agents exists, improved therapy often can be achieved at lower toxicity levels of the individual agents used. Chemotherapeutic drugs and radiation can have a positive interaction on the tumor cell in 3 ways: (a) each agent can have a differential level of effectiveness on different cancer cell populations; (b) the drug can sensitize the cell to radiation damage or, conversely, radiation can enhance drug action; and (c) spatial cooperation exists when drug action kills cells outside the radiation target area (4).

The study of combined-modality treatment of human cancer can be conveniently divided into 2 aspects: (a) study of combined agents on the molecular biology of the human cancer cell; and (b) study of combined agents on the human cancer cell within the activating, transporting, physiological environment of the human body. Many biochemical aspects of combined-agent therapy can be studied outside the physiological environment of the human body. The molecular effect of variables such as dose, time interval of dose fractionation, and time scheduling between agent administration can be conveniently studied in an in vitro or in vivo cell system.

In this study, we investigate the use of the athymic (nude) mouse-human brain tumor xenograft model for evaluation of combined-modality therapy. Nude mice have been well established as animal models to grow and treat human tumor xenografts (13, 14, 17). The advantage of the athymic mouse-human tumor xenograft model is that it permits an in vivo study of the molecular biological aspects of combined-agent therapy on the human cancer cell in a mammalian system which has some of the properties of the human physiological environment.

MATERIALS AND METHODS

Athymic (nude) mice of NIH Swiss background were used in these experiments and were supplied by the Animal Genetics and Production Branch of the National Cancer Institute. All mice were housed in cages held in Bioclean laminar-flow racks. Food, bedding, cages, and drinking water were autoclaved. Water was acidified (pH 2.8).

The tumor cells used in this study were BN-2, a human cerebellar medulloblastoma cell line originally established by McAllister et al. (11) as line TE-671; and BN-3, a human glioblastoma multiforme which was established as line U-251 MG by Pontén (15) and Bigner et al. (3). They were further characterized by Slagel et al. (18) using lactate dehydrogenase isozyme analysis and have maintained the human phenotype during serial heterotransplantation in nude mice. Initial growth and chemotherapy data have been established in one of our
laboratories (7). A tissue culture line was established as a monolayer from the tumor grown in mice. This tumor line was maintained in minimum essential medium plus 10% fetal calf serum. Cells were harvested by scraping and were counted by trypan blue exclusion. Mice were anesthetized with sodium pentobarbital and inoculated with \(1 \times 10^6\) BN-2 cells in 0.025-ml volume in the right cerebral hemisphere, using a 26-gauge needle with a sleeve set for 3-mm penetration. In experiments where the BN-3 tumor was implanted, \(2 \times 10^6\) viable cells were used for inoculation.

Combined-modality treatment was performed with procarbazine (NSC 77213), AZQ (NSC 182686), BCNU (NSC 409962), and \(\gamma\)-radiation using a Picker \(\gamma\)Co unit. Compounds were stored at \(-20^\circ\), and the injection solution was prepared immediately prior to use with sterile 0.9% sodium chloride solution. Dose levels used were 400, 450, and 500 mg/kg/injection for procarbazine, 10 and 20 mg/kg for BCNU, and 7.5 mg/kg for AZQ. Injections of procarbazine were made i.p. 30 mm before irradiation and were given every 5 days thereafter for a total of 3 treatments. Injections of AZQ were made i.p. 30 min before radiation and were given every 5 days thereafter for a total of 3 injections. Single i.p. injections of BCNU were made 40 to 60 min before radiation for combined treatment of BN-2 and 20 to 30 min after radiation for treatment of BN-3. Valium, 25 mg/kg, was used as an anesthetic and was given to all mice. \(\gamma\)-Radiation was localized to the head with lead shielding. At a distance of 80 cm, the dose was 125 rads/min. Levels of 320, 480, 540, 640, 800, 1000, and 1316 rads were given as single doses. Treatments were initiated 3 days after tumor implantation.

Mouse death day was recorded, and mean death day for each of 4 groups (tumor control, chemotherapy, radiation, and combined modality) was calculated. Deaths occurring either at a very early time after tumor implantation or at a late time after the majority of mouse deaths in a group had occurred were tested for exclusion using a modification of Grubb's statistical method for the detection of outliers (20). We considered these deaths to be due to causes other than tumor growth and treatment interaction. The use of this statistic makes the model more sensitive and the analysis of variance more efficient for different treatment modality effects (5).

