Radiosensitization of C6 glioma by thymidine and 41.8°C hyperthermia

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The cytotoxic, antiproliferative, and radiosensitizing effects of thymidine (a nucleoside metabolite) were studied using the C6 glioma cell line in vitro. Radiosensitization by a combination of thymidine and 41.8°C hyperthermia was also evaluated. Thymidine concentrations above 100 µg/ml completely inhibited C6 proliferation while concentrations of 100 to 1000 µg/ml (for up to 24 hours) decreased C6 cell survival to as little as 7.4% compared to untreated control cells. Radiosensitivity was enhanced by the administration of thymidine alone (400 µg/ml x 24 hours before irradiation); sensitization by 41.8°C hyperthermia alone (1 hour ending immediately before irradiation) was less pronounced. Thymidine and hyperthermia together produced greater radiosensitization than did heat alone or thymidine alone. These data support the further investigation of thymidine as a neuro-oncology radiosensitizer.

KEY WORDS • brain neoplasm • glioma • thymidine • hyperthermia • radiosensitization

In view of these considerations, thymidine, a nucleoside metabolite, may deserve further attention as a neuro-oncology radiosensitizer. Thymidine blocks repair of radiation injury by inhibiting poly (adenosine diphosphate (ADP)-ribose) polymerase, a deoxyribonucleic acid (DNA) repair enzyme. Thymidine also prevents DNA repair by inhibiting ribonucleotide reductase, a key enzyme regulating deoxyribonucleotide pool balance. During continuous thymidine infusions, cerebrospinal fluid thymidine levels are 29% of the simultaneous serum levels, which are 500 to 1300 µg/ml. In this regard, poly (ADP-ribose) polymerase is inhibited 50% at just 10 µg thymidine/ml. Thymidine lacks neurotoxicity (except for reversible mental status changes at extremely high doses) and high-dose continuous thymidine infusions have been well tolerated clinically for as long as 29 days. Parenthetically, it should also be noted that thymidine itself has significant antiproliferative and tumoricidal efficacy, although these effects have not been studied in neoplasms of glial origin.

The present experiments tested whether clinically achievable thymidine concentrations would have significant antiproliferative and cytotoxic effects in the C6 rat glioma cell line in vitro and whether clinically relevant thymidine concentrations would sensitize C6 cells to ionizing irradiation. As thermal radiosensitization of malignant gliomas is now being pursued in the clinical setting, the possibility was also investigated that hyperthermia and thymidine together would cause greater radiosensitization than when using these sensitizers separately.

Material and Methods

Tumor Cell Preparation

For this study, C6 cells were grown in Ham's F10 medium supplemented with 2.5% fetal bovine serum, 15% donor horse serum, 100 U penicillin/ml, and 100 µg streptomycin/ml. Unless otherwise indicated, “medium” means this mixture.

Single-Agent Thymidine Experiments

Thymidine cytotoxicity was assessed by adding thymidine-containing medium (0 to 1000 µg thymidine/
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ml for 0 to 24 hours) to tissue culture plates containing exponentially growing C6 cells. Cells were then harvested with trypsin, counted, washed twice with fresh drug-free medium, diluted depending on expected survival, and plated in 6-cm diameter tissue culture dishes in a medium containing 5% fetal bovine serum and 15% donor horse serum. After 14 days, colonies (≥ 2 mm) were stained with methylene blue and counted visually.

In proliferation experiments, exponentially growing C6 cells were plated in 2.5-sq cm tissue culture multiwells in 0.5 ml medium/well. The cells were fed daily with thymidine-containing medium and were periodically harvested with trypsin. Cell numbers were determined with the aid of a hemacytometer and the percent of cells excluding trypan blue was determined.

