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Chemosensitivity testing of primary cultures of Merkel cell cancer

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Twenty-seven tumor specimens from patients with Merkel cell carcinoma (MCC) were tested for chemosensitivity against a battery of nine cytotoxic drugs in a short-term antimetabolic assay measuring inhibition of thymidine incorporation. Dose–response curves were constructed by plotting drug concentration in μg/ml versus % control [^{3}H]thymidine incorporation. Specimens were considered 'sensitive' to a drug if, at the approximate peak plasma concentration (PPC), the inhibition of [^{3}H]thymidine was greater than 50% when compared with untreated control primary cultures. The assay revealed a 'sensitive' tumor in 19 of 20 specimens and 16 of 17 patients had a tumor that was 'sensitive' to at least one drug tested in the assay system. The highest sensitivity in order of frequency was found with doxorubicin, epirubicin, cyclophosphamide, etoposide and cisplatin. At least 40% of the tumors were 'sensitive' to these five drugs. Cyclophosphamide was chosen as the most active drug (at PPC) in 10 of 19 assays (53%), etoposide in seven of 17 (41%), doxorubicin in four of 19 (21%), chlorambucil in one of 12 (8%) and cisplatin in one of 18 (5%) of assays. Though our results are preliminary, we have identified for the first time a range of cytotoxic drugs which appear effective against MCC *in vitro*. Our main task now is to determine whether our *in vitro* predictive assay will correlate with clinical benefit to the patient.

Key words: Cell cultures, chemosensitivity, Merkel cell carcinoma, predictive assay.

Introduction

Merkel cell cancer (MCC) is increasingly recognized as a highly malignant skin cancer with a recurrence rate of approximately 60% following surgical resection, even when the primary skin lesion is small (less than 2 cm in size) and surgical margins are histologically clear.[1] Recent reports suggest that wide-field, high dose post-operative radiotherapy can reduce the risk of local recurrence[2] which is believed to be the result of intradermal lymphatic permeation by tumor cells. However, a propensity for loco-regional lymphatic and hematogenous dissemination is responsible for the high mortality rate documented for patients with MCC.[2]

Data on the chemosensitivity of MCC are scarce and are limited to a handful of case reports.[3–6] Although clinical response is seen following the administration of several cytotoxic agents (including doxorubicin, cisplatin, etoposide, cyclophosphamide and vincristine), the choice of drugs for an individual patient is often empirical. Furthermore, patients who demonstrate an initial response to a cytotoxic drug not infrequently develop resistance to further administration soon afterwards; other patients run a progressively downhill course despite cytotoxic treatment.

In Queensland, which has the highest incidence of skin cancer in the world, we have developed a large experience in treating patients with MCC. Faced with the dilemma of the apparent chemosensitivity of MCC, difficulties in treatment because of its highly aggressive course, and an increasing number of patients referred with this diagnosis to our center over the past 5 years, we have studied the effects of cytotoxic drugs on MCC in a short-term *in vitro* chemosensitivity assay. Here, we report the results on 27 tumor specimens from 18 patients.

Materials and methods

Tissue specimens

Fresh tumor specimens of histologically-proven MCC were transported immediately from the operating theatre to the laboratory in sterile containers. All procedures were performed in a class II biohazard cabinet and have been described previously. Briefly, tumor tissue was washed in Hanks balanced salt solution (HBSS, Flow

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Laboratories, Australia) containing double strength penicillin, streptomycin and fungizone. Cell suspensions were prepared from tumor tissue by mechanical disaggregation using crossed scalpels. The preparation was gently passed through a 94 μm pore size tissue sieve (Bellco Glass, Vineland, NJ) to separate tissue debris. The resultant solution was then sucked up and down repeatedly in HBSS until multicellular clumps were broken up into single cells. Viability of the resuspended tumor cells was tested by Eosin-Y dye exclusion. In most cases the cell preparation was excellent from the fleshy tumors and resulted in single cell suspensions of greater than 80% viability. In the presence of heavy red blood cell contamination and/or poor cellular viability, the suspensions were passed over a Ficoll-Paque gradient. Five specimens had their volume measured by a simple $l \times b \times h$ assessment and this was used to derive a viable cell yield at the end of the cell preparation procedure.

