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Radiation Response of Two Human Malignant Melanomas Grown in Athymic Nude Mice

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Abstract—*The radiation response of two human malignant melanomas (V.N. and G.E.) irradiated as solid tumours in athymic nude mice was studied by measuring single cell survival in soft agar. Survival curves were determined for cells from tumours irradiated either in air-breathing mice or in mice killed by cervical dislocation* 15 min *before irradiation. The hypoxic fractions, determined from the vertical displacement of these two curves, were* 12–17% *for V.N. melanoma and* 26–36% *for G.E. melanoma. The* D_0*-values were* 3.43 Gy (*air-breathing mice*) *and* 3.05 Gy (*dead mice*) *for V.N. melanoma and* 3.00 Gy (*air-breathing mice*) *and* 3.35 Gy (*dead mice*) *for G.E. melanoma. Survival curves of cells irradiated in dead mice showed* $n=4.6$ *and* $D_q=4.6$ Gy *for V.N. melanoma and* $n=2.0$ *and* $D_q=2.2$ Gy *for G.E. melanoma.*

INTRODUCTION

MALIGNANT melanomas are generally considered to be resistant to radiation [1–3]. The biological basis of this resistance is not well understood. The explanation of the radioresistance has mainly been sought in two fields:

(1) Survival curves of melanoma cells irradiated under aerobic conditions *in vitro* are often wide-shouldered, i.e. characterized by high extrapolation numbers and high 'quasithreshold doses' [4–7]. Hornsey [8, 9] suggested that the poor response of melanomas to conventional fractionated radiotherapy (30 × 2.0 Gy) might be explained by these wide-shouldered survival curves. Clinical investigations have indicated that the radiotherapy of melanomas may be improved by the use of fractionation regimes employing high individual dose fractions [3, 10].

(2) The radioresistance may also be explained by factors other than the shoulder of the survival curves of melanoma cells. Particularly, tumour cell hypoxia may be of great importance [11]. Guichard *et al.* [12] reported an extremely high hypoxic fraction of 85% in a human melanoma grown in nude mice. On the other hand, Rofstad [13] found only 5–10% in another human melanoma grown in nude mice.

The purpose of the present work was to study the radiation response of two human malignant melanomas, grown and irradiated as xenografts in athymic nude mice [14, 15]. The radiation response was measured as single cell survival in soft agar.

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MATERIALS AND METHODS

Animals

Six- to eight-weeks old BALB/c/nu/nu/Bom and NMRI/nu/nu/Han mice of both sexes were used in the experiments. The animals were kept in a special room at 27°C with automatically regulated 12-hr light and dark periods, under conventional but strict conditions.

Tumour material

V.N. malignant melanoma was taken from a subcutaneous metastasis on the right side of the abdomen of a 29-year-old woman in 1978. Histologically, the tumour was composed of solid trabecules and nests of relatively large cells with hyperchromatic vesicular nuclei surrounded by partly abundant eosinophilic cytoplasm. Areas with more spindle-shaped cells were also found. The cytoplasm contained

little or no melanin. Numerous mitoses were found. The patient had received several preoperative treatments with DTIC and CCNU.

G.E. malignant melanoma was taken from a recurrent (following two previous surgical resections) tumour on the left lower leg of a 51-year-old woman in 1977. Histologically, the tumour was composed of densely packed cells with atypically hyperchromatic nuclei surrounded by varying amounts of cytoplasm. The cytoplasm contained little or no melanin. Abundant necrosis and numerous mitoses were found.

Transplantation method

Immediately after surgery, the tumour tissue was cut into pieces approximately 2 × 2 × 2 mm in size and implanted subcutaneously on the back of the animals. This method was also used for serial transplantation. Passages 13–18 of V.N. melanoma and 16–20 of G.E. melanoma were used in the experiments described in this paper. The tumours started growing 1–2 weeks after implantation and reached a size suitable for experiments (7–10 mm in diameter) 3–4 weeks after implantation. The tumours were surrounded by a layer of connective tissue and did not show invasive growth. The tumours were histologically indistinguishable from the original tumour material.

