

Tumour-Cord Parameters in Two Rat Hepatomas that Differ in Their Radiobiological Oxygenation Status

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Summary. Tumour cords have been examined quantitatively in two rat hepatomas, 3924A and H-4-II-E, that differ in their radiobiological oxygenation status (oxygen enhancement ratio for growth delay [tumour clamped: tumor 'in air'] was 1.35 for 3924A and only 1.08 for H-4-II-E). The average thickness of tumour cords in 3924A was 118 μm and only 69 μm in H-4-II-E. The migration rates across the cords of the two tumours were approximately the same (1.7 and 1.4 $\mu\text{m} \cdot \text{h}^{-1}$) but for any given distance from the subtending blood vessel, the proportion of histologically-dead cells within the cord was always higher for H-4-II-E. Volume for volume, H-4-II-E contained four times as much vascular space as 3924A but it is suggested that the poor *quality* of this vasculature in H-4-II-E contributed to its relative radioresistance.

Introduction

The Morris hepatoma H-4-II-E has been reported to be resistant to the action of ionising radiation [9] and to several cytotoxic drugs of different therapeutic classes [7]. Such a broad spectrum of resistance is unusual. Radiation resistance in tumours occurs often through lack of oxygen, while *one* component of resistance to cytotoxic drugs is failure of the cytotoxic moiety to reach all cells, through spatial considerations [11]. The histological feature known as the 'tumour cord' has been proposed as one possible site for both forms of resistance. The neoplastic cells of a number of human and animal tumours grow as cylindrical cords or cuffs that separate capillaries from areas of gross necrosis. Thomlinson and Gray [16] showed for a series of human lung tumours, that the thickness of tumour cords correlated well with the expected diffusion length of oxygen out from the subtending capillary. The cells most remote from the blood vessel have been considered as candidates for the source of hypoxic radioresistance that can be demonstrated by radiobiological assay, e.g., delay in growth of the tumour [15]. We describe here the characteristics of cords in

hepatoma H-4-II-E and compare them with those of 3924A, another rapidly-growing Morris hepatoma in the same strain of rat, but which has a quite different radiobiological response.

Materials and Methods

Animals and Tumours

Female rats of the inbred ACI strain (Laboratory Supply Co., Indianapolis, IN) were used at a weight of 120–140 g. The rats were caged individually in a room lighted from 08:00 to 20:00 h and were provided with rat chow and water *ad libitum*.

Fragments of 3924A were implanted by trocar injection and cell suspensions of H-4-II-E by needle injection, into the subcutis of the dorsal flank of the rats. Tumour size was measured daily using calipers. For the radiobiological studies, tumours were treated at an average volume of 200 to 300 mm³. For cord analysis, tumours were examined at 1,000 to 15,000 mm³.

Radiation

Tumours were irradiated with 250 kV X-rays filtered by 0.5 mm of Cu and 1 mm of Al. Graded, single doses were delivered at a dose-rate of 2.80 Gy · min⁻¹. The body of the ether-anaesthetised host was shielded by lead. The air-breathing animals were irradiated either with the tumour lying freely in the beam path, or with the tumour blood-supply occluded by a 'D'-shaped clamp 10 min before irradiation (longer times were not found to confer any additional protection). Eight to 10 rats were irradiated at each dose-point.

Experimental

(i) Tumour growth delay. The time taken by tumours in untreated and treated rats to reach a size eight times the treatment volume (8 Vo), was measured. For individual tumours, values of two parameters were calculated: (a) absolute growth delay, i.e., time to 8 Vo (treated) minus time to 8 Vo (controls; i.e., anaesthetised, clamped and sham-irradiated), and (b) relative growth delay, i.e., time to 8 Vo (treated) divided by time to 8 Vo (controls). (ii) Morphometry. The relative volumes of different histological compartments were measured by the method of Chalkley [2]. Five hundred 'strikes' were registered at random in each of six untreated tumours. (iii) Cord analysis. All tumours were unirradiated. Rats were injected intraperitoneally with 50 µCi of tritiated thymidine (³H]-TdR, sp. act. 3 Ci mM⁻¹; Schwarz-Mann, Orangeburg, NY). For each tumour line, a group of six animals was killed 1 h after injection. Groups of two animals were killed at increasing intervals up to 64 h after injection. Tumours of the two lines were excised, fixed in neutral formalin and processed identically.