Thymidine, Hyperthermia, and Irradiation Experiments

Tissue culture plates containing exponentially growing C6 cells were drained and re-fed with medium containing 0 or 400 µg thymidine/ml and incubated for 23 hours (5% CO2/95% air). The cells were then harvested (using 0 or 400 µg thymidine/ml media), counted, placed in loosely capped disposable 15-ml centrifuge tubes, and allowed to equilibrate with incubator gases for 15 minutes. Next, the tubes were tightly sealed, placed for 1 hour in continuously shaking waterbaths (37.0° or 41.8°C), removed from the baths, and promptly irradiated with a 137Cs source at 7.60 Gy/min. Finally, the cells were washed twice and plated. Mean plating efficiency was 52.2% ± 7.0% (± standard error of the mean) for all experiments. The temperature of 41.8°C was selected because of strong evidence that 42.0°C appears to be the maximum safe temperature for avoiding irreversible thermal injury of normal brain.

Data Analysis

The experiments were performed in duplicate and repeated twice. Radiation survival curves were analyzed using the single-hit multitarget model.2 5 For the irradiation experiments, test statistics were computed by comparing the difference in estimated terminal slopes to the estimated standard error of the difference. All p values are two-sided.

Results

Figure 1 shows the antiproliferative efficacy of thymidine (0 to 500 µg/ml) against C6 cells. Complete inhibition of C6 proliferation occurred at thymidine concentrations over 100 µg/ml. Figure 2a demonstrates that 24-hour exposure to clinically achievable thymidine concentrations causes significant cytotoxicity in C6 cells. Thymidine cytotoxicity (400 µg/ml) increases with longer thymidine exposures for at least up to 24 hours (Fig. 2b).

Figure 3 shows the effects of thymidine and 41.8°C hyperthermia on the radiosensitivity of exponentially growing C6 cells. Interestingly, heat alone only modestly enhances radiosensitivity in this cell line (p = 0.1028 based on terminal slopes); that is, D0, an inverse measure of the terminal slope, decreases from 2.53 ± 0.43 (curve 1) to 1.84 ± 0.32 Gy (curve 2). Thymidine alone causes more pronounced radiosensitization (D0 = 1.58 ± 0.26 Gy; p = 0.0290 for curve 3 vs. curve 1). Thymidine and hyperthermia together cause greater
radiosensitization \((D_0 = 1.09 \pm 0.18 \text{ Gy}; p < 0.0001\) for curve 4 vs. curve 1) than when only hyperthermia is used \((p = 0.0010\) for curve 4 vs. curve 2) or when only thymidine is used \((p = 0.1180\) for curve 4 vs. curve 3). In contrast to the effects on terminal slopes and \(D_0\), thymidine and/or hyperthermia have little impact on the survival curve shoulder effect (Fig. 3).

**Discussion**

Figures 1 and 2 provide the first evidence that clinically relevant thymidine concentrations have significant antiproliferative and cytotoxic effects against tumors of glial origin. Similar thymidine exposures inactivate many tumors in vitro with preferential injury of neoplastic cells as opposed to normal cells.\(^{10,16,19}\) Continuous thymidine infusions also produce tumor regressions of many human tumor xenografts in nude mice.\(^{11,12}\) To date, Phase II clinical studies of thymidine have only been pursued in patients with lymphoid malignancies\(^{3,8}\) and acute myelogenous leukemia,\(^{3}\) in whom responses have been seen.

The data in Fig. 3 show that clinically relevant thymidine concentrations sensitize C6 cells to irradiation and that radiosensitization is greater when heat and thymidine are both used. The difference between thymidine plus heat and thymidine alone is only significant at the \(p = 0.1180\) level. This statistical result reflects the unexpectedly modest thermal radiosensitization in this cell line. As a result, thymidine radiosensitization is heavily weighted relative to thermal radiosensitization, making it difficult to demonstrate the differences between curves 3 and 4 (Fig. 3).

It is of interest to note that we have also studied the interactions of thymidine and hyperthermia with the chemotherapeutic agent carboplatin using the C6 cell line. In these experiments, enhancement of carboplatin killing by hyperthermia and thymidine together greatly exceed enhanced by hyperthermia alone or thymidine alone (unpublished data). Thus, the use of thymidine as part of a combined modality approach to malignant gliomas could be multifaceted.

It is concluded that clinically relevant thymidine concentrations have antiproliferative and cytotoxic activity in C6 glioma cells. More importantly, as thymidine induces significant radiosensitization in C6 cells, the application of thymidine to glioma radiotherapy deserves further investigation.

**References**

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