Irradiation procedure

The irradiation procedure is described in detail elsewhere [16], and only a shortened description is therefore given here. The tumours were irradiated locally at a source–skin distance of 80 cm by applying a tangential radiation field from a 5000 Ci ^{60}Co therapy unit (TEM, Mobaltron 80). The mice were immobilized in a holder and partly immersed in a water phantom, whereby the tumours were positioned under water and at a distance from the outer surface of the phantom wall greater than that corresponding to the ionization maximum of the radiation used. The mouse-holders and the phantom wall were made of Perspex. The temperature of the water was kept at 30°C. Under these conditions, the uncertainty in the absolute dose-rate at the ionization maximum was less than 3% and the variation in dose within each tumour was less than 10%.

Tumours were irradiated either in (a) air-breathing, non-anaesthetized mice whereby normal oxygenation of the tumour cells was assumed, or in (b) mice killed by cervical dislocation 15 min before irradiation, whereby all tumour cells were assumed to be hypoxic during irradiation. The tumours were removed from the mice for assay in soft agar within 10 min after irradiation.

Preparation of single cell suspensions

Single cell suspensions were prepared from the tumours without the use of enzymes. The tumours were finely minced in culture medium [Ham's F12 medium with 20% foetal calf serum, 250 mg/l penicillin and 50 mg/l streptomycin (Gibco-Biocult, Glasgow)] using a scalpel and a pair of tweezers. The resulting suspensions were filtered through 30 μm filters (Nytal, Schweizerische Seidengazefabrik AG). Unstained cells were counted in a hemocytometer viewed under a phase contrast microscope. Cells having an intact and smooth outline with a bright halo were counted as viable. The cell suspensions were diluted to appropriate concentrations in culture medium. Heavily irradiated (HR) cells were obtained by giving a cell suspension 300 Gy X-rays at a dose rate of 25.2 Gy/min.

Soft agar colony assay

The soft agar technique used for measurement of single cell survival of irradiated tumours is based on that reported by Courtenay [17] and Courtenay and Mills [18]. It is described in detail elsewhere [13] and a shortened explanation suffices therefore here.

A 5% agar solution was made by boiling powdered agar (Bacto agar, Difco) with bidistilled water for 10 min and autoclaving at 120°C for 20 min. The solution was then cooled to 44°C in a water bath and diluted 1:10 in culture medium.

Blood was taken from August rats by heart puncture. After centrifugation, the serum and the buffy coat were removed. The red blood cells (RBC) were rinsed three times with phosphate buffered saline, resuspended in culture medium to the original blood volume and stored at 4°C for up to 3 weeks. The RBC suspension was heated to 44°C for 1 hr to destroy remaining nucleated cells [17, 18] and diluted 1:2 in culture medium immediately before adding to the agar.

Soft agar was made by mixing 6 vol of 0.5% agar solution, 3 vol of tumour cell suspension and 1 vol of RBC suspension (or 6 vol of 0.5% agar solution, 2 vol of tumour cell suspension, 1 vol of HR cell suspension and 1 vol of RBC suspension). Immediately after mixing, 1 ml volumes of soft agar were seeded in glass tubes, six tubes for each tumour cell suspension. The tubes were then put into crushed ice for the agar to set, flushed with a

gas mixture of 5% O_2, 5% CO_2 and 90% N_2 and sealed. The flushing was repeated 2 hr later. The tubes were incubated at 37°C for 4–5 weeks (V.N. melanoma) or 5–6 weeks (G.E. melanoma). Tumour cells given large doses of radiation were incubated for a longer time than unirradiated cells and cells given small doses of radiation. Culture medium (2 ml) was added to the tubes 5 days after seeding and changed weekly. The tubes were flushed with the gas mixture described above after each medium change and additionally once between each medium change.

