The in-vitro chemosensitivity of three cell lines derived from the VM/DK spontaneous murine astrocytoma

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SUMMARY Three cell lines, VM/Dk P497 P540 and P560 derived from the VM spontaneous murine astrocytoma have previously been fully characterised and found to differ in their degree of astrocytic differentiation. The in vitro chemosensitivity of the three lines has been investigated using the ³⁵S-methionine uptake assay. Differential chemosensitivity was found to exist between the cell lines. The pattern of chemosensitivity in relation to astrocytic differentiation was complex but the least differentiated cell line, P497, tended to be the least chemosensitive.

Wilson and Bates¹ have proposed that the ideal brain tumour model for chemotherapy studies should be "a serially transplantable glioblastoma in the mouse". In addition Wilson² has suggested that an ideal animal model for human glioma should be spontaneously arising, of glial origin, capable of intraparenchymal growth, uniformly fatal within a reasonable time period, capable of in vitro growth, transplantable both intracranially and subcutaneously in syngeneic animals and correlate with the therapeutic sensitivities of human gliomas. The choice of agents in current use for the chemotherapy of glioma has largely depended on results obtained from experimental models. Few, if any of these fulfil the criteria for an ideal therapeutic model of human glioma.

The inbred VM strain of mice has a high incidence of spontaneous astrocytomas.³ From one of these spontaneous murine astrocytomas (SMA) three permanent cell lines P497, P540 and P560 have been derived.⁴ These cell lines have been extensively characterised both in vitro and in vivo and have been shown to retain astrocytic features.⁵⁻⁸ They express the two astrocytic immuno-markers glial fibrillary acid protein (GFAP) and glutamine synthetase.⁷ In addition, transmission electron microscopy and immunocytochemistry have revealed that the lines show variation in their degree of astrocytic differ-

entiation. Cell line P560 contains 10 nm filaments in 98% of cells and shows a strong positive cytoplasmic reaction for GFAP in vitro and is considered the best differentiated of the three lines.⁶ The least differentiated cell line P497 contains 10 nm filaments in only 15% of cells and is only weakly positive when stained for GFAP in vitro. Cell line P540 is of intermediate differentiation.

As an initial stage in the development of an in vitro—in vivo therapeutic model of glioma based on the VM cell lines we have investigated the in vitro chemosensitivity against a wide range of cytotoxic agents to determine if differential sensitivity exists among them and whether this is related to their degree of astrocytic differentiation.

Materials and methods

Cell Lines The cell lines VM/Dk P497, P540 and P560 were originally the gift of Dr DD Bigner, Duke University Medical Centre, USA. These cell lines were stored at the Institute of Neurology in the liquid nitrogen freezer. Chemosensitivity assays were performed on cell lines P497, P540 and P560 at in vitro passage levels 34-39, 40-45 and 24-29, respectively. These passage levels were chosen since they fell within the range for which pathological characterisation had been performed.⁷

Maintenance of cells in culture The methods used for the maintenance of cell cultures have previously been described.⁷

Drugs Drugs used in this study together with the range of concentrations are given in table 1. Drugs were dissolved in Ham's F10 medium (without foetal calf serum) apart from the nitrosoureas which were dissolved in absolute ethanol and C-PLAT which was dissolved in normal saline. Stock

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Table 1 Chemotherapeutic agents and range of concentrations used in chemosensitivity assays

Drug	Abbreviation	Concentration range on microtitration plate ($\mu\text{g/ml}$)
Procarbazine	PCB	1000-4
Vincristine	VCR	10^{-3} - 12×10^{-6}
CCNU*	CCNU	40-0.013
BCNU†	BCNU	40-0.013
Vindesine	VDS	10^{-3} - 12×10^{-6}
Adriamycin	ADM	10^{-10} - 10^{-4}
Cis-platinum	C-PLAT	10^{-10} - 10^{-4}
Bleomycin	BLM	10^{-10} - 10^{-4}
5-Fluorouracil	5-FU	10^{-10} - 10^{-4}

*1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea

†1,3-bis(2-chloroethyl)-1-nitrosourea

solutions were stored at -70°C . Drug breakdown was not determined but assumed to be minimal at this temperature. Drug solutions were all diluted in Ham's F10 plus 10% v/v foetal calf serum with 50 IU/ml penicillin and 50 $\mu\text{g/ml}$ streptomycin and buffered with 20 mM Hepes.

