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Radiation Studies on Multicellular Tumour Spheroids Derived From Human Neuroblastoma: Absence of Sparing Effect of Dose Fractionation

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Abstract—In vitro *experiments were carried out to compare the effects of single-dose and split-dose irradiation on a cell line (NB1-G) derived from human neuroblastoma and grown as multicellular tumour spheroids (MTS). The radiation response was evaluated in terms of regrowth delay; estimates of* in situ *cell survival were made by back-extrapolation of regrowth curves. These studies showed no significant difference in the effectiveness of single as compared to split dose irradiation i.e. no sparing effect of fractionation. If MTS constitute a realistic model for micrometastases* in vivo, *these results provide a radiobiological rationale for hyperfractionated treatment regimes in the adjuvant radiotherapy of neuroblastoma.*

INTRODUCTION

MULTICELLULAR tumour spheroids (MTS) constitute an *in vitro* model for human cancer which has several novel features. In particular, it has been proposed that MTS resemble, and may provide a realistic model for micrometastases during the avascular phase of their development [1]. In this paper, studies are reported of the response to radiation of MTS derived from human neuroblastoma. This neoplasm is commonly radioresponsive but prone to early dissemination [2] and relapse with chemoresistant disease. Total Body Irradiation with bone marrow rescue, (following surgery and chemotherapy) seems a rational approach to radical therapy [3]. In the design of treatment regimes, knowledge of the radiobiological properties of neuroblastoma micrometastases (or an appropriate model such as MTS) may be of value. In this paper, consideration is given to the relative effectiveness of single-dose as compared to split-dose irradiation in sterilizing neuroblastoma cells. It has been suggested that the well-known sparing effect of fractionation in the irradiation of normal tissues, especially late-responding normal tissues, may provide a therapeutic advantage, if tumours are less likely to be spared [4–6]. Only a small number of studies have been reported [7–9] on the sparing effect of fractionation in the radiation response of human tumours or appropriate models. The present paper seeks to address this question in the case of human neuroblastoma.

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MATERIALS AND METHODS

Cell line

The cell line, (NB1-G) was obtained by growth in monolayer culture of cells released by enzymatic disaggregation of a human tumour xenograft implanted in athymic nude mice. The xenograft originated from tumour fragments obtained by surgical excision of a stage IV abdominal neuroblastoma in a 2-year-old boy. NB1-G cells in culture synthesize catecholamines, have neurosecretory granules visualizable by electron microscopy and an aberrant but identifiably human karyotype. *In situ* DNA hybridization studies have revealed the presence of multiple copies (~20–24) of the human oncogene N-myc.

Spheroid culture

Cell suspensions were obtained by trypsinization of monolayer culture and used to provide cells for initiation of MTS cultures using the 'agar underlay' method [10]. 10^6 cells were used per 25 cm^2 flask base-coated with 1% Noble agar, containing 5 ml medium (MEM) incorporating 15% foetal calf serum. The flasks were incubated in a humid atmosphere with 7% CO_2 content at 37°C. Small spheroids could be harvested individually using a

Pasteur pipette within a few days. Spheroids (diameter ~250 μM) were selected and transferred to individual agar-coated wells of 24 well test plates (Linbro). The plates were then incubated as above with weekly addition of fresh medium (0.5 ml).

Determination of spheroid growth curves

The growth of MTS in test plate wells was quantified by thrice weekly measurement of cross-sectional area of individual MTS using an automated scanning system similar to that described by Twentyman [11]. The cross-sectional area measurements were subsequently transformed to estimates of volume assuming spherical geometry.

Spheroid growth curves were obtained by taking the medium volume for the MTS in each experimental group on each day of measurement.

Irradiation procedures

All MTS were individually transferred to wells of test plates prior to irradiation. The irradiations were carried out using a 4 MeV 'Dynaray' linear accelerator, at ~200 cGy/min with 1.3 cm Perspex 'build up' to ensure maximum deposition of energy within each well. For single dose irradiation, 50–350 cGy were given. For split dose irradiation, doses in the range 25–175 cGy were given on each of two occasions; at the time of the single dose irradiations and 6–7 hr thereafter. Control plates were removed from the incubator at times of irradiation but were not irradiated.

Estimation of growth delay

With median growth curves constructed, the growth delay for MTS in each experimental group was derived graphically as the time for median volume to reach 10 × the original volume.

