Characterization of radiation resistant hypoxic cell subpopulations in KHT sarcomas. (I) Centrifugal elutriation

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Summary Studies were performed to determine the location, with respect to cell cycle phase, of the radiobiologically hypoxic cells in KHT sarcomas. Cells dispersed from solid KHT tumours were separated into subpopulations at different stages of the cell cycle by centrifugal elutriation. Flow cytometric analysis of the DNA content of these subpopulations indicated that the degree of synchrony that could be achieved was ≥95% for G1, S, and G2/M. The approach to locate and characterize hypoxic cells was based on the premise that due to their lack of oxygen such cells would preferentially survive radiation treatment. Consequently KHT sarcomas were irradiated in situ either in dead animals or in animals breathing air. Following treatment, the tumours were dissociated, the cells elutriated into the various phases of the cell cycle and clonogenic cell survival was determined. Complete dose-response curves were determined for cells in the G1, S and G2/M cell cycle phases. The various cell cycle subpopulations obtained from tumours irradiated while mice breathed air, all demonstrated survival curves with a break and final slope which paralleled that of the corresponding anoxic cell survival curve. From these curves the proportion of hypoxic tumour cells in the G2 phase was calculated to be 10.1±1.7%. Because the elutriated S and G2/M enriched cell fractions were to some extent contaminated by cells from other phases of the cell cycle, the percentage of hypoxic S and G2/M tumour cells was estimated to range from 0-7% and 0-5% respectively. However, since G1 cells comprised the majority of all the neoplastic cells in these tumours, the data suggest that hypoxic cells in KHT sarcomas are found primarily in this cell cycle stage.

Materials and methods

Animals and tumour line

The KHT sarcoma (Kallman et al., 1967), a tumour maintained in vivo, was used in all experiments. This tumour was passaged approximately every 2 weeks by preparing single cell suspensions from solid tumours using a mechanical dissociation procedure (Thomson & Rauth, 1974) and injecting these cells i.m. into the hind limbs of 8-14 week old female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME). Tumours were used when they reached a size equivalent to a weight of 0.5-0.7 g.

Irradiation

The mice were irradiated whole body using a 137Cs source operating at a dose rate of 5.5 Gy min⁻¹. The animals were not anaesthetized and were irradiated either while breathing air or under anoxic conditions. In the latter case, the mice were killed 10 min before tumour irradiation. Immediately following the radiation treatment, single cell suspensions were prepared from the tumours using a combination of mechanical and enzymatic (0.2% trypsin+DNase) dissociation procedures (Thomson & Rauth, 1974).

Synchronization by centrifugal elutriation

Cell populations in various phases of the cell cycle were obtained directly from solid KHT sarcomas by centrifugal elutriation as has been described in detail elsewhere (Keng et al., 1981; Siemann et al., 1981). Briefly, prior to use, the elutriator system was sterilized by autoclaving and then flushing with 70% ethanol. Approximately 1-4 x 10⁸ cells were suspended in 19.5 ml of alpha-minimal essential medium plus 0.5 ml of DNase and loaded into the separation chamber at a rotor speed of ~4000 r.p.m. and a flow rate of 35 ml min⁻¹. During the separation procedure, the elutriator system and the elutriation fluid (alpha-minimal essential

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medium plus 10% foetal calf serum) were held at 4°C. The rotor speed was then decreased in increments to ~2000 r.p.m. with a variable number of 40 ml fractions being collected at every interval. For each fraction collected, the cell number and cell volume distribution were determined using a Coulter Channelizer system. FCM analysis was used to determine the percentage of separated cells in the G1, S, and G2M phases of the cell cycle as has been previously described (Keng et al., 1981; Siemann & Keng, 1986). Briefly, FCM measurements were made with an EPICS V dual laser (argon and krypton) flow cytometer (Coulter Electronics, Inc.), using a TERAK 8600 minicomputer for data storage and analysis. Cells were fixed in 70% ethanol and stained with mithramycin (1.0 mg ml⁻¹) according to the methods of Crissman and Tobey (1974). DNA histograms were analyzed using the model of Fried and Mandel (1979) implemented as the ‘C CYCLE’ program on the TERAK 8600 system. Data obtained previously with the KHT sarcoma have demonstrated a close agreement between the estimates of cells in the various phases of the cell cycle based on FCM analysis and titrated thymidine uptake (Keng et al., 1981). In these experiments, the percentage of non-neoplastic cells in each separated fraction was scored from cytopsin centrifuge prepared slides stained with Wright’s and Giemsa stains (Siemann et al., 1981).