Autoradiographs of 5 μm -thick tumour sections were prepared and the cell nuclei were stained by the Feulgen reaction. Tumour cords were scored which were cut through in longitudinal section and for which the capillary lining and the row of pyknotic cells adjacent to the necrosis were approximately parallel. Cord thickness or radius, was measured between the capillary endothelium and the first pyknotic cell at the viable/necrotic interface. The distance between endothelial walls of the subtending capillary was also measured, half of this distance being taken as the vessel 'radius'. The tumour cord was then divided into zones of 100 μm (parallel to the vessel) by 20 μm (at right angles to the vessel), zone 1 adjacent to the capillary, zones 2, 3 . . . n, progressively further away. Parameters measured for each zone were (a) [^3H]-TdR Labelling Index (LI), (b) Mitotic Index (MI), (c) Necrotic Index (NI), i.e., the proportion of pyknotic or karyorrhectic cells relative to all viable plus dead cells, (d) Cellular Density (CD), i.e., the number of histologically-intact cells per zone. The value of each parameter was obtained for an individual cord, and a mean value and standard error was calculated from the 12 to 20 cords scored in each tumour. In total, 1,500–2,000 cells were scored per zone.

Results

Tumour Growth Delay

Untreated, H-4-II-E tumours grew from 200 mm^3 to 8 Vo in 4.58 ± 0.23 days, 3924A in 4.30 ± 0.24 days. To achieve a given absolute growth delay (up to 20

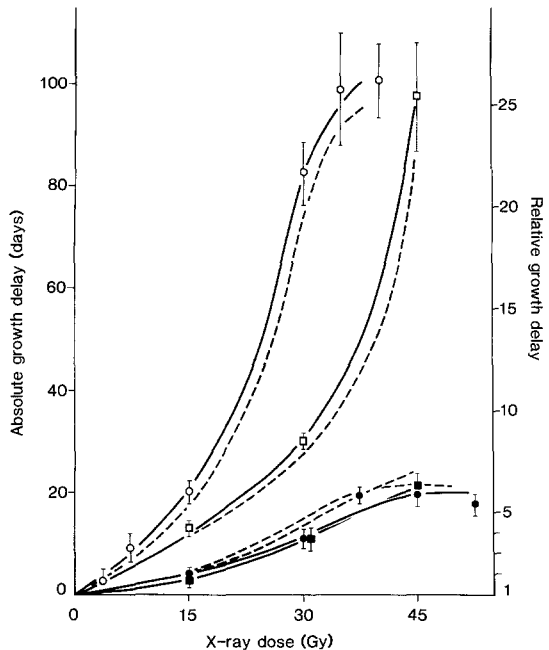


Fig. 1. Delay in growth of 3924A (irradiation in air \circ , clamped \square) and of H-4-II-E (air \bullet , clamped \blacksquare), as a function of radiation dose. Symbols and solid lines are for absolute growth delay, dashed lines for relative growth delay. Errors as ± 1 SE, for eight to 10 tumours

days), required approximately four times as much radiation dose for H-4-II-E than for 3924A, when both tumours were treated unclamped (Fig. 1). When plotted as relative growth delay, i.e., taking into account the slightly different control growth rates, about 2.5 times as much dose was required for H-4-II-E. These differences in 'intrinsic radiosensitivity' are to be the subject of a separate report and will not be further elaborated on here. The Oxygen Enhancement Ratio (OER), i.e., the ratio of doses for a given growth delay, tumour clamped : tumour in air, was calculated for both tumours at relative growth delays of 2, 3, and 4. Mean values were 1.35 ± 0.07 for 3924A and significantly lower ($p < 0.05$) at 1.08 ± 0.03 for H-4-II-E.

