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Radiation and Heat Sensitivity of Cells from Human Melanoma Xenografts. Lack of Correlations with Tumour Growth Parameters*

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Abstract—*Five human malignant melanomas grown in athymic nude mice were studied. Tumour volume-doubling times were determined from Gompertzian growth curves, vascular volumes from stereological analysis of 2-μm thick tumour sections and DNA histograms by flow cytometric analysis. Single-cell suspensions prepared from the tumours were exposed to radiation or heat (42.5°C; pH 7.4) under aerobic conditions in vitro and the colony-forming ability of the cells was assayed in soft agar. Tumours with short volume-doubling times tended to show higher fractions of cells in S-phase and higher vascular volumes than those with long volume-doubling times. The radiation and the heat sensitivity of the melanoma cells, i.e. the D_0-values, were probably not positively correlated with the tumour volume-doubling time, the fraction of cells in S-phase or the vascular volume, or with each other either. The variation in radiation and heat sensitivity among cells from the different melanomas appears not to be due to external factors, but reflects, rather, intrinsic cellular differences.*

INTRODUCTION

THE GROWTH characteristics as well as the response to therapy of human tumour xenografts in the athymic nude mouse have been extensively studied at our institute. The studies have been concentrated on five different malignant melanomas with similar histological appearance. These melanomas showed individual, characteristic volume-doubling times at a given tumour volume [1]. DNA histograms, obtained with flow cytometry, showed that the fraction of cells in the different phases of the cell cycle varied significantly among the different melanomas [1]. The five melanomas also showed individual, characteristic vascular volumes, ranging from 0.9 to 2.2 per cent of the histologically intact tumour volume [2]. Cells from the melanomas treated under aerobic conditions *in vitro* showed survival curves with D_0-values varying with a factor of about two when exposed to X-rays [3] and with a factor of about thirty when exposed to hyperthermia (42.5°C) at pH 7.4 [4]. The major results from these studies and a short description of the experimental procedures used are presented in the present communication.

The main purpose with the work was to analyse whether there is a positive correlation between the different growth parameters of the melanomas, and between these growth parameters and the sensitivity of the cells to radiation and hyperthermia. The correlation between the radiation and the heat sensitivity of the cells is also analysed.

MATERIALS AND METHODS

Tumours

Five different human melanomas (E.E., E.F., G.E., M.F., V.N.), derived from metastases of patients at The Norwegian Radium Hospital, were used in this work. The melanomas were transplanted into nude mice (BALB/c/nu/nu/BOM) without adaptation to *in vitro* culture conditions. Histologically the tumours were similar. Both cells and nuclei varied greatly in size and shape.

The tumours were grown serially in nude mice (BALB/c/nu/nu/BOM and NMRI/nu/nu/Han)

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by implanting fragments approximately 2 × 2 × 2 mm in size subcutaneously into the flanks of recipient mice. Passages 20–40 of the tumours were used in the present work. The tumour volumes were about 200 mm^3 when the experiments were carried out. Light- and electron-microscopic examinations showed that the serially transplanted tumours retained very nearly the morphology of the parent tumours. However, the volume-doubling times of the transplanted tumours were shorter than those of most tumours in man, probably mainly due to a lower cell loss factor [1], indicating that they might to some extent be different from the original tumours in the patients. The growth rate of the G.E. melanoma increased abruptly during the passages 12–17 to a new, constant level. All experiments on the G.E. melanoma reported in the present work were carried out after the growth rate was stabilized at the new level.

Growth

Tumour growth was followed by measuring two perpendicular diameters. The tumour volumes were calculated as:

$$V = \pi \cdot (\text{mean diameter})^3/6.$$

Gompertz curves were fitted to the volumetric growth data and the tumour volume-doubling times at $V = 200\ mm^3$ were calculated. Details have been reported elsewhere [1].

DNA histograms were obtained by flow cytometric analyses of single-cell suspensions prepared from the tumours. Cell debris and injured cells were separated from intact cells in the suspensions by centrifugation on a 15% metrizamide cushion. The cells were stained in a mithramycin solution [5] and the 457-nm line of a 4-W argon laser was used for excitation of mithramycin fluorescence, which was detected at wavelengths longer than 515 nm. The flow cytometer [6] and the experimental procedure [7] have been described in detail elsewhere.

