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Radiation and Drug Response of the Rat Glioma RG2*

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Abstract—*A clonogenic cell assay was developed for the chemically induced rat glioma RG2 that allows* in vivo, in vitro, *and* in vivo *to* in vitro *studies of cell survival after experimental therapy. RG2 monolayer cells were resistant to BCNU up to high concentrations. The radiation survival curves were characterized by a* D_0 *of* 2.4 gray *and* $n = 2.2$ *for monolayer cells, a* D_0 *of* 3.5 gray *and* $n = 1.3$ *for cells irradiated as brain tumors in air-breathing rats, and a* D_0 *of* 5.9 gray *and* $n = 1.2$ *for cells irradiated as brain tumors in nitrogen-asphyxiated rats. There was no evidence of a radiobiologically hypoxic fraction of cells in the brain tumors, but their radiosensitivity was definitely smaller than that of monolayer cells.*

INTRODUCTION

VARIOUS chemically and virally induced glioma models have been developed [1, 2]. However, for experimental tumor therapy it is essential to quantify tumor cell survival by the colony formation assay (CFA). Only with the 9L rat brain tumor was this method developed and widely used [3]. This tumor is a gliosarcoma, sensitive to BCNU, and has an unconventional radiobiology in contrast to other solid tumors [4–6]. Because most human malignant gliomas are resistant to chemotherapy and their radiobiology is widely unknown, it seems warranted to use another animal brain tumor comparatively or alternatively. We have developed the rat glioma RG2 as an *in vivo* to *in vitro* model, and studied its response to chemo- or radiotherapy.

MATERIALS AND METHODS

Origin of the cell line

Primary brain tumors were induced by a single transplacental exposure of CDF-Fischer rat fetuses to 50 mg/kg of *N*-ethylnitrosourea on the 20th day of gestation [1]. The malignant glioma which appeared 7 months later in a male rat of the offspring was transplanted subcutaneously on syngeneic rats. After the first passage, the tumor was further propagated in cell culture. The cell line was cloned, designated RG2 and has recently been characterized [7]. During the following passages in culture, the cell feature and the growth characteristics did not change significantly.

Culture conditions

Cells of passage Nos 60–100 of the cloned tumor line were used. Initially the cell line was carried in Earle's minimum essential medium (MEM) supplemented with 2% fetal bovine serum (FBS), 5% newborn bovine serum (NBS), glutamine (4 mmol/l) and penicillin–streptomycin. For the experiments presented in this paper, 10% FBS was used as serum component, non-essential amino acids were added and the antibiotics were changed to gentamycin (50 μg/ml) (complete medium; CM). Cells (1×10^6) were seeded in 25-cm^2 tissue culture flasks (Falcon Plastics, Oxnard, CA, U.S.A.). The monolayers were trypsinized with trypsin 0.05%–EDTA 0.02% in PBS (Boehringer, Mannheim, F.R.G.). The trypsinization was stopped after 5 min by the addition of CM. The cell suspensions were counted with a Coulter counter. To assure the absence of mycoplasma, cells were routinely screened using the method of Chen [8].

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Treatment in culture

Cell monolayers were treated 48 hr after seeding during the exponential phase of growth. Dilutions of BCNU ('CARMUBRIS', Bristol Arzneimittel, Bergisch Gladbach, F.R.G.) were made in 1 ml of MEM which was added to 4 ml of CM in the culture flasks to achieve the final dilution. Cells were maintained at 35–37°C, pH 7.2–7.4. After a 2-hr exposure, the drug-containing medium was decanted, and the monolayers were washed twice with MEM and trypsinized.

The cell monolayers were irradiated at room temperature with a Siemens-Stabilipan 200 kV X-ray machine, operated at 20 mA, filtration 0.5 mm Cu and 1 mm Al, FSD 42 cm, and dose rate 0.73 gray/min. The monolayers were trypsinized within 10–15 min after irradiation.

Colony formation assay

The procedure of the assay has been described for another cell line [9]. Briefly, single-cell suspensions were plated in 60-mm Petri dishes (Greiner, Solingen, F.R.G.) for colony formation.

In selected experiments, the medium in the Petri dishes was supplemented with varying concentrations of FBS. Varying numbers of heavily irradiated (40 gray) autologous feeder cells were added and the plates were incubated for varying periods of time in order to optimize the assay.