Morphometry

The % volumes ($\pm 1SE$) of four histological compartments in H-4-II-E and 3924A are shown in Table 1. In relative terms, there was twice as much gross necrosis in 3924A than H-4-II-E and only one quarter the vascular space.

Cord Analysis

(i) Cord and vessel radius. To determine whether these parameters varied with tumour size, mean values for individual tumours were plotted against the weight of the tumour at excision. Cord radius remained constant over a 30-fold range of weight (Fig. 2, upper panel). Notably, the overall mean radius of cords in H-4-II-E was only 60% that of 3924A (69 ± 3 vs. $118 \pm 3 \mu\text{m}$). The data for blood vessel radius were scattered (Fig. 2 lower panel) but yielded overall means of $23 \pm 2 \mu\text{m}$ for 3924A and 38 ± 3 for H-4-II-E. (ii) Cell Density and Necrotic Index. The cells of H-4-II-E were markedly smaller than those of 3924A (Fig. 3, upper panel). The ratio of CD's (a function of cell area) was 1.8 : 1. As the cells were circular in cross section, this would imply a ratio of cell volumes of 3.6 : 1. Approximately six to seven cell layers spanned the width of the cord in 3924A and eight to nine layers in H-4-II-E. There was a greater degree of histologically-defined cell death *within* cords in H-4-II-E, for a given distance

Table 1. Relative volumes of four histological compartments in hepatomas H-4-II-E and 3924A, measured by morphometry

	% Volumes	
	H-4-II-E	3924A
Parenchyma	56 \pm 5	47 \pm 4
Gross necrosis	26 \pm 4	49 \pm 5
Vasculature	16 \pm 3	4 \pm 1
Connective	2 \pm 1	0.1 \pm 0.1
Ratio of $\frac{\text{parenchymal vol.}}{\text{vascular vol.}}$	3.5 \pm 0.7	11.8 \pm 3.1

Fig. 2. Radius of tumour cords (upper panel) and subtending vessels (lower panel) in individual tumours of different weight. 3924A (■, □); H-4-II-E (●). Errors as ± 1 SE, for 12 to 20 cords within a tumour

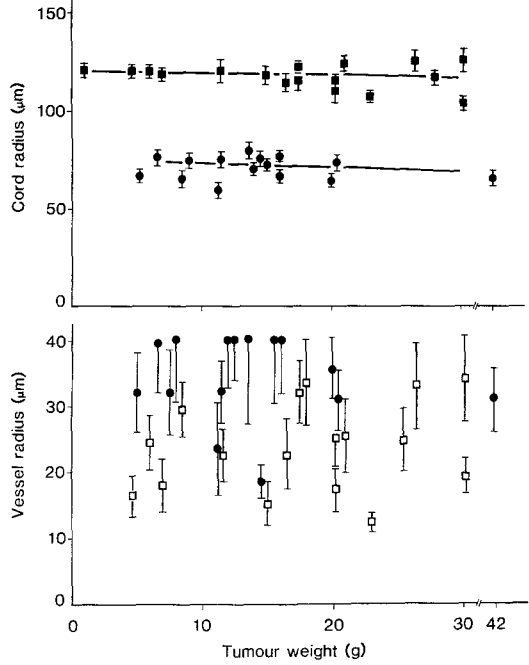
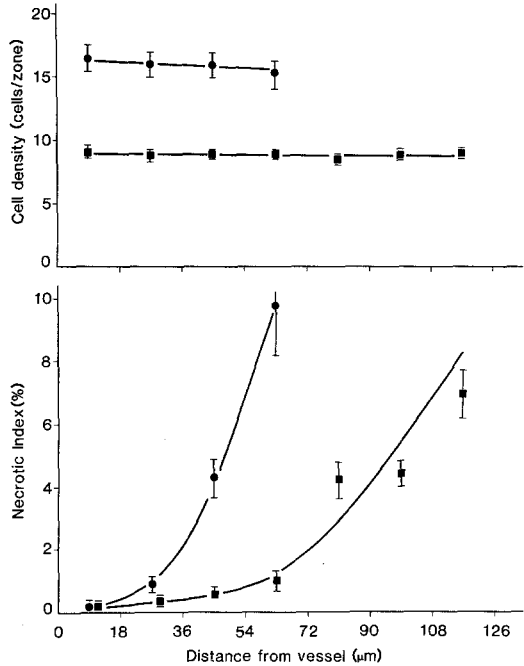