Vascularization

The vascular system of the tumours was filled with a contrast medium [100 ml 0.9% saline, 5 g gelatin, 50 g Pb_3O_4, 1 ml detergent (Joy/Salo), 5 000 U heparin]. Histological sections 2 μm thick were prepared from the tumours. Due to the contrast medium, the vessels appeared in the sections as dark circles or ellipses, depending on whether they were cut at a right or an oblique angle. The vascular volume of the tumours was obtained by stereological analysis of the sections. The sections were examined at a magnification of ×400 by the use of a projecting light microscope and a counting frame 20 × 20 cm in size. The vessel profiles which were projected within the counting frame were counted and classified with respect to vessel diameter. Five diameter classes, 5–15 μm, 15–25 μm, 25–35 μm, 35–45 μm and larger than 45 μm, were used. The vascular volumes were calculated from these measurements. The experimental procedure and the mathematics involved have been described in detail previously [2].

Irradiation and heating

Immediately before treatment, single-cell suspensions were prepared by mechanical dispersion of the tumours in culture medium [Ham's F12 medium with 20% foetal calf serum, 250 mg/l penicillin and 50 mg/l streptomycin (Gibco-Biocult, Glasgow)]. The cell concentration was determined by the use of a haemocytometer viewed through a microscope with phase-contrast optics. Cells having an intact and smooth outline with a bright halo were counted as viable. Further details have been reported elsewhere [3, 4].

A Siemens 'Stabilipan' X-ray unit, operated at 220 kV, 19–20 mA and with 0.5 mm Cu filtration, was used for irradiation. The cell suspensions, which were irradiated under aerobic conditions at a dose-rate of 2.0 Gy/min, were kept in glass Carrel flasks during exposure, as previously described [3].

The single-cell suspensions were kept during heating in glass tubes with ground glass stoppers. Stop-cock grease was applied in the joints to make the tubes gas-tight. Immediately before heating, the tubes were flushed (5% CO_2 in air) and carefully sealed. The pH of the cell suspensions was 7.4. The tubes were immersed in a water-bath thermostatically kept at 42.5°C. The cell suspensions reached the temperature in the water-bath within a few minutes. Further details of the procedure have been reported previously [4].

In vitro *colony assay*

The colony-forming ability of the cells was assayed in soft agar. The soft agar was prepared from powdered agar (Bacto agar, Difco), suspensions of melanoma cells and red blood cells from August rats according to the procedure described previously [8]. Aliquots of 1 ml of the soft agar were seeded in glass tubes. Immediately afterwards, the tubes were flushed with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2 and carefully sealed. The tubes were incubated at 37°C for 3–6 weeks and colonies containing more than 40 cells were counted. Experiments have shown that the number of colonies with more than 40 cells cannot be enhanced by increasing the incubation times beyond those used here. The plating efficiencies were 5–15% (G.E.), 15–40% (E.E.,

M.F., V.N.) and 50–80% (E.F.). Details of the procedure have been reported elsewhere [8].

RESULTS

The growth parameters of the melanoma xenografts are summarized in Table 1. The tumour volume-doubling times were determined from Gompertz growth curves based on the mean volume of 8–20 individual tumours [1]. The fractions of cells in S-phase were determined by analysis of DNA histograms of 5–8 individual tumours [1], while the vascular volumes were based on measurements on 4–6 individual tumours [2]. Since vessels were observed almost exclusively in areas with histologically intact tissue and very seldom in necrotic areas [2], the vascular volumes per unit histologically intact tumour volume are presented in Table 1.

In Fig. 1 the tumour volume-doubling time is plotted vs the fraction of cells in S-phase (panel a) and vs the vascular volume per unit histologically intact tumour volume (panel b). The melanomas with short volume-doubling times tend to show higher fractions of cells in S-phase and higher vascular volumes than those with long volume-doubling times. However, statistically significant correlations were not achieved with the five melanomas studied ($P = 0.1$ in both panel a and b).