Clonogenic cell survival assay

Following elutriation, the cells from the various fractions collected were counted in a haemocytometer and a number of dilutions were prepared. The cells were mixed with 10% lethally irradiated tumour cells in 0.2% agar containing a terminalimal medium supplemented with 10% foetal calf serum and plated into 24-well multidishes. In about two weeks, the surviving cells formed colonies which were counted with the aid of a dissecting microscope. Because unelutriated cell suspensions as well as the various elutriated cell fractions, contain different host to neoplastic cell ratios (Siemann et al., 1981), survival values were calculated on the basis of the number of neoplastic cells actually plated as determined from differential counts performed on cytopsin slides (Siemann et al., 1981).

Results

Figure 1a shows the radiation response of anoxic KHT sarcoma cells as a function of cell cycle stage. To induce anoxia, tumour-bearing mice were killed 10 min prior to irradiation. The KHT sarcomas then were dissociated, the cells elutriated and cells from each fraction were (i) prepared for FCM and morphological analysis and (ii) plated for clonogenic cell survival. The data, for the 3 doses evaluated in detail, all indicated the most radiation-resistant cells to be in the late G1 and early S phases. Early G1 cells showed relatively greater radiation sensitivity and late S and G2M cells were particularly sensitive. For comparison, the showed the tumours of air-breathing animals were irradiated with 10, 17.5 or 25 Gy and the radiation response across the cell cycle was determined (Figure 1b). These doses were chosen to give approximately similar levels of survival as were attained under anoxic conditions. However, the pattern of radiation sensitivity seen when KHT cells were irradiated in situ in air-breathing mice was different; particularly in the G1 cell cycle stage (Figure 1a vs. 1b). Cells in late S and G2M were still the most radiosensitive but now cells in the G1 phase were most radiation resistant (Figure 1b).

The fraction of hypoxic cells in the various cell cycle phases was subsequently evaluated. Tumour cell survival curves for KHT sarcomas irradiated with single doses of radiation ranging from 0–35 Gy under air-breathing or anoxic conditions are illustrated in Figure 2a. The data shown represent the response of the total cell population obtained from KHT tumours. Complete dose response curves also were determined for G1, S and G2M cells irradiated in vivo prior to cell separation. These are depicted in Figures 2b, 3a and 3b, respectively. Under both treatment conditions, the neoplastic cells in the elutriated cell fractions used for cell survival evaluations had a purity, with respect to cell cycle, of ≥95% for the G1 phase and 70–75% for cells in S or G2M. The proportion of non-neoplastic host cells in these elutriated fractions was typically <10% for the G1 fraction and <5% for the S and G2M fractions. Because the radiation treatment was given prior to tumour dissociation and cell elutriation, in all figures, each datum point represents a separate elutriation experiment. A straight line was fitted by linear regression to each set of data in Figures 2 and 3 for doses ≥15 Gy. For every cell cycle phase enriched fraction, analysis of covariance (Snedecor & Cochran, 1973) found the slope of the anoxic data not to be significantly different from the slope of the air-breathing

![Figure 1](image1.png)

**Figure 1** Cell survival of irradiated KHT sarcoma cells in various phases of the cell cycle. (a) Tumours irradiated in dead mice (anoxic conditions) (redrawn from Siemann and Keng (1984)). (b) Tumours irradiated in air-breathing mice. Data shown are the mean ± s.e. of 3–5 experiments.

![Figure 2](image2.png)

**Figure 2** Cell survival following a range of radiation doses for (a) unelutriated KHT sarcoma cells or (b) cells in the G1 phase. Tumours were irradiated under anoxic or air-breathing conditions prior to cell elutriation. Data shown are the mean of 3–5 experiments ± s.e. Individual points represent single experiments.
data. However, when the intrinsic radiation sensitivities of cells in the various cell fractions were compared, cells in the G2M enriched fraction were, as has been previously reported by us (Keng et al., 1984; Siemann & Keng, 1984), once again the most radiosensitive.

When the tumours of air-breathing mice were irradiated, the G1, S and G2M elutriated subpopulations all demonstrated cell survival curves with an initial sensitive component and resistant ‘tail’ at higher doses (Figures 2 and 3). This tail was parallel to the cell survival curve of the various cell cycle subpopulations obtained when tumours were irradiated under anoxic conditions. From these data an estimate was made of the proportion of hypoxic cells in the various cell cycle phases in the tumours of air-breathing mice (Table I). This was done by calculating cell survival ratios under air-breathing and anoxic conditions for the same radiation dose. The data demonstrate that KHT sarcomas typically possess ~10% hypoxic cells, a finding in agreement with that of others working with this tumour (Hill & Bush, 1977; Hill, 1980). These hypoxic tumour cells were not, however, equally distributed throughout the cell cycle (Table I). The majority were found in the G1 stage with relatively fewer in the other phases of the cell cycle (see Discussion).

