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Melphalan Concentration and Distribution in the Tissues of Tumour-bearing Limbs Treated by Isolated Limb Perfusion

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Levels of melphalan (L-phenylalanine mustard) were measured in the tissues of tumour-bearing limbs treated by isolated limb perfusion (ILP). 41 samples of melanoma tissue, normal fat and skin were excised from 15 patients during ILP. A high performance liquid chromatography assay was used to measure melphalan concentrations. Levels of melphalan were higher in tumour than in fat ($P < 0.01$, Wilcoxon signed-ranks test), and not significantly different from levels in adjacent skin. In 2 cases there was significant regional toxicity in the treated limb, but this was not related to the levels of melphalan measured in the tissues of the limb. It is encouraging that the concentrations of melphalan which were achieved in large necrotic nodules by ILP were similar to those in well-perfused normal skin.
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INTRODUCTION

ISOLATED LIMB perfusion (ILP) is a form of regional chemotherapy for cancer which involves the exposure of a tumour-bearing limb to a high concentration of anticancer agent, while sparing the patient from systemic toxicity. In the management of malignant melanoma, ILP with melphalan is an effective method of control for locoregional advanced disease, and may be an effective adjuvant to surgery in the treatment of high-risk primary lesions [1]. Melphalan is a bifunctional alkylating agent which acts on tumour cell DNA. The drug must therefore penetrate tumour masses in order to be effective.

It has been shown that ILP can successfully achieve high levels of melphalan in the perfusate which circulates within the treated limb, with low levels in the systemic circulation [2, 3], but there have been no published studies to show how melphalan distributes within the tissues of the tumour-bearing limb during ILP.

The aim of this study was to measure the levels of melphalan in the tissues of tumour-bearing limbs treated by ILP.

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PATIENTS AND METHODS

Clinical method of ILP

External iliac ILP of the lower limb for malignant melanoma was performed by a method based on standard techniques [4, 5]. Fluorescein is added to the perfusate and observation with ultraviolet light confirms adequate perfusion and satisfactory isolation. Melphalan was given in a dose of 1.75 mg/kg body weight, when the calf skin temperature reached 37.5°C. ILP lasted 1 h, according to our protocol for therapeutic perfusion. The flow of perfusate was adjusted to the maximum achievable rate during ILP.

Samples of perfusate were drawn at 5-min intervals for melphalan assay by high performance liquid chromatography (HPLC). The area under the perfusate concentration–time curve (AUC) for each patient was derived by the trapezoidal rule.

Ethical Committee approval was obtained for blood and tissue sampling. All patients gave informed consent for the procedures.

Tissue samples

In 15 patients, samples of malignant melanoma tissue, as well as normal fat and skin, were excised from accessible sites during and after ILP. When tissue was obtained it was immediately placed in a universal container and immersed in liquid nitrogen. Thereafter the tissue was stored at −20°C for the minimum possible time until melphalan assay. At the time of assay, tissue samples were thawed, weighed, minced with scissors and scalpel, then homogenised using an Ultra Turrax homogeniser in a volume of ice-cold 0.01M NaH_2PO_4/1 mmol/l EDTA at pH3.1. Care was taken to minimise heating of specimens during preparation. Samples were further homogenised using a Potter 'S' homogeniser. The final volume was measured and duplicate 1-ml samples of homogenate from each specimen were used for assay and subsequent analysis was as for plasma specimens, including the use of internal standard.

In vitro tissue specimens were also 'spiked' with melphalan to measure recovery by the extraction method used. The reported results are adjusted to take account of 87% recovery.

Melphalan assay by HPLC

The sensitive and specific HPLC assay which we used is based on the method described by Chang *et al.* [6].

10 μg of dansyl proline (Sigma) is added to a 1-ml or less portion of the thawed homogenate or plasma sample. In the analysis dansyl proline acts as an internal standard, a substance which is chemically similar to melphalan (the losses of which parallel the losses of melphalan) but which generates a distinct peak on the chromatogram.