Table 1. Growth parameters (mean values and standard errors) of human melanoma xenografts

Melanoma	Tumour volume-doubling time (days)	Fraction of cells in S-phase (%)	Vascular volume (%)
E.E.	4.4	21 ± 2	1.5 ± 0.2
E.F.	21.6	13 ± 2	0.9 ± 0.1
G.E.	4.2	16 ± 2	2.1 ± 0.1
M.F.	20.0	14 ± 2	1.5 ± 0.1
V.N.	6.2	17 ± 2	2.2 ± 0.2

The parameters of the survival curves for cells from the melanomas exposed to radiation or hyperthermia (42.5°C; pH 7.4) under aerobic conditions *in vitro* are presented in Table 2. The survival curves have been presented and discussed in detail previously [3, 4].

Figure 2 shows the radiation sensitivity and Fig. 3 the heat sensitivity, i.e. the D_0-values from Table 2, as a function of the growth parameters presented in Table 1. Since the D_0-values for cells exposed to hyperthermia varied with a factor of about thirty they were plotted on a logarithmic scale. Statistical analysis of the data showed that the radiation and the heat sensitivity are probably not positively correlated with the tumour volume-doubling time ($P = 0.4$ and 0.5 respectively), the fraction of cells in S-phase ($P = 0.5$ and 0.3 respectively) or the vascular volume per unit histologically intact tumour volume ($P = 0.2$ and 0.6 respectively). The radiation sensitivity is probably not positively correlated with the heat sensitivity either ($P = 0.5$; Fig. 4).

DISCUSSION

Tumour growth

The melanomas with short volume-doubling times tended to show higher vascular volumes per unit of histologically intact tumour volume than those with long volume-doubling times (Fig. 1b).

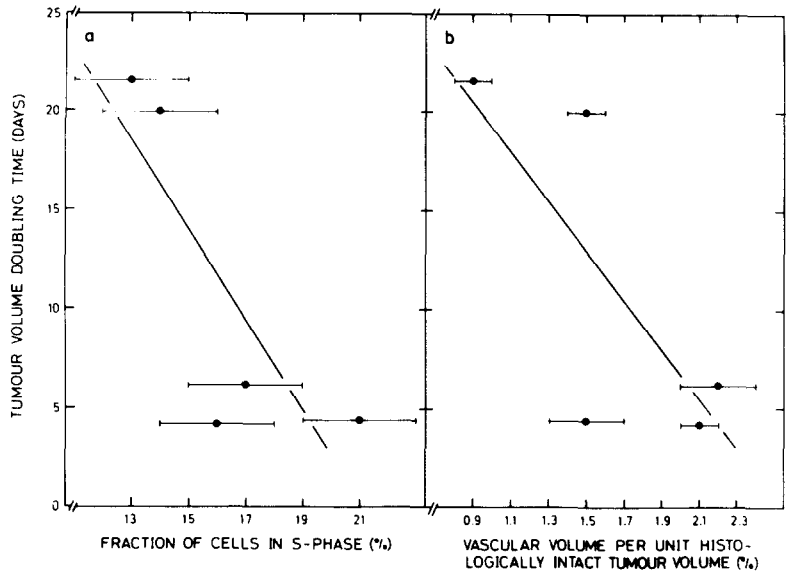

Fig. 1. Tumour volume-doubling time vs (a) fraction of cells in S-phase and (b) vascular volume per unit histologically intact tumour volume for human melanoma xenografts. The solid curves were determined by linear regression analysis. The correlation coefficients were (a) 0.76 *and (b)* 0.81.