Chemicals

Doxorubicin, cisplatin, carboplatin, vinblastine, chlorambucil and melphalan were obtained from Sigma (St Louis, MO); epirubicin was obtained from Farmitalia Carlo Erba (Milan, Italy); etoposide from Bristol Meyers (Madrid, Spain). Cyclophosphamide was obtained in the form of 4-hydroperoxycyclophosphamide (ASTA Z-7557) and was kindly provided by Dr J Pohl (ASTA Pharma AG, Germany). RPMI 1640 medium was obtained from Flow Laboratories (Paisley, UK).

Cytotoxic drug treatment and cell culture technique

The cell suspension was adjusted with culture medium (RPMI 1640 with 10% fetal calf serum) to obtain 1×10^6 cells/ml. Antibiotics were not used in cell cultures during the chemosensitivity assays. Tumor cell incubation was carried out in 96-well Millititer filtration plate (HA, Nitrocellulose growth surface, Millipore, Bedford, MA). Care was taken to ensure that the tumor cells were evenly distributed during transfer of 100 μl aliquots of the cell suspension to the culture plates. Cultures were preincubated for 30 min at 37°C in an atmosphere of 5% CO_2/95% air before the addition of cytotoxic drugs. Depending upon cell numbers obtained from the tumor specimens, up to nine cytotoxic drugs (cisplatin, carboplatin, vinblastine, epirubicin, doxorubicin, cyclophosphamide in the form of 4-hydroperoxycyclophosphamide, chlorambucil, melphalan and etoposide) were tested. At least two cytotoxic drugs were tested in triplicate cultures at three concentrations and were freshly prepared before each experiment. Peak plasma concentrations (PPCs) for the drugs used in this study were ascertained by reference to published levels.[7,8] 10 μl of ten times concentrated stocks were added to the test cultures to yield final concentrations in the range of 0.25–25 μg/ml for cisplatin, 1–100 μg/ml for carboplatin and 4-hydroperoxycyclophosphamide, 0.05–5 μg/ml for epirubicin, doxorubicin and etoposide, 0.2–20 μg/ml for chlorambucil and melphalan, and 0.1–10 μg/ml for vinblastine. The experiment was controlled by the inclusion of (1) undrugged cultures, (2) background wells to measure non-specific label binding and (3) a positive cell poison to kill cells (10 μl of 100 μg/ml mercuric chloride). Both control and drug-treated cells were cultured for 20 h at 37°C before the wells were pulsed with 50 μl of 5 μCi/ml methyl-[^{3}H]-thymidine (Amersham, UK) for another 4 h.

Cell harvest

The plates were harvested using a Millititer Plate filtration device. Wells were washed with 100 μl of Dulbecco's phosphate buffered saline, followed by 2×100 μl washes with ice cold 5% trichloroacetic acid and a final wash with 100 μl of 95% ethanol. After drying, individual wells were punched out and prepared for scintillation counting in a LKB-Wallac Rackbeta liquid scintillation counter.

Calculation of results

Results for drug-treated wells were expressed as a percentage of control incorporation of tritiated thymidine in untreated wells. Dose-response curves were constructed by plotting drug concentration in μg/ml versus % control [^{3}H]thymidine incorporation. Specimens were considered 'sensitive' to a drug if, at the approximate PPC, the inhibition of [^{3}H]thymidine was greater than 50% when compared with untreated control cultures.

For an assay to be considered valid the following quality control criteria had to be met: control wells >300 d.p.m. incorporated [^{3}H]thymidine, and background and positive cell poison cultures

Table 1. Reasons for an unsatisfactory result from the assay

	Evaluable results*	Contamination	Technical fault	Insufficient cells
Multiple specimens from four patients	7	2	2	2
Single specimens from 14 patients	13	1	0	0

*Successful assay in 17 out of 18 patients (94%). 13 single specimen patients plus four multiple specimen patients. Successful assay in 20 out of 27 specimens (74%). 13 single specimens plus seven multiple specimens.

<100 d.p.m. The standard error of the means of triplicate cultures were routinely 10–15%.

Results

Technical/methodological considerations

Primary cultures were established from 27 tumor specimens received from 18 patients, (13 specimens from four patients; single specimens from 14 patients). Three specimens were contaminated, two were unsuccessful because of difficulties in cell harvesting and two yielded insufficient cells to set up the minimum criteria for a valid assay (Table 1). An evaluable assay result was obtained in 74% of specimens and in 94% of the patients.