### RESULTS

#### Treatment of Medulloblastoma Xenograft

Four experiments were performed using procarbazine as the chemotherapeutic agent in combined-modality treatment of the BN-2. The results of these experiments are seen in Table 1. Four drug-radiation dose combinations were used. Drug dose ranged from 400 to 500 mg/kg/injection, and radiation dose ranged from 480 to 1050 rads. Procarbazine alone at these levels had a minimal effect over the control group. Radiation treatment given alone had no effect at 480 rads, minimal effect at 640 rads, and a progressively positive effect at 800 and 1050 rads. Combined treatment of procarbazine given 20 min prior to radiation had a significantly positive effect in all 4 experiments and was superior to either single-modality treatment in all but the 1050-rad radiation treatment, where it showed an equal effect.

The mean death day, standard deviation, probability of a treatment being different from the control using Student's 2-tailed \(t\) test, and percentage of increased life span are summarized in Table 1. The mean death day for the combined modality group is significantly increased over the control group \((p < 0.001)\). Radiation-only treatment at 1050 rads had a significantly increased life span \((p < 0.001)\). AZQ was used in 2 experiments. Drug dose of 7.5 mg/kg/
 Combined therapy of an intracerebral nude mouse-human medulloblastoma (BN-2) xenograft using BCNU and γ-radiation

The mice were implanted with 1 x 10^5 cells intracranially.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BCNU (mg/kg/injection)</th>
<th>Radiation (rads)</th>
<th>No. of mice</th>
<th>Survival time (days)</th>
<th>Increase in life span (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>9</td>
<td>39.9 ± 3.5c</td>
<td>0.02</td>
<td>-12</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>20</td>
<td>8</td>
<td>35.3 ± 3.7</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>480</td>
<td>9</td>
<td>40.0 ± 5.5</td>
<td>0.4</td>
<td>6</td>
</tr>
<tr>
<td>Combined</td>
<td>20</td>
<td>480</td>
<td>41.6 ± 4.8</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>8</td>
<td>26.8 ± 1.7</td>
<td>0.09</td>
<td>11</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>20</td>
<td>8</td>
<td>28.4 ± 3.4</td>
<td>0.0001</td>
<td>32</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>640</td>
<td>8</td>
<td>33.9 ± 4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>20</td>
<td>640</td>
<td>33.0 ± 3.5</td>
<td>0.0001</td>
<td>29</td>
</tr>
</tbody>
</table>

* Treatment was given 3 days after tumor implantation, BCNU was administered by i.p. 40 to 60 min before radiation to the head.

* Percentage of the untreated control mice.

* Mean ± S.D.

Table 3
Combined therapy of an intracerebral nude mouse-human glioblastoma (BN-3) xenograft using BCNU and γ-radiation

The mice were implanted with 2 x 10^5 cells intracranially.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BCNU (mg/kg/injection)</th>
<th>Radiation (rads)</th>
<th>No. of mice</th>
<th>Survival time (days)</th>
<th>Increase in life span (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>8</td>
<td>30.4 ± 3.4c</td>
<td>0.007</td>
<td>32</td>
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<tr>
<td>Chemotherapy</td>
<td>10</td>
<td>8</td>
<td>40.0 ± 7.7</td>
<td>0.22</td>
<td>14</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>540</td>
<td>8</td>
<td>34.5 ± 5.0</td>
<td>0.0001</td>
<td>150</td>
</tr>
<tr>
<td>Combined</td>
<td>10</td>
<td>540</td>
<td>7</td>
<td>76.0 ± 9.3</td>
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</tr>
<tr>
<td>Experiment 2</td>
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</tr>
<tr>
<td>Control</td>
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<td>9</td>
<td>29.7 ± 2.6</td>
<td>0.51</td>
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</tr>
<tr>
<td>Chemotherapy</td>
<td>10</td>
<td>8</td>
<td>32.0 ± 2.5</td>
<td>0.0005</td>
<td>48</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>800</td>
<td>7</td>
<td>43.9 ± 2.6</td>
<td>0.0001</td>
<td>110</td>
</tr>
<tr>
<td>Combined</td>
<td>10</td>
<td>800</td>
<td>9</td>
<td>65.0 ± 13.0</td>
<td></td>
</tr>
</tbody>
</table>

* Treatment was given 3 days after tumor implantation. BCNU was administered by the i.p. route 20 to 30 min after radiation to the head.

* Percentage of the untreated control mice.

* Mean ± S.D.

injection given on a schedule of every 5 days for 3 doses had a minimal effect increasing life span over control. Radiation at 320 rads or at 480 rads, as well as combined treatment of AZQ given 30 min prior to radiation at these levels, had minimal effects (increased life span < 25%).

Two experiments were performed using BCNU as the chemotherapeutic agent (Table 2). The drug dose of 20 mg/kg/injection given as a single injection had no effect as compared to the control. Radiation at 480 rads also had no effect on increased life span. However, radiation at 640 rads had a significant effect on life span. This radiation dose combined with BCNU did not increase the effect.