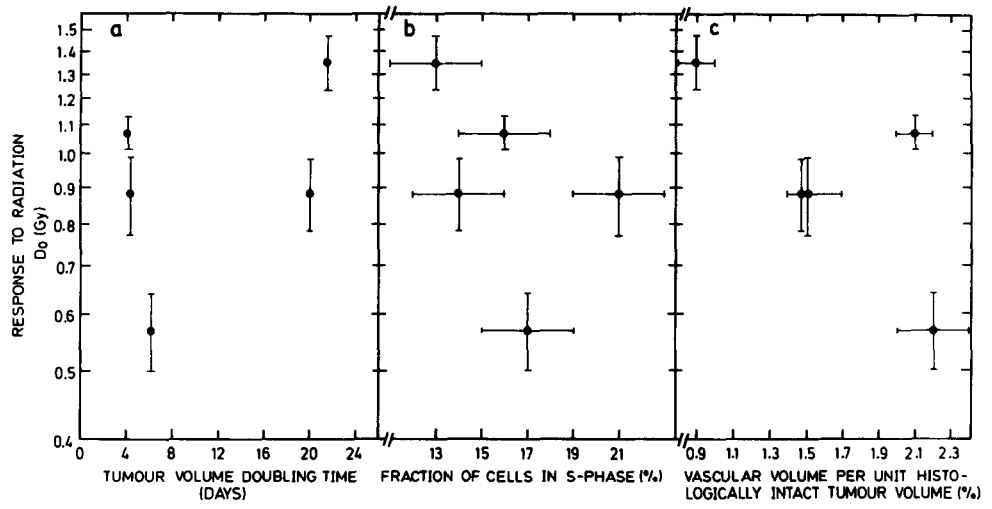

Fig. 2. Radiation sensitivity of cells from human melanoma xenografts vs (a) tumour volume-doubling time, (b) fraction of cells in S-phase and (c) vascular volume per unit histologically intact tumour volume.

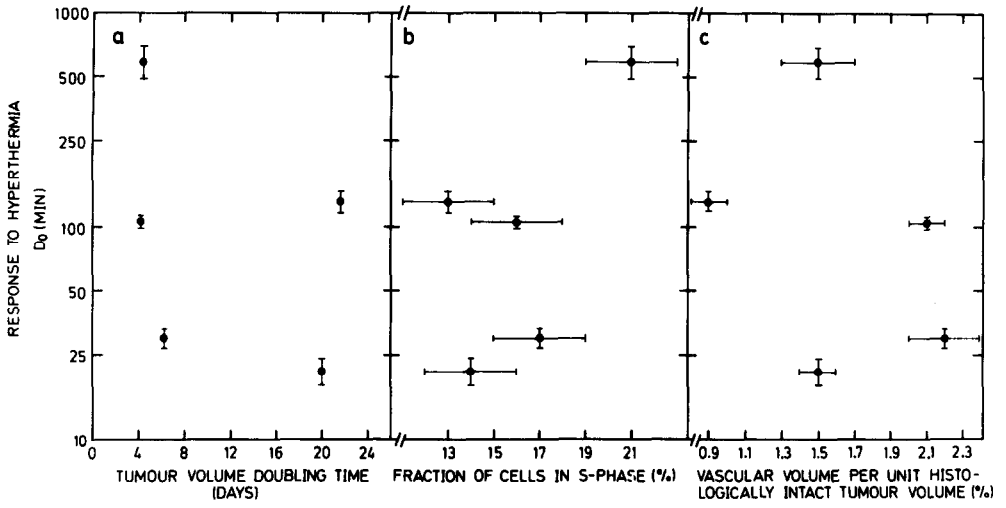

Fig. 3. Heat sensitivity of cells from human melanoma xenografts vs (a) tumour volume-doubling time, (b) fraction of cells in S-phase and (c) vascular volume per unit histologically intact tumour volume.

Table 2. Parameters (mean values and standard errors) defining the exponential portion of survival curves of cells from human melanoma xenografts exposed to radiation and hyperthermia (42.5°C; *pH* 7.4) *under aerobic conditions* in vitro

Melanoma	Radiation D_0 (Gy)	Radiation n	Hyperthermia D_0 (min)	Hyperthermia n
E.E.	0.88 ± 0.11	21^{+36}_{-13}	590 ± 100	1.0*
E.F.	1.35 ± 0.12	$3.8^{+2.0}_{-1.4}$	131 ± 14	1.0*
G.E.	1.07 ± 0.06	$1.4^{+0.4}_{-0.3}$	105 ± 6	1.0*
M.F.	0.88 ± 0.10	18^{+22}_{-10}	21 ± 3	$7.6^{+7.6}_{-3.8}$
V.N.	0.57 ± 0.07	78^{+126}_{-48}	30 ± 3	$6.6^{+2.9}_{-2.0}$

*Exponential curves forced through the origin were fitted to the experimental data.

