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A Comparison of the Chemosensitivity of a Primary Tumour and its Metastases Using a Human Tumour Xenograft

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Abstract—*Clonogenic cell survival curves were constructed for melphalan treatment of primary and secondary tumours of a human xenograft in immune deprived mice. The small metastases were shown to be more sensitive to melphalan than the larger primary tumours. Experiments with radiolabelled melphalan suggested that the greater sensitivity of the small tumours was due to better drug penetration.*

INTRODUCTION

THE RELATIONSHIP between the size of a tumour and its sensitivity to chemotherapy is of critical importance in devising a rational strategy for cancer therapy. It is generally held that tumours of small bulk will be more readily cured by a given chemotherapeutic schedule and it is argued that smaller tumours have a higher fraction of actively proliferating cells and, therefore, are likely to be more sensitive to proliferation dependent cytotoxic drugs [1, 2].

Experimental animal tumours have been used to investigate this relationship and, in general, small metastases have been observed to have steeper cell survival curves than the primary tumours from which they are derived [3–5]. This may be due to greater sensitivity resulting from a more rapid proliferative rate but correlations between cell kinetics and chemosensitivity of tumours of different sizes have not been convincingly shown [5]. Alternatively, changes in the metabolic status of tumour cells related, for example, to vascular insufficiency, are known to modify chemosensitivity [6]. Finally, reduced drug induced cell kill may be due to the inability of drugs to penetrate larger tumours [7, 8], due to poor blood supply [9].

We have investigated the relationship between size and chemosensitivity of a human tumour growing in immune deprived mice. Reports of metastases occurring consistently in serial passage of human tumours as xenografts are unusual [10], but, using an improved method of immune-suppression [11], an anaplastic human tumour growing intramuscularly in the hind legs of mice has been found to metastasise reproducably to the aorto-iliac lymph nodes of a high proportion of animals in each passage.

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MATERIALS AND METHODS

The human tumour xenograft used in these studies (designated HX32) was derived from a biopsy of a peritoneal deposit of a presumed carcinoma of the pancreas [12, 13]. It has been maintained in immune-deprived CBA/lac mice and the experiments described in this report were carried out in passages 20–25 during which time it was shown to have retained a human karyotype.

CBA/lac mice were immune-deprived by thymectomy and treatment with cytosine arabinoside (200 mg/kg) followed two days later by 900 rad of whole body irradiation, a non-lethal combination [11]. Tumours were passaged by i.m. injection of approximately 10^5 tumour cells into the hind legs.

Mice bearing i.m. and metastatic tumours were treated with doses of melphalan by i.p. injection and the tumours removed 18 hr later. Three or four mice were used for each dose point in each experiment and lymph

nodes from all animals pooled to provide sufficient tumour material.

Single cell suspensions were prepared by mechanical disaggregation and enzymatic digestion with collagenase and trypsin, according to the method of Courtenay *et al.* [13]. Cells were counted in a haemocytometer using lissamine green dye under phase contrast microscopy. Bright cells which excluded dye were regarded as viable. Non-tumour cells were excluded by size and morphology only. Cell suspensions were mixed with soft agar and introduced into Millipore® agar diffusion chambers which were incubated in the peritoneal cavities of pre-irradiated C57 mice according to the method of Smith *et al.* [12]. The surviving fractions of the colony forming cells were calculated from the ratio of the plating efficiency of treated cell suspensions to that of control cell suspensions.

Drug uptake into the primary and metastatic tumours was investigated using ^{14}C-labelled melphalan (Radiochemicals, Amersham, spec. act. 3.6 mCi/mM). Approximately 7.5 Ci of labelled melphalan were added to unlabelled melphalan (Alkaran, Wellcome) to a total dose of 15 mg melphalan/kg. The solution was injected into tumour bearing mice, either i.p. or i.v. Samples of nodes, primary tumours, necrotic centres of primary tumours and the periphery of primary tumours were taken at specified times after treatment and immediately frozen at −20°C. Samples were subsequently completely oxidised using an Oxymat electric furnace (Intertechnique Limited) and the ^{14}C labelled carbon dioxide collected in alkaline scintillant. This was counted in an automated scintillation counter (Intertechnique Limited).

The results are expressed as the content of melphalan plus metabolites per gm of tumour. These values were derived from comparison of the radioactivity of tumour specimens to that of standards of known melphalan content obtained by oxidising a measured volume of the radiolabelled melphalan solution. This method makes no allowance for metabolism of melphalan in the test samples.