Discussion

We have previously performed experiments which determined the survival across the cell cycle of cells grown as KHT sarcomas (Siemann & Keng, 1984). In order to assess the radiation response solely due to cell cycle position and to avoid the complicating effects that variations in the cellular oxygenation state have on cell survival, the radiation doses were given under conditions of uniform oxygenation. These were achieved by either irradiating the cells anoxically in dead mice (Figure 1b) or oxically in vitro after dissociation of the tumours (Siemann & Keng, 1984). Under both treatment conditions, cell survival increased through G1 and early S prior to decreasing continuously through mid- to late S and into G2M (Figure 1a). In contrast, when KHT sarcomas were irradiated under air-breathing conditions radiation resistance remained constant through G1 to mid-S and then decreased in late S and G2M (Figure 1b). This latter pattern of radiation sensitivity suggested that perhaps hypoxic conditions in the tumour were most strongly influencing the response of cells in the G1 phase of the cell cycle.

In order to determine the cell cycle location of hypoxic cells, complete dose response curves for each cell cycle phase were derived from KHT tumours irradiated under anoxic or air-breathing conditions (Figures 2 and 3). From these, the proportion of radiobiologically hypoxic tumour cells could be calculated (Table I). Using covariance analysis the proportion of hypoxic cells in the unseparated cell suspension and the G1 fraction was found to be 10.1 ± 1.2% and 10.1 ± 1.7% respectively. Since the isolated G1 fraction was ≥95% pure (Table I), the presence of hypoxic G1 cells in solid KHT sarcomas seems certain. Exact computation of the hypoxic cells in the elutriated S and G2M fractions is however more complicated. Although the 70–75% purity achieved in these fractions is as good as has been reported for cells isolated directly from solid tumours, it is possible that the cells making up the remaining 25–30% of the S and G2M fractions could be influencing cell survival. For example, the S enriched fraction is contaminated by 15–20% G1 cells and the G2M enriched fraction contains ~20% S cells (Keng et al., 1981). If the contaminating cells in these fractions, or even a portion of these cells, were hypoxic, then the estimate of hypoxic S or G2M cells in KHT tumours could be severely affected. For example, if all the hypoxic cells in the S and G2M enriched fractions were in the contaminating populations, then the actual percentage of hypoxic S or G2M hypoxic cells could be zero. Alternatively, if none of the contaminating cells in the S and G2M enriched fractions were hypoxic, then the percentage of hypoxic cells in these two cell cycle phases can be calculated from Figure 3 to be 5.8 ± 1.0% and 3.9 ± 0.9% respectively. The present data do not readily allow us to distinguish between these two possibilities. Consequently, while there is little doubt about the presence of hypoxic G1 tumour cells, it is not possible to accurately determine the actual percentage of hypoxic cells in the S and G2M phases. Given the uncertainties discussed above, the percentage of hypoxic cells in these latter two phases could range from 0–7% (S) and 0–5% (G2M).

In these investigations, cell suspensions prepared from KHT sarcomas typically were found to contain 60–70% cells in G1, 20–30% cells in S and 10–20% cells in G2M. Since at least 10% of the G1 cells were hypoxic at the time of irradiation, this suggests that the majority of all the hypoxic cells in KHT sarcomas are in this phase of the cell cycle. This conclusion is further supported by some of our recent investigations of hypoxic cells in this tumour model using a cell separation technique which depends on the diffusion properties of the fluorochrome Hoechst 33342 (Chaplin et al., 1985; Olive et al., 1985), and allows cells to be separated by a cell sorter on the basis of their Hoechst 33342 staining intensity. When applied to KHT sarcomas, ~90% of the neoplastic cells in the fraction containing the 10% most dim cells, were in the G1 phase (Siemann & Keng, in preparation). These findings, along with the elutriation results presented here, strongly imply that hypoxic cells in the KHT sarcoma are located predominantly in one phase of the cell cycle, namely G1. The results further suggest that acute hypoxia, a consequence of intermittent opening and closing of blood vessels (Brown, 1979; Sutherland & Franko, 1980), is not a dominant form of hypoxia in these tumours.
Otherwise, if hypoxia in this model were primarily the result of random intermittent changes in blood flow, then a more uniform distribution of hypoxic cells throughout the cell cycle would have been expected. While the results do not rule out completely some contribution of acute hypoxia, they are more closely aligned with chronic hypoxia being of greater importance in KHT sarcomas.

In summary, delineation of the types of hypoxia in solid tumours is of considerable importance. The presence of acute and/or chronic hypoxia in tumours may have significant impact on the choice of therapies to overcome radiation resistance due to hypoxia. Studies of tumour cell sub-populations may provide valuable information in this area.

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