After incubation, the agar lumps were placed on a sectioned glass plate with a 2 mm high rim and carefully squeezed out with a glass cover. Colonies were counted using a stereomicroscope. Cells giving rise to colonies consisting of more than 40 cells were counted as viable.

RESULTS

A reliable colony assay for measuring single cell survival requires constant plating efficiency (PE) as a function of the number of cells seeded. Unirradiated cells from V.N. melanoma showed no significant variation in PE when seeded in concentrations from 200 to 2000 cells per tube. Cells seeded in concentrations of 5000 and 10,000 cells per tube showed significantly lower PEs than cells seeded in concentrations of 200–2000 cells per tube (Fig. 1a). Unirradiated cells from G.E. melanoma showed no significant variation in PE when seeded in concentrations from 200 to 10,000 cells per tube (Fig. 1b).

In some control experiments, HR cells were added to the soft agar to examine their influence upon the PE. Addition of HR cells to secure a large excess of radiation-inactivated cells in all samples in dose–survival experiments can be omitted only if HR cells do not affect PE of unirradiated cells. Addition of 10^3–10^5 HR cells per tube did not affect the PE of unirradiated cells from either V.N. (Fig. 1c) or G.E. (Fig. 1d) melanomas. Thus, HR cells were not added to the soft agar in the present dose–survival experiments.

The number of cells seeded and the number of colonies obtained in the dose–survival experiments were kept within the ranges of concentration independence as shown in Fig. 1. PE of unirradiated cells in different experiments was 17–81% for V.N. melanoma and 6–27% for G.E. melanoma. Figure 2 shows survival curves of cells from V.N. (panel a) and G.E. (panel b) melanomas irradiated as solid tumours in nude mice. Each point represents the colony-forming fraction of cells from an irradiated tumour relative to that of an unirradiated one. The tumours were either irradiated in air-breathing mice (open circles) or in dead mice (closed triangles). By the method of least squares, exponential curves were fitted to the data in the range 7.5–20 Gy for cells from tumours irradiated in air-breathing mice, and in the range 10–25 Gy for