Fig. 3. Cellular Density (upper panel) and Necrotic Index (lower panel) for cell populations at different average distances from the capillary of a cord. Errors as ± 1 SE, for 15 to 18 tumours. Symbols as for Fig. 2



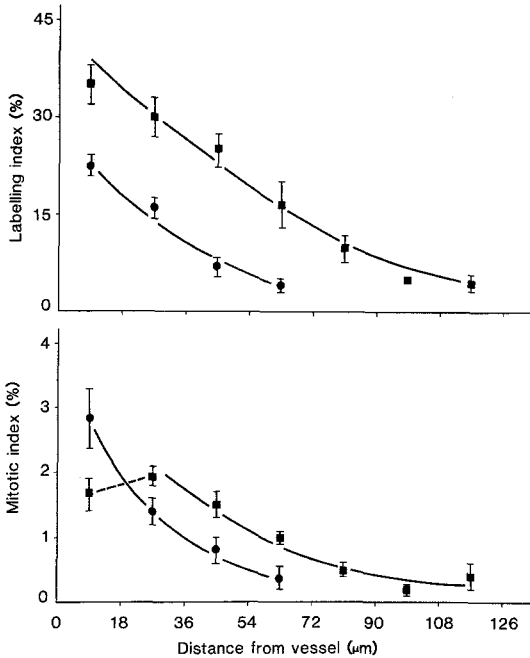


Fig. 4. 1 h- Labelling Index (upper panel) and Mitotic Index (lower panel) within a cord. Details as for Fig. 3

from the vessel (Fig. 3, lower panel). The ratio of distances for the same NI was approximately 1 : 2. (iii) Mitotic and Labelling Indices. Both MI and LI fell with increasing distance from the vessel (Fig. 4). Values for H-4-II-E were always lower (except for the first MI point, which is unexplained). The growth fraction of cells in the various zones were calculated from these data, using the methods of Steel [10] and the following values for parameters of the cell cycle: 3924A, average cell cycle time (T_c) = 27.4 h, duration of the S phase (T_s) = 9.3 h, duration of the G₂ phase (T_{G_2}) = 3.7 h [8]; H-4-II-E, T_c = 17.2 h, T_s = 6.6 h, T_{G_2} = 3.8 h (Moore, unpublished). Growth fractions were:

	Zone 1	Zone 4	Zone 6
3924A:	1.2 ± 0.3	0.53 ± 0.21	0.15 ± 0.10
H-4-II-E:	0.61 ± 0.15	0.10 ± 0.09	

The value of 1.2 for zone 1 for 3924A is (insignificantly) higher than the maximum possible growth fraction of 1. For this tumour, the values used for parameters of the cell cycle were for the overall tumour [8]; it may be that T_c for cells in zone 1 of 3924A is somewhat shorter than the average (as has been shown for other tumours [4]). This would lower the calculated growth fraction. (iv) Cord transit time. The time taken for the LI of the last row of cells in the cord to reach the same values as the first row, gives an estimate of the transit time of a cell cohort across the cord (Fig. 5). In 3924A, this took approximately 52 h, i.e., a migration rate of $1.7 \mu\text{m} \cdot \text{h}^{-1}$. For H-4-II-E the corresponding values were