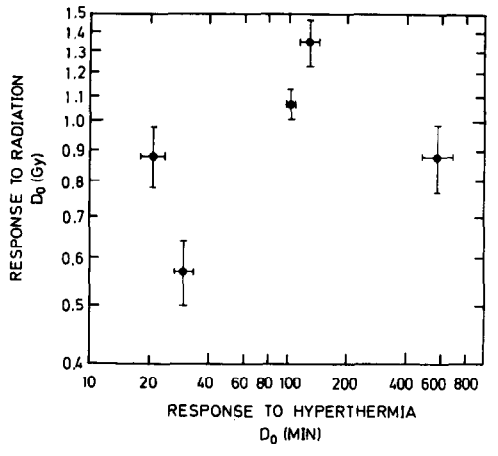

Fig. 4. Radiation sensitivity vs heat sensitivity of cells from human melanoma xenografts.

Previous calculations, based on the duration of the cell-cycle phases of human melanoma xenografts and on the relation between the tumour volume-doubling time and the fraction of cells in S-phase (Fig. 1a), indicated that the melanomas with short volume-doubling times have both higher growth fractions and lower cell-loss factors than those with long volume-doubling times [1]. Poor vascularization may thus result in low growth fractions and high cell-loss factors of the melanomas. Consequently, the rate of volume growth of the melanomas may, at least in part, be limited by the ability of the vascular system to supply the tumours with oxygen and nutrients.

Response to radiation and hyperthermia

A large number of experimental and clinical investigations has indicated that hyperthermia used in combination with radiation may be useful in the treatment of cancer [9]. A main concern is that radioresistant tumours may also be resistant to hyperthermia, and that only radiosensitive tumours may respond well to hyperthermia. Studies of experimental animal tumours have thus indicated that tumour cure by hyperthermia alone can only be obtained with particularly radiosensitive tumours [10]. However, a positive correlation between the radiation and the heat sensitivity *in vitro* of the present melanoma cells was not found (Fig. 4). Similar observations have also been made from studies of cells of established lines [11, 12]. Thus if the response of tumours *in situ* to radiation and to hyperthermia is positively correlated, this would seem likely to be a consequence of extracellular factors.

The radiation (Fig. 2a) and the heat (Fig. 3a) sensitivity of the tumour cells appeared not to correlate positively with the tumour volume-doubling time. This does not mean that the therapeutic response *in situ* of melanomas cannot be correlated with the tumour volume-doubling time. In fact, there is some evidence that the radiation response of human tumours may be related to the rate of growth prior to treatment. Breur [13] studied lung metastases in patients with different histopathological types of cancer and showed that the tumour shrinkage following radiotherapy was largest in the most rapidly growing metastases. Also, Tubiana *et al.* [14], based on a review of the literature, have suggested that rapidly growing tumour types may respond better to radiotherapy than slowly growing ones. The latter observations may be due to lower fractions of hypoxic cells and/or more efficient reoxygenation rather than higher cellular radiosensitivity in the rapidly than in the slowly growing tumours.

In vitro studies of synchronized cell populations have shown that cells are often resistant to radiation [15] but sensitive to heat [16–18] when treated in the S-phase. For the present melanomas the D_0-values for cells exposed to radiation did not increase (Fig. 2b) and the D_0-values for cells exposed to hyperthermia did not decrease (Fig. 3b) with increasing fraction of cells in S-phase. Thus the variation in radiation and heat sensitivity among cells from the different melanomas cannot be attributed to differences in the distribution of cells in the cell-cycle. Consequently, the radiation and the heat sensitivity of cells of malignant melanomas can probably not be predicted from DNA histograms or labelling index data.

Studies of cells in culture have shown that the radiation and the heat sensitivity of cells may depend on the supply of nutrients [19, 20]. The supply of nutrients to cells in solid tumours may be related to the vascular volume of the tumours. However, the radiation and the heat sensitivity of the present melanoma cells appeared not to be positively correlated with the vascular volume (Figs. 2c and 3c). Consequently, it is not likely that the variation in radiation and heat sensitivity among cells from the different melanomas was due to variations in nutritional supply either.