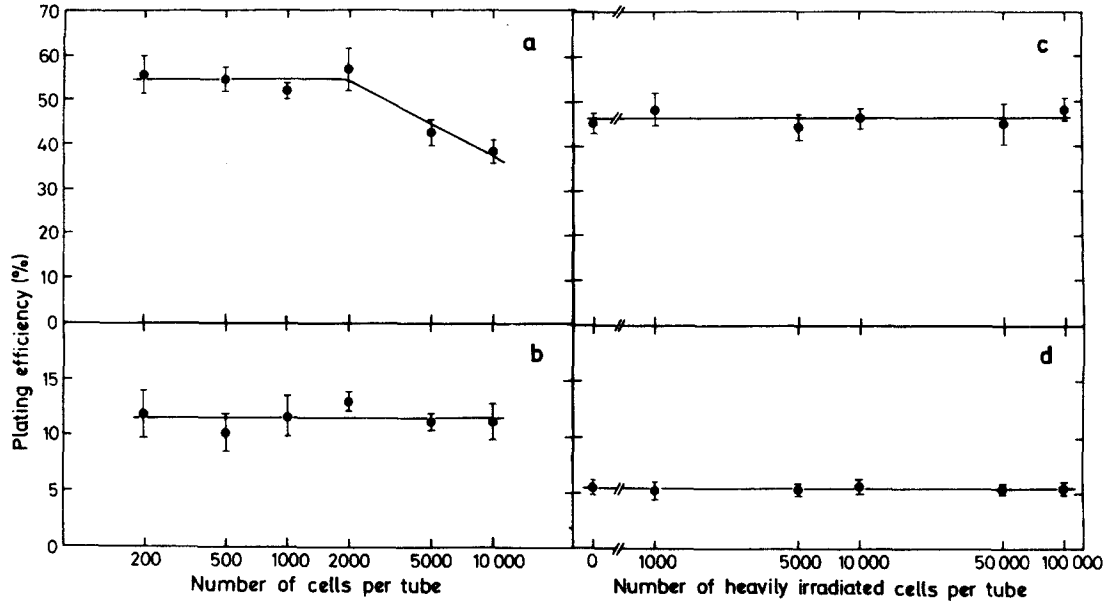

Fig. 1. Plating efficiency in soft agar for cells from V.N. (a *and* c) *and G.E.* (b *and* d) *malignant melanomas as a function of the number of unirradiated cells seeded per tube* (a *and* b) *and the number of HR cells seeded per tube in addition to* 500 *unirradiated cells* (c *and* d). *The points and the vertical bars represent the mean values and the standard deviations calculated from the number of colonies in six tubes.*

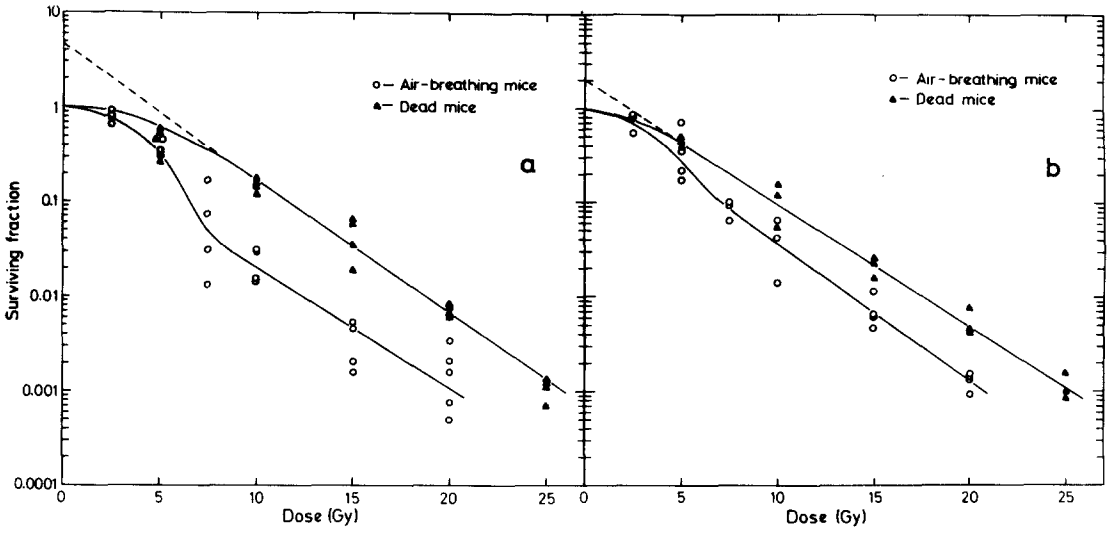

Fig. 2. Survival curves of cells from V.N. (a) *and G.E.* (b) *malignant melanomas irradiated as solid tumours in nude mice. The tumours were irradiated either in air-breathing mice* (○) *or in dead mice* (▲). *Each point represents the surviving fraction of cells from one irradiated tumour, calculated from the mean number of colonies in six tubes with cells from this tumour and six tubes with cells from an unirradiated tumour.*

cells from tumours irradiated in dead mice. Parameters derived from the exponential parts of the curves (D_0, n and D_q) are given in Table 1.

The D_0-values of the survival curves of cells from tumours irradiated in air-breathing and in dead mice were not significantly different, either for V.N. or for G.E. melanoma. Assuming equal response to radiation for acute and chronically hypoxic cells, the hypoxic fractions were determined from the vertical displacement of these two curves to be 12–17% for V.N. melanoma and 26–36% for G.E. melanoma.

DISCUSSION

Guichard *et al.* [12] reported a hypoxic fraction of 85% and Rofstad [13] one of 5–10% in human malignant melanomas grown in nude mice. The two human melanomas studied in the present work exhibited hypoxic fractions of 12–17% and 26–36%. The present values, together with the one determined by Rofstad [13], indicate that human melanomas do not always exhibit extremely high fractions of hypoxic cells. In addition, Hill and Stanley [19] determined the hypoxic fraction of the B16 melanoma to be 12%. This value is in the lower range of hypoxic fractions of murine experimental tumours as listed by Kallman [20]. The results mentioned above indicate that the magnitude of the hypoxic fraction probably does not alone give the explanation of the radioresistance of melanomas.