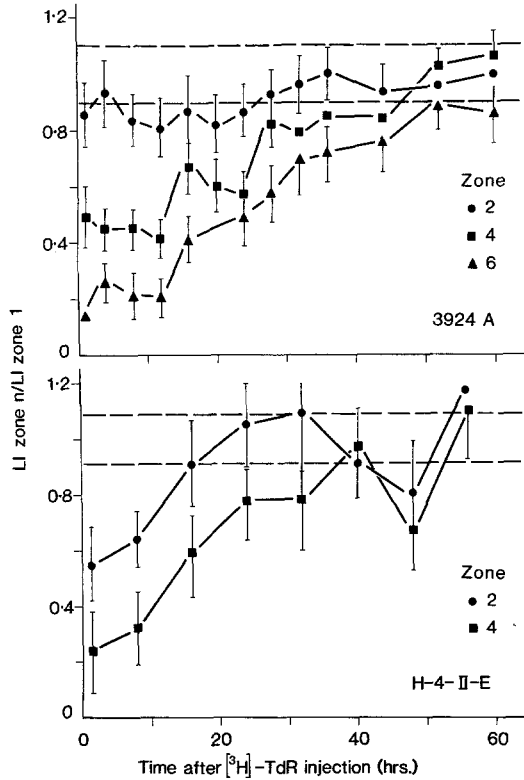


Fig. 5. Ratio of Labelling Indices in zones 2, 4, and 6 to LI in zone 1 of cords, at different times after injection of ³H-TdR. Dashed lines are ± 1 SE for LI in zone 1. Error bars ± 1 SE

approximately 40 h and $1.4 \mu\text{m} \cdot \text{h}^{-1}$. Expressed in terms of cell rows, one row of 3924A cells entered the necrotic zone approximately each 8 h, while for H-4-II-E the Figure was 5 h.

Discussion

It is generally believed that the inflection of curves of tumour growth delay after irradiation in air, reflects grossly a transition between regrowth of the tumour from a cell population that is largely ‘well-oxygenated’ (measured at lower doses of radiation) to regrowth from surviving cells (after higher doses) that have the characteristics of radiobiological hypoxia [15]. Such an inflection may be present in the response of 3924A at a dose of 35 Gy and a growth delay of 100 days (Fig. 1). However, this dose is close to curative ($\text{TCD}_{50} = 40 \text{ Gy}$; Hopkins, unpublished) so that the flattening of the growth delay curve may equally well have resulted from all (non-cured) tumours regrowing from a single cell, when growth delay should be relatively independent of dose. H-4-II-E appears to consist very largely of radiobiologically-hypoxic cells (OER 1.1; Fig. 1), a result consistent with that for clonogenic assay of this tumour (monotonic survival curve for treatment in air-breathing rats, Do 4.11 Gy, $n = 1.31$; [9]).

The average thickness of tumour cords in 3924A was 1.7 times that for H-4-II-E. For the 15 different rodent tumours for which we have been able to find quantitative values for cords (Moore, unpublished), mean cord thickness was 102 μm . Only one other tumour, the murine carcinoma KHU, had a smaller cord radius than H-4-II-E (54 μm ; [5]). The average thickness of cords in a tumour, is sensitive to the oxygenation status of the host. Tannock and Steel [14] found for a mammary tumour grown in mice, that the mean thickness of cords was reduced significantly (by 12%) as the percentage of O_2 in the air breathed by the mice, was lowered from 21 to 10%. Similarly in *in vitro* tumour spheroids, reducing the O_2 content of the gas mixture reduced the thickness of the outer shell of viable cells (e.g., [3]). Thus the characteristics of cords in a tumour may reflect the oxygen physiology of that tumour, mediated via the tumour vasculature and the metabolic properties of the tumour cells.

The relative vascular volume in H-4-II-E was four times that of a 3924A tumour of the same size (Table). However, that vasculature in H-4-II-E supported only 3.5 times its own volume of parenchyma, compared to 11.8 times in 3924A (Table 1). The volume of a parenchymal *cell* in 3924A was approximately 3.6 times that for H-4-II-E (see Results) suggesting that the two sets of vasculature support the same relative number of viable parenchymal cells, i.e., $11.8 \div 3.6 = 3.3$, and 3.5 respectively.