All treatment was done after the assay had been optimized, and treated and control cells were plated in medium containing 10% FBS and 5×10^4 irradiated feeder cells. The time of incubation was 10–12 days in a humidified 95% air–5% CO_2 environment at 35–37°C and pH 7.2–7.4. After the incubation the plates were stained with crystal-violet; groups of more than 50 cells were counted as colonies. The colony-forming efficiency (CFE) was calculated as the ratio of the number of colonies observed to the number of cells plated. The surviving fraction (SF) of cells was calculated as the ratio of the CFE of treated cells to the CFE of untreated cells.

Brain tumor transplantation

Three to four-month-old inbred male CDF-Fischer rats (Ch. River, Wilmington, MA, U.S.A.) were anesthetized with i.m. injections of 0.08 ml of 'HYPONORM' (fentanyl dihydrogencitrate and fluanison, Janssen Pharmaceutics, Beerse, Belgium) and placed in a stereotactic head holder (Kopf, Tujunga, CA, U.S.A.). The scalp was opened and a hole was drilled in the skull at a point 3 mm to the right of the midline and 3 mm posterior to the coronar suture. Cells (1×10^5) trypsinized from exponentially growing monolayers were injected in a volume of 0.015 ml into the region of the basal ganglia at a depth of 6 mm beyond the skull through a 28-gauge needle attached to the syringe of a microinjection unit. The hole and the scalp were closed with 'HISTO-ACRYL' (Braun-Dexon, Melsungen, F.R.G.).

With 7 animals the onset of tumor symptoms was observed and the survival time was measured. After death the brains were removed and studied microscopically. With the other rats radiation experiments were done.

Irradiation of brain tumors in vivo *and evaluation of treatment*

Nine days after tumor implantation, unanesthetized air-breathing rats fixed in small boxes, or nitrogen gas-asphyxiated rates were placed under the Siemens-Stabilipan X-ray machine described above. Single doses of X-rays were administered as total-body irradiation. For each set of experiments, sham-irradiated air-breathing or nitrogen-asphyxiated tumor-bearing rats were used as controls. The air-breathing rats were killed in ether 10–15 min after the irradiation. The brain tumors were aseptically excised, placed into Petri dishes, weighed, minced into fine fragments and trypsinized with 10 ml of trypsin 0.25% in PBS (Boehringer, Mannheim, F.R.G.) under continuous agitation with a magnetic stirrer at 35–37°C for 15 min. The cell suspensions were decanted through a 80-μm stainless steel mash, CM was added and the cells were counted under phase-contrast microscopy. Thereafter, the cells were seeded into Petri dishes at several dilutions for the CFA under the conditions described above.

Analysis of the radiation survival data

The survival curves and their parameters were calculated by a linear least-squares regression analysis of the logarithmic means of the surviving fraction of cells from individual brain tumors or monolayers in the exponential portion of the curves. The regression equation for all lines had a correlation coefficient of 0.9 or greater.

RESULTS

Cell growth in culture

After seeding of cells in culture flasks, proliferation started after approximately 6 hr (Fig. 1). Unfed cell monolayers showed exponential growth up to 72 hr, with a cell doubling time of approximately 24 hr. When cell density ap-

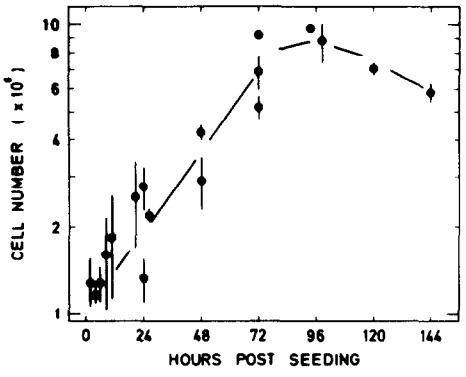

Fig. 1. Growth curve of unfed RG2 cell monolayers after seeding of 1×10^6 *cells in 25-cm² tissue culture flasks. Each point represents the mean number (± S.D.) of cells harvested from 3 culture flasks.*

proached 9×10^6 cells a short plateau occurred. Thereafter, the growth curve declined due to cell detachment and deterioration.

Optimization of the CFA

With 2% FBS in the medium of the Petri dishes the CFE was low (Fig. 2A). The addition of 5% NBS did not enhance the CFE (data not shown). With 5–15% FBS a CFE of approximately 30% could be achieved, while larger concentrations of FBS decreased the CFE. A further increase of the CFE could be achieved by the addition of heavily irradiated autologous feeder cells to the dishes. When 1×10^4–1×10^5 feeder cells were added, the CFEs were 60–80% (Fig. 2B). Higher feeder cell numbers again decreased the CFE.