4 volumes of acetonitrile (BDH, HPLC grade) with 1% hydrochloric acid (BDH, Analar grade) are then added to the plasma in 15-ml conical centrifuge tubes. The sample is vortex-mixed immediately for 15 sec and then centrifuged at 2000 r.p.m. for 10 min to precipitate protein. The clear supernatant is then transferred to 30 ml vials, and the volume is reduced to 300 μl or less under vacuum using a Buchler vortex evaporator (approximately 25 min at 30°C). The volume of the sample is then made up to 500 μl with 20% methanol (BDH, HPLC grade). The samples are transferred to autosampler vials, sealed and loaded in the autosampler.

The samples are injected by an Altex autosampler (Model 500, Beckman RIIC) which has a 100 μl loop (50 p.s.i.). An Altex solvent programmer (Model 420, Beckman RIIC) and an Altex solvent pump (Model 100A, Beckman RIIC) deliver the mobile phase at 1.5 ml/min to a 250 × 4 mm stainless steel column (Waters), packed with μBondapak C18 (Waters). The elution buffer consists of 50 ml 0.01 mol/l NaH_2PO_4 and 50 ml methanol titrated to pH 3.0 with phosphoric acid (BDH, Analar grade).

Eluted melphalan and dansyl proline were detected by a ultraviolet detector (Model LC-UV, Pye Unicam) set at 261 nm wavelength. The ultraviolet spectrophotometer range was 0.01 or 0.02 A.U. The recorder was set at 1 mv and the chart run at 10 cm/h.

The data was processed by a recorder–integrator (Model DP88, Pye Unicam) which measures the areas under peaks on the elution chromatogram. The t_R (retention time) value for melphalan was 6.68 min, and for dansyl proline was 9.57 min.

Standard curves were generated which confirmed the accuracy of the method over the range of melphalan concentrations 0.2–200 μg/ml (correlation coefficient, $r = 0.9894$). The coefficient of variation (S.D./mean × 100) for 20 identical samples at 2 μg/ml in plasma was 4.2%.

Table 1. Tissue concentrations of melphalan at 55 min during ILP, and postoperative toxicity

Patient	Melphalan (μg/g) Tumour	Fat	Skin	Toxicity*
(a)	4.83	1.02	5.36	—
(b)	2.29	0.885	2.2	Grade III
(c)	3.76	0.48	4.95	—
(d)	0.66	1	1.43	—
(e)	1.68	1.045	—	—
(f)	7.85	2.11	5.4	—
(g)	2	1.19	—	—
(h)	3.04	1.56	—	—
(i)	4.64	0.87	2.82	Grade IV
(j)	3.2	1.43	1.9	—
(k)	2.8	0.96	3.19	—
(l)	0.74	3.4	4.5	—
(m)	6.125	—	3.25	—
(n)	6.5	3.5	7.16	—
(o)	0.88	1.3	3.78	—

Statistical comparisons by Wilcoxon signed-ranks test: tumour vs. fat, $P < 0.01$; tumour vs. skin, not significant.
*Toxicity by Wieberdink grade [7].

RESULTS

41 tissue specimens for melphalan assay were taken from 15 patients with recurrent melanoma during ILP after 55 min of perfusion.

The measured tissue levels of melphalan are shown in Table 1. The levels in tumour were significantly higher than levels in surrounding fat ($P < 0.01$, Wilcoxon signed-ranks test), and not significantly different from levels in adjacent normal skin.

Table 2 summarises this physio-pharmacological data. The median AUC was 1442 μg.min/ml (range 988–4011), and the median flow rate was 350 ml/min (range 188–675).