RESULTS

Metastases were noted in the aorto-iliac lymphnodes of about 70% of the animals in each passage, at a time when the primary tumours were approximately 2 cm in diameter, the maximum size tolerated by the mice. Nodes ranged from 1 to 3 mm in diameter and were usually bilateral.

Cell suspensions were readily prepared from the primary tumours and from the lymph nodes. Cell yields were about 10^6 viable cells per gm of tumour. Cell counts were performed in duplicate and did not differ by more than 10% even for low cell yields from the small lymph nodes. Plating efficiencies were between 20 and 30% and there was no significant difference between those of the primary and secondary tumours.

Figure 1 shows clonogenic cell survival curves for the large i.m. leg tumours (2 cm) and the small metastatic tumours in the aorto-iliac nodes (2 mm). The accumulated results of three experiments are shown. Inspection of the data suggests that the small metastatic tumours were more sensitive to melphalan. The shapes of the dose response curves were not precisely defined by the data. However, this work was part of a larger unpublished

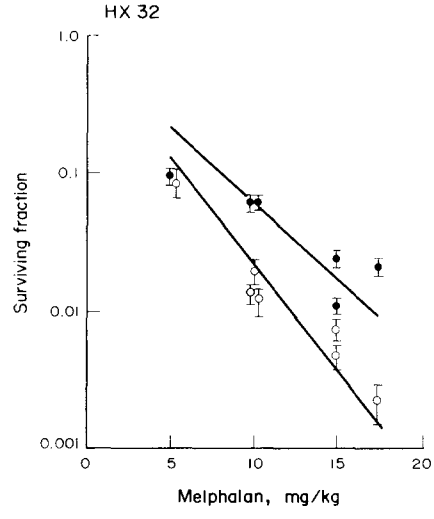

Fig. 1. Cell survival curves for large primary intramuscular tumours (●) and small metastatic tumours in lymph nodes (○) treated with melphalan.

study in which we have accumulated a substantial amount of data on clonogenic cell survival in xenografts treated with melphalan. These data indicate an exponential dose–response relationship and we have, therefore, fitted exponential curves to the data in Fig. 1. Lines were drawn by linear regression analysis including the origin as a date point and the slopes of the curves compared using Student's *t*-test. The calculations were performed using a computer programme kindly made available to us by Dr. J. L. Millar. This comparison confirms a significant difference between the two curves ($P<0.02$).

The uptake of melphalan plus metabolites by the small metastatic tumours and the large intramuscular tumours at a dose of 15 mg/kg to the mouse is shown in Fig. 2. The accumulated data from three experiments in which the drug was given by the i.p. route are shown. There was a wide scatter in the data in each experiment. Despite this scatter, a clear difference was shown between the uptake into the small metastatic tumours and into the large tumours. In other experiments, this difference was also apparent when the drug was given i.v. In Fig. 3, the drug uptake into the periphery and necrotic centre of the primary tumours is shown. The uptake into the periphery is higher than that into the necrotic centre, although it is still less than that into the nodal tumours.

Histological examination of the tumours in the aorto-iliac nodes show no areas of central necrosis in marked contrast to extensive necrosis seen in the primary intramuscular tumours.

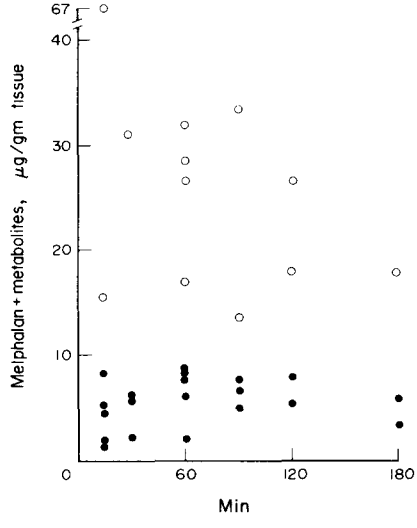

Fig. 2. Tissue content of melphalan and metabolites after 15 mg/kg *melphalan given by i.p. injection* (3 *expts*). ○—*Small nodal tumours*, ●—*large primary tumours.*

DISCUSSION

These experiments indicate that small metastatic deposits of this human tumour xenograft are more sensitive to melphalan than large i.m. primary tumours, although the difference is small. The slope of the survival curve is increased by a factor of about 1.4. This difference in sensitivity could be due either to better drug penetration or to a higher rate of cellular proliferation in the smaller tumours. The statistical analysis of the difference between the two curves assumed