RESULTS

Spheroid growth curves

Figure 1 shows growth curves for control and irradiated MTS constructed as described above. Control growth curves follow a characteristically exponential form (volume doubling time ~ 2–3 days) until a diameter of ~800–1000 μM, after which growth slows progressively. Growth curves for irradiated MTS displayed a static or regression phase before resumption of growth with growth curves becoming parallel to those of controls. The lateral displacement of irradiated from control curves is seen to increase progressively with dose. Curves were constructed of growth delay vs. total radiation dose for both single-dose and split-dose radiation groups and are shown together in Fig. 2. As may be seen, there is no significant difference between these two curves, indicating no sparing effect of dose fractionation as evaluated in terms of growth delay.

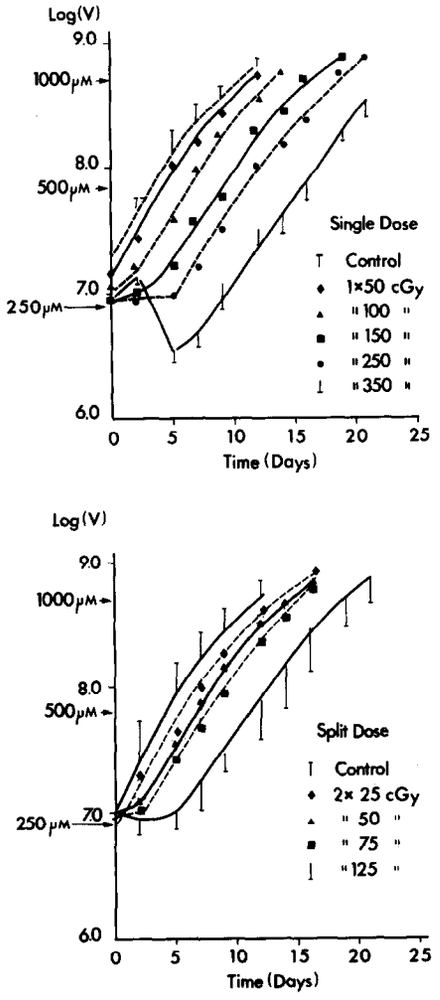

Fig. 1. Growth curves for neuroblastoma MTS irradiated with (a) *single doses* (b) *split doses of* 4 MeV *X-rays. The Y-axis represents median spheroid volume, in cubic microns, on a logarithmic scale to base* 10. *The volumes which on this scale correspond to spheroid diameters of* 250, 500 *and* 1000 μM *are also shown.*

Estimation of cell survival in situ

The theoretical assumptions underlying the analysis of spheroid regrowth curves to yield estimates of cell survival *in situ* have been presented previously [12]. Regrowth curves may theoretically

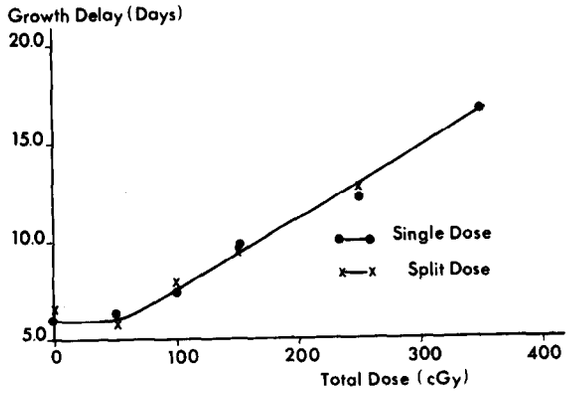

Fig. 2. Regrowth delay (time to reach 10 × *original volume) as a function of total dose for neuroblastoma MTS subjected to single or split dose X-irradiation.*

allow estimation of cell survival *in situ* provided growth of surviving cells continues as in the unirradiated case [13]. With this assumption, regrowth curves may be interpreted as simple displacements of the control growth curve, the displacement providing a measure of the magnitude of cell kill assumed to have taken place. The surviving fraction (S) for each experimental group may then be estimated as the ratio of the 'effective volume' V_E on day zero after irradiation obtained as the Y-axis intercept of the back-extrapolated regrowth curve, to the 'initial volume' (V_0) as measured immediately before irradiation (see Fig. 3 for additional explanation). Cell survival data estimated as described are shown in Fig. 4. The data from the single and split dose experiments can be fitted by a single survival curve; the multitarget parameters for the best-fitting equation are: $D_0 \sim 90$ cGy, $D_Q \sim 45$ cGy, $n \sim 1.6$.