Treatment of Glioma Xenograft. Two experiments were performed using BCNU as the chemotherapeutic agent in combined modality treatment of the glioblastoma xenograft BN-3. The results of these experiments are seen in Table 3 and in the Kaplan-Meier plots illustrated in Chart 1. The Kaplan-Meier plot, in which the data are shown without outlier exclusion, illustrates the increased survival of the combination group over the radiation-only or chemotherapy-only treatment groups. The effect of combined treatment was increased when compared to a decreasing radiation dose. At a dose of 10 mg/kg/injection given as a single treatment, BCNU had a minimal effect on increased life span over the control group, as seen in Table 3. γ-Radiation was given (whole head) at dose levels at 540 and 800 rads. The dose of 540 rads had little effect, but at 800 rads, radiation had a statistically significant effect and resulted in 48% increased life span. However, when either of these levels of radiation was combined with a 10-mg dose of BCNU per kg given 20 min after radiation, the combined modality groups had a large increase in life span of 110%, which was statistically very significant (p < 0.0001). Since the 800-rad dose also gave a statistically significant increase in life span, we tested whether it was significantly different from the combined modality results using the Student’s t test. The combined-modality group with 800 rads gave a very significantly increased life span (p < 0.0001) over the radiation-only treatment group.

DISCUSSION

Since procarbazine has an aromatic acetone group and is a derivative of methylhydrazine, in common with radiation sensitizers (1, 12), it might be expected to increase tumor cell
Combination Radiation-Drug Therapy Using Nude Mouse Xenografts

Alkylation reaction depends on the reactivity of the cellular DNA with BCNU and the extent to which DNA repair reactions remove the chloroethyl monoadducts. The second slow reaction results in interstrand DNA cross-links which correlate with cytotoxicity. Radiation combined with BCNU at short time intervals between treatments is likely to interact with the initial alkylation reaction. Radiation administered at long (possibly 2 to 6 hr in vitro) treatment intervals is likely to affect the DNA interstrand cross-linking reaction.

In our experiment, radiation was administered 15 to 30 min before BCNU treatment. We suggest that the mechanism of treatment improvement which occurred was related to radiation enhancement of the BCNU alkylation reaction or indirectly by inhibition of the DNA repair reactions. The molecular mechanisms by which radiation can potentiate the alkylation reaction or interstrand cross-link reaction must be investigated before the mechanism of BCNU-radiation therapeutic interaction can be fully understood. The nude mouse-human tumor xenograft model is particularly suited for evaluation of cooperative drug-radiation effects.

ACKNOWLEDGMENTS

We would like to thank Joanne Ries for helpful discussion on the statistics of outlier analysis and Y. Murayama, Chairman of Radiation Medicine, for assistance and use of Picker 6Co unit.

REFERENCES

15. Ponten, J. Neoplastic human glial cell cultures in: J. Fogg (ed.), Human death when combined with radiation. A recent study using Escherichia coli B/r showed that procarbazine enhanced radiosensitization under anaerobic conditions about 1.4 times at subtoxic concentrations of 1 mm (16).

The molecular mechanism by which procarbazine and radiation combine to produce enhanced damage of tumor cell DNA or other biochemical pathways has not been experimentally documented. In fact, little is known about the biochemical basis for the antitumor activity of procarbazine, although it seems clear that an oxidative metabolite of procarbazine is the active compound affecting DNA and protein synthesis (22). This metabolite is probably an alkylating intermediate (6, 21) capable of methylating biopolymers.

Combined studies using BCNU and radiation have been carried out in experimental animals using the intracerebral 9L tumor rat model (2). A significant increase in life span was obtained in tumors treated with the combination of BCNU and X-irradiation. This therapeutic enhancement was critically dependent on the time interval between BCNU and X-ray treatment. After 5 hr, the increase in survival was only 2 or 6 hr before irradiation.

Analysis of the interaction of BCNU and radiation in cell survival experiments with 9L cells suggested that maximum interaction occurred when drug treatment precedes radiation by 5 hr (9). This time will undoubtedly vary with cell populations having different characteristics, i.e., drug transport into the cell nucleus, drug-DNA reactivity, and DNA monoadduct repair capability. In considering the mechanism of enhanced therapeutic interaction between BCNU and radiation at the DNA molecular level, it is important to review the BCNU-DNA alkylation reaction. The initial reaction is rapid and results in DNA base chloroethyl monoadducts. These react slowly, over a period of hours, with a second nucleophilic site to form intrastrand and interstrand cross-links (8, 10). The kinetics of the


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