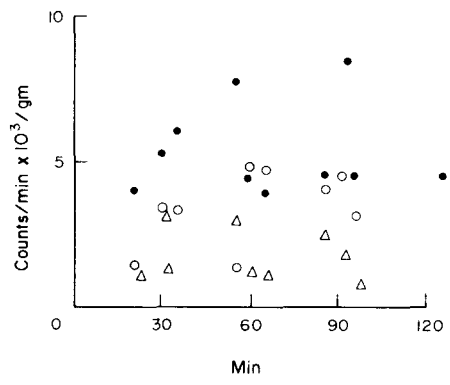

Fig. 3. Tissue content of radiolabel after 15 mg/kg *melphalan given i.p.* (1 *expt*). ●—*Periphery of large primary tumours*, △—*necrotic centre of large primary tumour*, ○—*whole tumour. The melphalan content of the periphery of these tumours was lower than that of the small nodal tumours shown in Fig. 2.*

exponential cell survival based upon our experience with melphalan and the other xenograft lines (unpublished observations). The 'fit' of these lines to the data in the present study was only approximate and we feel that no more detailed interpretation of the shapes of the curves is justified.

Clonogenic cell survival curves may be subject to a variety of well recognised artifacts. In particular, the timing of tumour removal after treatment must be chosen carefully to allow for completion of drug action but to precede significant cell loss from the tumours or the beginning of cell repopulation. In some tumour systems there may be an increase in surviving fraction with time after treatment, believed to be due to the repair *in vivo* of damage to cells which is not repaired in the assay system [14]. In the xenograft system employed in this study, no changes in surviving fraction were observed between 3 and 24 hr, and so these artifacts are unlikely to have been significant when cell survival was estimated at 18 hr.

A difference in slope between the cell survival curves of large and small tumours has been described in experimental murine tumours [4, 15]. The difference may be large as in the case of BCNU treatment of Lewis lung tumour [4] or much smaller as was the case with cyclophosphamide in the same tumour [15]. Whether the size of the difference observed depends upon the drug used, the tumour tested or a combination of the two, is not known. We are unaware of previous studies in which this question has been studied using melphalan.

The results of the experiments with radiolabelled melphalan suggest that drug penet-

ration was better into the smaller tumours and this is likely to explain the difference in sensitivity. The absence of necrosis in the small tumours supports the better perfusion of these tumours. Better drug penetration of small metastases has been demonstrated by Donelli *et al.* [8] in an experimental animal tumour.

Larger volumes of the primary tumours than the lymph nodes were used to estimate melphalan content. This could have produced errors by allowing a greater proportion of contaminating normal tissues to be included with the nodes. However, content of radiolabel in the primary tumours was not dependent upon the mass of tumour used and so it is unlikely that this error significantly altered the results.

The concentrations of melphalan plus metabolites in the nodes are 3–4 times higher than those in the primary tumours but the data are too scattered to allow accurate measurement of pharmacokinetic parameters such as the area under the curves. The difference would appear to be greater than that necessary to explain the small difference in cell survival but this conclusion is unreliable in view of the wide scatter in the data and the uncertainty about the degree of metabolism of melphalan in the tumours.

The growth of a human tumour as a xenograft involves a complex relationship between the tumour cells and the host. On the one hand, the cells are presumably subject to an immune response directed against tumour and species specific antigens and, on the other hand, the host is induced to provide a stroma and blood supply to the tumour. The latter provision is quite remarkable and implies that tumour angiogenic factors are not species specific. Our present study suggests that an adequate blood supply maintained with tumour growth is likely to be an important determinant of the relationship between tumour size and chemosensitivity. However, the study was performed in a xenograft tumour and, while this has the advantage of demonstrating the relevance of the conclusions to human tumour cells, the blood supply of the tumour is murine. The system may have few advantages over experimental murine tumours for studying those aspects of tumour behaviour determined by blood supply. In general, the xenograft system may have advantages for studying aspects of chemosensitivity of tumours determined by cell type but less for aspects determined by the host-tumour relationship.

Nevertheless, it seems reasonable to conclude that these experiments support the suggestion that treatment of small volume disease, as in the adjuvant chemotherapy situation, may have the advantage not only of a lower body burden of tumour cells, but also of slightly greater sensitivity of the tumours to some treatments.

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