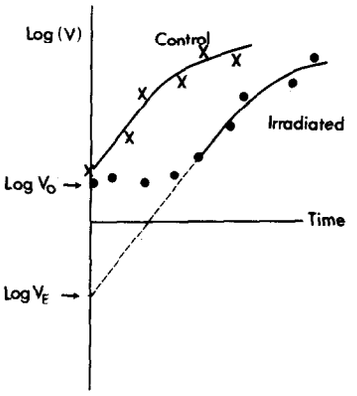

Fig. 3. General principle of estimation of cell surviving fraction from spheroid regrowth curves. Provided the regrowth curve becomes parallel to the control growth curve the effect of treatment may be interpreted as due to cell kill leading to lateral displacement of the regrowth curve from the control. Extrapolation of the regrowth curve to zero time yields an estimate (V_E) *of the 'effective volume' from which the spheroid appears to have regrown* i.e. *it represents the volume of viable cells following treatment. The ratio* V_E *to the measured volume* V_0 *(representing the volume of all cells initially present) provides a measure of the cellular surviving fraction* (S) *immediately following treatment.*

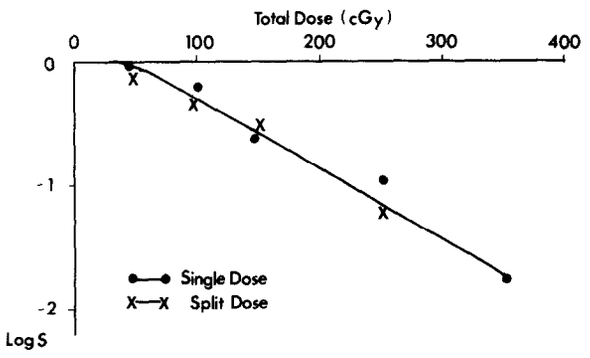

Fig. 4. The cellular surviving fraction (S), *as estimated from spheroid regrowth curves, plotted log-linearly against total dose for neuroblastoma MTS subjected to single or split dose X-irradiation.*

DISCUSSION

Division of a radiation dose in the range 150–350 cGy into two equal fractions spaced 6 hr apart makes little difference to the response of a line of tumour spheroids derived from a human neuroblastoma xenograft (Figs. 1 and 2). The spheroids irradiated by the split doses appear more sensitive than those exposed to single doses. This result might be explained in terms of cellular redistribution throughout the mitotic cycle but is within experimental uncertainties in the data. No evidence has been found for any substantial interfraction repair capacity of these MTS. Several reports have now appeared on the single-dose radiosensitivity of human neuroblastoma cells in monolayer culture [14–17] or as MTS [12, 17, 18]. They reveal a significant heterogeneity in the *in vitro* radiosensitivity of human neuroblastoma cells (with D_0 values ranging from 66 to 155 cGy). Characteristically, however, neuroblastoma cells appear to have single-dose survival curves with little or no initial shoulder. Data for NB1-G spheroids indicate a radiosensitivity in the middle of the range reported for other cell lines and with only a small shoulder apparent on the single-dose survival curve. The split-dose data are consistent with negligible capacity for the accumulation of sublethal damage. Several workers have proposed that a systematic difference may exist in the repair capacities of tumour cells and target cells in normal tissues and that a therapeutic advantage might result from the use of small doses per fraction (hyperfractionation) by which normal tissues should be preferentially spared [4–6]. However, human tumour cells have recently been found to differ significantly in their radiobiological properties *in vitro*, especially in the low dose region [19, 20]. It is likely therefore that tumour cell-normal tissue differences will not be the same in all cases and it will be important to determine directly the interfraction repair capacities for particular tumour types.

In the present paper, no significant repair capacity has been found for human neuroblastoma MTS subjected to one or two doses of radiation in the total dose range 50–350 cGy. The contribution of factors such as redistribution has not been assessed, and experiments with larger numbers of fractions will be necessary to confirm the absence of the sparing effect of fractionation in a clinically relevant context. However, the present findings may be taken as providing a radiobiological rationale for the use of hyperfractionated treatment schedules in the adjuvant radiotherapy of neuroblastoma for which the target micrometastases may have similar radiobiological properties to MTS *in vitro*.

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