There are, however, some uncertainties inherent in the experimental determination of hypoxic fractions. First, if either hypoxic or well oxygenated subgroups of cells are selected by the preparation of cell suspensions or by the survival assay, the hypoxic fractions determined will be artificially high or low. Second, because the vascular network of tu-

Table 1. Parameters (mean values and standard errors) defining the exponential portions of survival curves of cells from V.N. and G.E. malignant melanomas irradiated as solid tumours in nude mice

Tumours	D_0 (Gy)	n	D_q (Gy)
V.N. malignant melanoma			
Air-breathing mice	3.43±0.50	0.37±0.27	
Dead mice	3.05±0.15	4.6±1.6	4.6±0.9
G.E. malignant melanoma			
Air-breathing mice	3.00±0.22	1.0±0.4	
Dead mice	3.35±0.21	2.0±0.7	2.2±1.0

mour transplants is derived from the host [21], a human tumour xenograft in nude mice does not necessarily attain the same oxygen supply and hence exhibit the same fraction of hypoxic cells as the tumour in the patient. However, as referred to above, human melanomas grown at the same site in nude mice show a very wide range of hypoxic fractions. This indicates that the hypoxic fractions are not determined by the host alone, but also, at least in part, by the parenchymal melanoma cells.

D_0-values of survival curves of cells from V.N. and G.E. human melanomas irradiated as solid tumours in air-breathing and in dead mice ranged from 3.00 to 3.43 Gy. Corresponding D_0-values determined by Rofstad [13] and Guichard *et al.* [12] for human melanomas ranged from 2.27 to 3.38 Gy. These values refer to hypoxic tumour cells. D_0-values of survival curves of malignant and non-malignant mammalian cells irradiated under aerobic conditions *in vitro* are mostly found to be in the range 1.0–2.0 Gy [22]. If oxygen is assumed to be a dose-modifying agent with an o.e.r. of approximately 3.0, the corresponding hypoxic D_0-values would be from 3.0 to 6.0 Gy. The D_0-values of survival curves of cells from human melanomas irradiated as solid tumours in nude mice referred to above are in the lower part of this range. Thus, these D_0-values do not provide an explanation of the radioresistance of melanomas. A justification of this *in vivo–in vitro* comparison is provided by Rofstad [13], who showed that D_0-values of survival curves of human melanoma cells irradiated either as solid tumours in nude mice or in suspension under extremely hypoxic conditions were not significantly different. However, McNally *et al.* [23] have determined the D_0-value of the survival curve of cells from a murine fibrosarcoma irradiated under hypoxic conditions *in vitro* to be 2.4 Gy, while that of the same cells irradiated as solid tumours *in vivo* and assayed *in vitro* was 4.0 Gy.

Values of n and D_q of survival curves of melanoma cells irradiated under aerobic conditions *in vitro* fall within wide ranges; D_q varies from 0.94 to 4.61 Gy, while n varies from 1.7 to 40 [4–7, 12, 13, 24–26]. The D_q-values of survival curves of cells from V.N. and G.E. melanomas irradiated in dead mice should, if oxygen is assumed to be a dose modifying agent, be divided by the o.e.r. to be comparable with those referred to above. If the o.e.r. is assumed to be 3.0, the 'aerobic' D_q-values can be estimated to be 1.5 Gy for V.N. melanoma and 0.7 Gy for G.E. melanoma. The values of n and D_q for V.N. melanoma are not among the highest for melanoma cells, but they fall well within the ranges referred to above. The values of n and D_q for G.E. melanoma show that the curve is not purely exponential, but the values are in the lower part of or even below the ranges referred to above.

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