The optimal time of incubation was 10–12 days, when the colonies of untreated cells reached diameters of 1–3 mm (Fig. 2C). After 7 days the CFE was equally high, but the colonies were rather small. With longer times of incubation the CFE decreased because the colonies deteriorated rapidly. Under optimized conditions with 10% FBS, 5×10^4 feeder cells and 10–12 days of incubation, the number of colonies was linearly related to the number of seeded cells within a range of 10–160 cells.

Brain tumor growth in vivo, *and* in vivo *to* in vitro

Seven tumor-bearing rats showed diminished motor activity, pareses and focal or generalized seizures, with a median interval of 12 (range 9–14) days after implantation of the tumor cells. The median survival time was 13 (range 11–15) days. Histological examination of the brains revealed large tumors located in the right basal ganglia which showed anaplastic tumor cells with frequent mitoses, giant cells, scarcely distributed blood vessels, focal

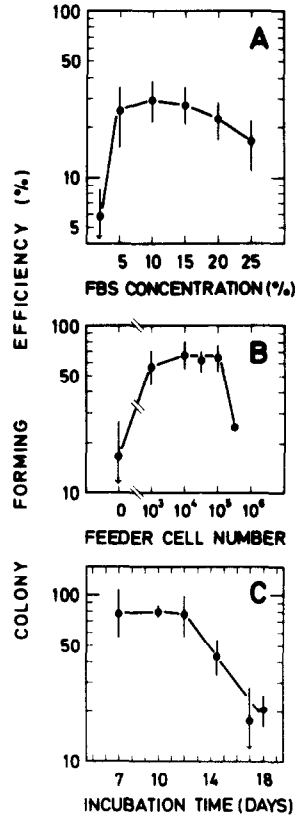

Fig. 2. CFE of RG2 cells as a function of the concentration of FBS (A), the number of feeder cells with 10% FBS (B) and the incubation time of the plates with 10% FBS and 5×10^4 *feeder cells (C). Each point represents the mean CFE (± S.D.) analysed from 16 Petri dishes in 2 separated experiments.*

necroses and infiltrative growth into the adjacent brain. The tumors were free of reticulin or collagen fibers.

The mean tumor weight for 10 untreated tumors which were excised 9 days after implantation was 24.5 (S.D. ± 8.4) mg. After trypsinization a mean cell yield of 2.6 (S.D. ± 0.9) × 10^5 cells per mg tissue was measured. When plated into Petri dishes the mean CFE of cells disaggregated from these tumors was 6.0 (S.D. ± 2.8)%. The conditions to achieve a high CFE were the same as for cells derived from monolayer cultures.

None of the parameters described above differed between animals killed with ether or by nitrogen gas asphyxiation.

Chemotherapy

The survival of RG2 monolayer cells was not affected up to a concentration of 10 μg/ml of BCNU for 2 hr (Fig. 3). SF was 0.845 (S.D. ± 0.030) at 20 μg/ml and decreased to 0.018 (S.D. ± 0.004) at 50 μg/ml.

Radiotherapy

The survival curve of cells irradiated *in vitro* showed a small shoulder region at doses be-

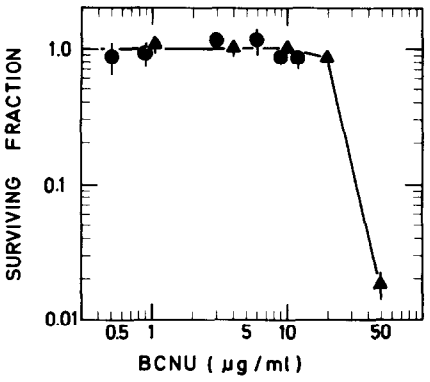

Fig. 3. Survival of RG2 monolayer cells after exposure to various concentrations of BCNU for 2 hr. *Each symbol represents the mean SF (± S.D.) analysed from* 3–10 *Petri dishes. Three separate experiments were done.*

tween 0.5 and 3 gray (Fig. 4, curve C). Thereafter, an exponential-type survival curve was observed which gave a D_0 of 2.4 gray and $n = 2.1$.

The slope of the survival curve of cells irradiated *in situ* in air-breathing rats was less steep than that of cells irradiated in culture: the D_0 was 3.5 gray and $n = 1.3$ (Fig. 4, curve A). The survival curve had no break up to a dose of 28 gray, and it never paralleled the curve for cells irradiated in nitrogen-asphyxiated rats with a D_0 of 5.9 gray and $n = 1.2$ (Fig. 4, curve N).