Five of the initial specimens in this series had their volume assessed by $l \times b \times h$ measurements from which a viable cell yield was calculated (Table 2). This varied from approximately 1×10^4 to 2×10^5 viable cells/mm^3 tissue. The mean yield was 5.5×10^4 cells/mm^3 tissue. To assess the chemosensitivity of cells in our assay system to two drugs at three concentrations with appropriate controls requires approximately 1×10^6 viable cells and our results suggest that a volume of approximately 18 mm^3 of tissue is required to achieve this cell number. This was obtained in four out of five specimens examined.

The representative dose–response relationships for a 'sensitive' and a 'resistant' tumor are shown in Figure 1.

Table 2. Viable cell yield from Merkel cell cancers

Specimen no.	Volume of tissue (mm^3)	Viable cell count	Viable cell yield (per mm^3)
1	8	1.4×10^6	1.75×10^5
2	125	8×10^6	6.4×10^4
3	100	7×10^6	7×10^3
4	63	5×10^5	8×10^3
5	512	1×10^7	1.9×10^4
Mean	$\sim 5.5 \times 10^4$		

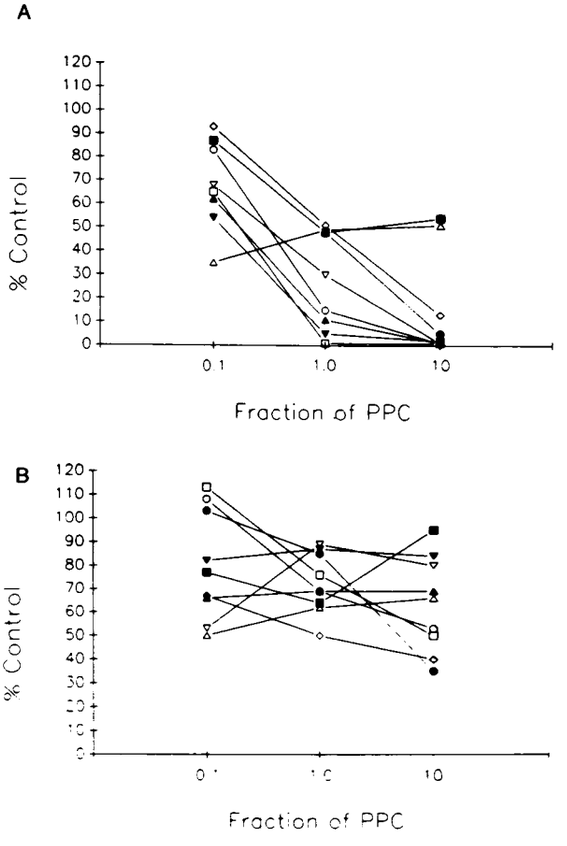

Figure 1. Chemosensitivity profiles of (A) 'sensitive' and (B) 'resistant' tumors. (○) Cisplatin, (●) carboplatin, (△) vinblastine, (▲) epirubicin, (□) cyclophosphamide, (■) chlorambucil, (▽) melphalan, (▼) doxorubicin, (◇) etoposide.

Chemosensitivity of primary cultures

Overall, the assay revealed a 'sensitive' tumor in 19 of 20 specimens and 16 of 17 patients had a tumor that was 'sensitive' to at least one drug tested in the assay system. The highest sensitivity, as defined by the assay, in order of frequency, was found with doxorubicin, epirubicin, cyclophosphamide, etoposide and cisplatin (Table 3). At least 40% of the tumors were 'sensitive' to these five drugs. The cytotoxic drugs least effective in the assay were vinblastine and carboplatin. We note with interest that only one tumor demonstrated a 'resistant' profile for all nine drugs used in our assay. In the patient in whom three sequential tumor specimens were available for testing and the results were evaluable, there was a change in sensitivity with time. Initially, the tumor specimen was found to be 'sensitive' to doxorubicin and cyclophosphamide, but the second specimen was only 'sensitive' to cyclophosphamide, and the third only to etoposide.