In conclusion, the present study indicated that the radiation and the heat sensitivity of the melanoma cells were not positively correlated with the tumour volume-doubling time, the fraction of cells in S-phase or the vascular volume, and not with each other either. Consequently, the observed differences in the radiation and the heat sensitivity of the cells were probably not due to external factors, but were, rather, a consequence of intrinsic differences among the cells.

REFERENCES

1. ROFSTAD EK, FODSTAD Ø, LINDMO T. Growth characteristics of human melanoma xenografts. *Cell Tissue Kinet* 1982, **15**, 545–554.
2. SOLESVIK OV, ROFSTAD EK, BRUSTAD T. Vascular structure of five human malignant melanomas grown in athymic nude mice. *Br J Cancer* 1982, **46**, 557–567.
3. ROFSTAD EK, BRUSTAD T. Radiation response *in vitro* of cells from five human malignant melanoma xenografts. *Int J Radiat Biol* 1981, **40**, 677–680.
4. ROFSTAD EK, BRUSTAD T. Effect of hyperthermia on human melanoma cells heated either as solid tumors in athymic nude mice or *in vitro*. *Cancer* 1982, **50**, 1304–1308.

5. CRISSMAN HA, TOBEY RA. Cell cycle analysis in 20 minutes. *Science* 1974, **184**, 1297–1298.
6. LINDMO T, STEEN HB. Flow cytometric measurement of the polarization of fluorescence from intracellular fluorescein in mammalian cells. *Biophys J* 1977, **18**, 173–187.
7. ROFSTAD EK, LINDMO T, BRUSTAD T. Effect of single dose irradiation on the proliferation kinetics in a human malignant melanoma in athymic nude mice. *Acta Radiol Oncol* 1980, **19**, 261–269.
8. ROFSTAD EK. Radiation response of the cells of a human malignant melanoma xenograft. Effect of hypoxic cell radiosensitizers. *Radiat Res* 1981, **87**, 670–683.
9. FIELD SB, BLEEHEN NM. Hyperthermia in the treatment of cancer. *Cancer Treat Rev* 1979, **6**, 63–94.
10. SUIT HD. Hyperthermic effects on animal tissues. *Radiology* 1977, **123**, 483–487.
11. GERWECK LE, BURLETT P. The lack of correlation between heat and radiation sensitivity in mammalian cells. *Int J Radiat Oncol Biol Phys* 1978, **4**, 283–285.
12. RAAPHORST GP, ROMANO SL, MITCHELL JB, BEDFORD JS, DEWEY WC. Intrinsic differences in heat and/or X-ray sensitivity of seven mammalian cell lines cultured and treated under identical conditions. *Cancer Res* 1979, **39**, 396–401.
13. BREUR K. Growth rate and radiosensitivity of human tumours. *Eur J Cancer* 1966, **2**, 173–188.
14. TUBIANA M, RICHARD JM, MALAISE E. Kinetics of tumor growth and of cell proliferation in U.R.D.T. cancers: therapeutic implications. *Laryngoscope* 1975, **85**, 1039–1052.
15. SINCLAIR WK. Cyclic X-ray responses in mammalian cells *in vitro*. *Radiat Res* 1968, **33**, 620–643.
16. WESTRA A, DEWEY WC. Variation in sensitivity to heat shock during the cell cycle of Chinese hamster cells *in vitro*. *Int J Radiat Biol* 1971, **19**, 467–477.
17. KIM SH, KIM JH, HAHN EW. The enhanced killing of irradiated HeLa cells in synchronous culture by hyperthermia. *Radiat Res* 1976, **66**, 337–345.
18. BHUYAN BK, DAY KJ, EDGERTON CE, OGUNBASE O. Sensitivity of different cell lines and of different phases in the cell cycle to hyperthermia. *Cancer Res* 1977, **37**, 3780–3784.
19. RAAPHORST GP, AZZAM EI. Dependence of heat and X-ray sensitivity of V 79 cells on growth media and various serum combinations. *Int J Radiat Biol* 1980, **38**, 677–683.
20. HAHN GM. Metabolic aspects of the role of hyperthermia in mammalian cell inactivation and their possible relevance to cancer treatment. *Cancer Res* 1974, **34**, 3117–3123.