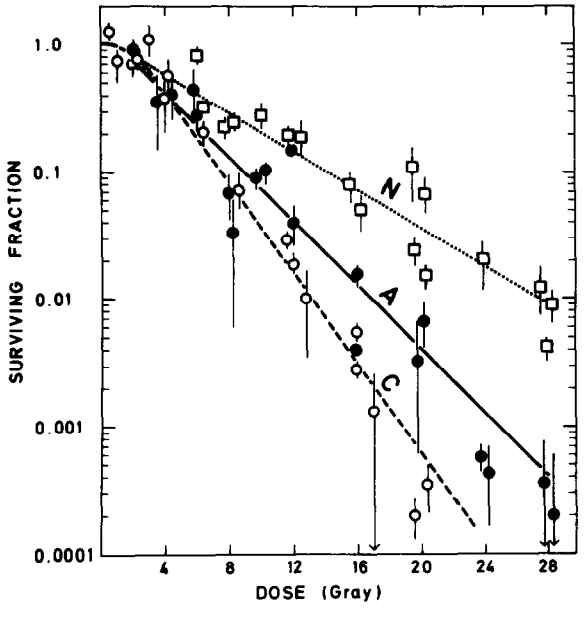

Fig. 4. Survival of RG2 cells after X-irradiation. Tumor cells were treated as monolayers (curve C; O–O), *as brain tumors in air-breathing rats (curve A;* ●—●) *or as brain tumors in nitrogen-asphyxiated rats (curve N;* □–□). *Each point represents an individual cell monolayer or tumor and each symbol gives the mean SF (± S.D.) analysed from* 4–15 *Petri dishes. The straight portion of the curves was fitted by a linear least-squares regression analysis of the logarithmic means of the points plotted at each dose.*

DISCUSSION

Animal models in part simulate the biology of human brain tumors and may provide a stepwise understanding of the partial resistance of human malignant gliomas to radiation or chemotherapy [10, 11]. The chemical-induced RG2 rat brain tumor is an anaplastic glioma; in contrast to the 9L gliosarcoma of the rat and many other malignant tumors of rodents, it has no sarcomatous features. The tumor is suited for studies of experimental therapy because cell survival can be quantified by the CFA. The CFE of untreated cultured RG2 cells is 60–80% if the culture conditions are optimized for medium constituents, feeder cells and incubation time. After implantation of 1×10^5 cells into the brains of syngeneic rats, tumors grow within 9 days and, if excised aseptically and trypsinized, cells from these tumors form colonies with a CFE of 6%. Thereby, the rat glioma RG2 is the only other brain tumor model next to the 9L tumor that includes the *in vivo* to *in vitro* system [3, 12].

In contrast to the 9L tumor, which is sensitive to BCNU and spirohydantoin-mustard [4, 13], RG2 cells are resistant to BCNU up to high concentrations and the model may be suitable for studying the potential resistance of brain tumors to nitrosoureas which has recently been addressed [14].

The radiosensitivity of RG2 cells irradiated as brain tumors in air-breathing rats (D_0 = 3.5 gray) was smaller than that of RG2 cells irradiated in culture ($D_0 = 2.4$ gray) but larger than that of RG2 cells irradiated as brain tumors in nitrogen-asphyxiated rats (D_0 = 5.9 gray). It has been speculated that due to fluctuations in tumor blood flow the mean oxygen tension in solid tumors may be between fully oxygenated and fully hypoxic, and the radiation response may then be intermediate [6]. However, with most solid tumors cell hypoxia is radiobiologically reflected in a break of the exponential part of the X-ray survival curve, indicating a *fraction* of radioresistant cells [15].

The radiation cell survival curves of both the intracranial 9L and RG2 tumors do not have such a break. Either *all* tumor cells are marginally hypoxic, as outlined above, or cell hypoxia is radiobiologically irrelevant with these tumors of glial origin: as an hypothesis, glial tumor cells may die from chronic hypoxia before levels of oxygen depletion are reached which produce substantial radioresistance.

Recently, the early recurrence of human glioblastomas after radiotherapy was linked to hypoxic cell radioresistance, and chemical

'radiosensitizers' were applied [10]. However, the survival and clonogenicity of chronically hypoxic cells have never been proved, neither for animal nor human glial tumors. The unconventional radiobiology of the two animal models is conspicuous, suggesting the absence of hypoxic cell radioresistance in tumors of glial origin. Therefore, both animal models may ultimately contribute to clarify whether the use of 'radiosensitizers' is of any benefit for the treatment of human malignant gliomas.

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