In two cases there was significant regional toxicity—case (b) suffered late blistering at a skin graft site (Wieberdink [7] grade

Table 2. Physio-pharmacological data for individual patients treated by ILP from whom biopsies were obtained to measure melphalan levels

Patient	Dose of melphalan (mg)	AUC (μg.min/ml)	Flow rate (ml/min)	Calf skin temperature (°C)
(a)	100	1130	200	37.2
(b)	140	1649	375	39.1
(c)	120	1275	333	37.8
(d)	70	1144	218	37.9
(e)	90	1204	413	38.7
(f)	135	4011	395	38.3
(g)	140	2720	300	38.0
(h)	130	1158	542	38.5
(i)	180	3117	360	37.6
(j)	100	1508	675	39.4
(k)	84	1442	275	37.1
(l)	100	1129	350	38.5
(m)	100	988	400	38.1
(n)	120	2128	325	38.1
(o)	90	2180	188	37.2

III toxicity), and case (i) suffered post-operative swelling of the limb with troublesome muscular pain, weakness and elevated creatine kinase (Wieberdink grade IV). All other cases in this series were uncomplicated.

DISCUSSION

In only one previous report have melphalan concentrations been measured in the limb perfusate and tissues of 3 patients having isolated limb perfusion [8]. The main findings were that more melphalan was detectable in tumour than in subcutaneous fat, and it was suggested that clinical response may be correlated with tumour concentration of melphalan [8].

The tissue concentrations of melphalan which we measured using a sensitive and specific HPLC assay seem to be lower than those calculated by Benckhuijsen and colleagues [9] but their estimates are derived from a selected subgroup of perfusate time–concentration curves and are expressed in terms of μg/ml of tissue water (approximately equivalent to 60% of tissue mass). Furthermore, as they admit, tumour uptake cannot be calculated from their estimates of total tissue uptake and, as others have shown, distribution within tumour masses may be uneven [10].

Initially we had hoped to obtain sufficient tissue samples for analysis of the time course of tissue levels of melphalan. However, many of our patients were referred after excision of recurrent melanoma and very few patients had multiple tumour nodules large enough for the melphalan assay. We were also restricted to sampling the accessible groin and thigh during ILP because of the heated blanket wrapped round the limb. It was therefore decided to take as many samples as possible at the one time point. Similarly, it was not possible to relate the levels of melphalan in tumour nodules to tumour response because few of our patients had tumour deposits to measure after ILP.

The variability of measured melphalan in our study probably reflects real differences in tissue levels. Our observations of the limb during ILP using ultraviolet light show that the distribution of fluorescence is initially very patchy, becoming more uniform in the foot and calf then spreading more proximally. This is related to distribution by the superficial femoral artery. The main determinant of the adequacy of proximal perfusion is inflow to the profunda femoris circulation. The variable anatomy of the profunda origin and its relationship to the tip of the arterial catheter thus explains differences in proximal tissue perfusion. The tissue levels in the distal part of the limb may well be higher than those we measured in the proximal part.

The assay used to measure melphalan in this study detects free drug in the tissues and it is not certain how this relates to uptake by cells, penetration of nuclei or alkylation of DNA and nucleo-protein. Perhaps simplistically, we believe that in ILP-effective tumour therapy will depend on concentration gradients driving the drug through penetration barriers. The distribution of drug in the tissues depends largely on microscopic vascularity thus the measured levels were lowest in fat. The vascularity of tumour nodules is known to be very variable and we are encouraged that the levels of melphalan in large, often necrotic, tumour nodules were similar to the levels in healthy skin.

We found no simple correlation between tumour levels of melphalan and dose, AUC, flow rate or skin temperature. In this study there was no clear relationship between levels of melphalan in normal tissues and regional toxicity. Other factors which may cause damage to the perfused limb include relative ischaemia, sensitivity of the vascular endothelium, compartment syndromes and nerve compression.

It is encouraging that isolated limb perfusion achieved levels of melphalan in large necrotic tumours which were higher than the levels in fat, and similar to the levels in well-vascularised healthy skin.

There was no relationship between levels of melphalan in normal tissues and regional toxicity.

Having established the methodology, tissue levels of melphalan may be studied in relation to tumour response, site of tumour and as a means of evaluating the effects of attempts to optimise the physiological conditions of bypass.

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