While most tumor specimens were 'sensitive' to several cytotoxic drugs (Table 3), not all specimens were exposed to the complete battery of nine cytotoxic drugs. Cyclophosphamide was chosen as the most active drug (at PPC) in 10 of 19 assays (53%), etoposide in seven of 17 (41%), doxorubicin in four of 19 (21%), chlorambucil in one of 12 (8%) and cisplatin in one of 18 (5%) of assays. In three assays both doxorubicin and etoposide were rated as producing equivalent inhibition of [^{3}H]thymidine uptake.

Discussion

A relatively large number of referrals has provided us with a unique opportunity to study the *in vitro* chemosensitivity of MCC, a highly lethal skin tumor for which the standard treatment at the Royal Brisbane Hospital consists of wide surgical excision followed by high dose radiotherapy to the site of excision and the primary echelon lymphatics.[2] One of us (JHK) has previously reported that MCC is sensitive to a range of cytotoxic drugs in the clinic;[3] in practice, patients with metastatic MCC are usually treated with the same drugs as are used for patients with small cell lung cancer. However, as many patients with disseminated MCC are elderly and frail, some modification of cytotoxic drug choice and dosage is often required.

The results of our *in vitro* chemosensitivity assay for Merkel cell tumor are important for several reasons. Firstly, we have demonstrated that the *in vitro* technique is simple, reproducible, and usually more successful than the results which we have reported for ovarian cancer specimens using the same methodology.[9,10] The small size of Merkel tumor cells allows adequate numbers of cells to be obtained as a single cell suspension even for small (less than 1 cm^3) operative specimens. In addition, Merkel cell tumors appear to have a low propensity towards spontaneous necrosis and disaggregation occurs easily. Dye exclusion results consistently demonstrate a high level of cellular viability in the resultant single cell suspensions. The validity of the assay, however, is yet to be established with *in vivo* clinical correlation; at present, most patients proceed directly to radical radiotherapy following surgical excision. Nevertheless, the results reported herein confirm our previous clinical observation that the most active drugs reported against MCC (and other small cell carcinomas) are those predicted by our assay.

The basis of our *in vitro* assay is the measurement of the effect of cytotoxic drugs by the incorporation of radioactive precursors into nucleic acids.[10] As many of the available cytotoxic drugs exert an effect at some stage in the metabolism of nucleic acids, the rate of nucleic acid synthesis may be used to detect metabolic cell death. It has previously been demonstrated that results of this assay correlate highly with those determined by the soft-agar clonogenic assay.[11] However, the short-term assay offers several advantages over the soft-agar assay, such as a higher successful culture rate, avoidance of clumping artefact, shorter time course for results (3 days) and a low false-negative rate. Volm *et al.*[12] and others[13] have used a similar short-term assay system to detect proliferation-dependent and induced tumor-resistance to cytotoxic drugs and

Table 3. Ranked sensitivity to individual drugs tested in assay

Assay result	Drugs tested in assay[a]								
	DO	EP	CY	ET	CP	ME	CH	CB	VB
'Sensitive' tumor									
no.	12	5	10	8	8	3	4	3	1
%	63	56	52	47	44	33	33	20	6
'Resistant' tumor									
no.	7	4	9	9	10	6	8	12	17
%	37	44	48	53	56	67	67	80	94
Ranking	1	2	3	4	5	6	6	8	9

[a] Key to drug abbreviation: DO, doxorubicin; EP, epirubicin; CY, cyclophosphamide; ET, etoposide; CP, cisplatin; ME, melphalan; CH, chlorambucil; CB, carboplatin; VB, vinblastine.

have reported a close correlation between *in vitro* sensitivity to doxorubicin and *in vivo* clinical response. Similarly, Sondak *et al.* have reported that cytotoxic combinations were not significantly better than the best single cytotoxic drug in either a clonogenic survival assay[14] or an antiproliferation assay.[15]

We have accumulated an increasing experience with this assay for MCC and, for the first time, we have defined a range of cytotoxic drugs, i.e. doxorubicin, epirubicin, cyclophosphamide and etoposide, which appear effective against MCC *in vitro*. The main task now is to determine whether our *in vitro* predictive assay will correlate with clinical benefit to the patient. If our *in vitro* results are validated, this assay will be useful for clinical management by enabling the clinician to select the most active chemotherapy drugs and/or avoid the use of ineffective drugs in patients who are so often elderly and frail.

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