In the capillaries serving tumour cords (Fig. 2) the ratio of vessel perimeter to vessel cross-sectional area was 0.09 for 3924A and only 0.05 for H-4-II-E (assuming the capillary to be a cylinder, this would also be the ratio of the surface area available for nutrient exchange, to the vessel volume). The capillaries of H-4-II-E in particular, are enlarged, tortuous and show invariable and marked signs of stasis. To our knowledge, the partial pressure of oxygen in the vessels of these two tumours had not been measured, but a crude value can be obtained from our quantitative data for tumour cords. Tannock [13] quotes the equations of Krogh that define the 'critical' distance from the centre of a blood vessel at which the oxygen tension in a metabolising tissue falls from the vessel partial pressure to zero. We assume here that the geometry of the central vessel in cords is essentially cylindrical so that the parameter we have measured as vessel 'radius' approximates to a true radius, and that this radius plus cord thickness represents the critical distance. The diffusion coefficient of oxygen was taken to be $7.2 \times 10^{-2} \text{ cm}^2 \cdot \text{h}^{-1}$ (as given in [13]). The rate of oxygen consumption (Q_{O_2}) of non-necrotic tissue slices of 3924A was $5.5 \mu\text{l} \cdot \text{mg dry wt}^{-1} \cdot \text{h}^{-1}$ [17]. We have been unable to find a value of Q_{O_2} for H-4-II-E, but that for its parental strain, H35, was $5.0 \mu\text{l} \cdot \text{mg dry wt}^{-1} \cdot \text{h}^{-1}$ [1]. Tannock [13] quotes values for the respiratory quotients of six rat tumours (including another hepatoma, 5123) and we have taken the mean of these ($5.5 \mu\text{l} \cdot \text{mg dry wt}^{-1} \cdot \text{h}^{-1}$) and the extreme values (1.3 and $11.8 \mu\text{l} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$) to calculate that the partial pressure of oxygen in the vessels of tumour cords would be: 3924A: 81 mm Hg (19, 173 mm Hg, using the extreme values of Q_{O_2}); H-4-II-E: 21 mm Hg (5, 45).

The mean value for 3924A lies between arterial and venous partial pressure for normal tissues and one would expect cells immediately adjacent to a vessel containing this oxygen concentration to be radiobiologically well-oxygenated

(e.g., [13]). The calculated oxygen partial pressure in H-4-II-E was fourfold lower than in 3924A, but still at a level where one might expect mammalian cells to be two to three times as sensitive to low-LET radiations as 'anoxic' cells. This might suggest that H-4-II-E contains a small fraction of radiobiologically well-oxygenated cells that are not being resolved by the growth delay assay.

With regard to the cellular kinetics of cords in the two tumours, the migration rates across the cords resemble those for other rodent tumours (e.g., [4]). Of potential therapeutic interest, was the inference that the growth fraction of cells immediately adjacent to the vessel in cords of H-4-II-E might be only 0.6. The calculation is sensitive to the measured value of average cell cycle time, but the latter was obtained using mitoses in the inner zones of cords. Measurements by others for this zone of tumour cords have suggested the GF to be normally 1, as for 3024A [4, 12], although recently a value of 0.41 has been inferred for a murine mammary carcinoma [6]. Out-of-cycle cells are relatively resistant to the action of a number of cytotoxic drugs and this, together with the indications of a sluggish blood flow, might account in part for the resistance of H-4-II-E to a spectrum of chemotherapeutic drugs.

These studies highlight the lack of correlation between simple measurement of vascular volume and the response to radiation therapy; there is four times as much vascular space in H-4-II-E than 3924A and the most remote cells of cords of H-4-II-E are almost twice as close to the source of oxygen (and diffusable cytotoxic drugs) as are those in 3924A, yet it is the former that is resistant and the latter sensitive.

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