Chemosensitivity Assay The chemosensitivity assay used was the ^{35}S -methionine assay which has been described in detail elsewhere.^{9,10} Briefly, monolayer cultures of P497, P540 and P560 were trypsinised in exponential growth phase and 96 well microtitration plates (Sterilin, UK) seeded with 2×10^3 cells per well. After 24 hours incubation at 37°C , medium was removed by suction and 0.2 ml of each drug dilution was added to quadruplicate wells of the plate. Two wells of each row were left free of drugs to act as controls. The drug containing medium was renewed 24 and 48 hours later to give a total drug exposure time of 72 hours. Drug solutions were then removed by suction and each well washed three times in HBSS and then filled with 0.2 ml of fresh growth medium. Following a recovery period long enough to include one population doubling time 0.1 ml of $2 \mu\text{Ci/ml}$ ^{35}S -methionine was added to each well and incubated for 4 hours. The plates were then washed in HBSS, fixed in methanol, extracted in ice-cold 10% trichloroacetic acid (BDH Chemicals, UK), washed in tap water and dried in methanol. Fifty microlitres of toluene based scintillation fluid (NE233, Nuclear Enterprises, UK) was added to each well and the plates dried by centrifugation. Autofluorograms were prepared by placing a sheet of X-ray film (Kodak, UK) under each plate and exposing them for 24-48 hours at -70°C . Quadruplicate determinations for each drug were performed on two separate occasions. The autofluorograms were analysed with a MR600 microplate reader (Dynatech, UK) which presents data as absorbance units. Using the absorbance reading for each well the incorporation of labelled amino acid was expressed as percentage of the mean of the control in that row and the dose of drug that inhibited protein synthesis by 50% (ID_{50}) determined graphically. The mean value of the ID_{50} for each drug was then calculated. Pairwise comparison of mean ID_{50} 's was performed using the Scheffé test.¹¹

For all experiments cell counts were made daily of replicate plates of each cell line to determine the population doubling time and to ensure that cultures remained in exponential growth throughout the assay.

Results

The population doubling times for the three cell lines P497, P540 and P560 were 27.5, 30.5 and 34.8 hours respectively.

The results are shown in the fig and table 2. It is clear that each cell line exhibits a different pattern of sensitivity to the nine drugs. Statistical analysis of the data was carried out using the Scheffé test which is a conservative method of performing multiple pairwise comparisons of means. P497 was significantly more resistant ($p < 0.05$) to PCB, 5-FU and VDS than P540, similarly, P497 was significantly more resistant to PCB, 5-FU and BLM than line P560. Cell line P560 was also more sensitive than P497 to C-PLAT and BCNU. Cell line P540 was significantly more sensitive than P560 to PCB, 5-FU and the vinca alkaloids, VCR and VDS but less sensitive to C-PLAT. Table 2

Fig The spectrum of in vitro chemosensitivity of the three VM/Dk cell lines.

Table 2

Drug	Mean ID ₅₀ ug/ml ± SE			
	P497	P540	P560	Fold difference*
PCB	543.0 ± 23.9	79.0 ± 5.0	218.0 ± 7.5	6.9
VCR	0.00018 ± 0.00004	0.00018 ± 0.00005	0.0012 ± 0.00013	6.7
CCNU	3.68 ± 0.27	2.25 ± 0.18	2.20 ± 0.35	1.7
BCNU	3.56 ± 0.39	1.75 ± 0.09	0.70 ± 0.06	5.8
VDS	0.0033 ± 0.0009	0.00014 ± 0.00001	0.0032 ± 0.0003	23.6
ADM	0.02 ± 0.002	0.014 ± 0.002	0.035 ± 0.0018	2.5
C-PLAT	0.17 ± 0.03	0.20 ± 0.02	0.043 ± 0.02	4.7
BLM	0.16 ± 0.036	0.031 ± 0.004	0.062 ± 0.01	5.2
5-FU	1.68 ± 0.26	0.03 ± 0.002	0.19 ± 0.02	56.0

*Least to most sensitive

also shows the number of fold difference in the ID₅₀ between the least and most sensitive cell line. The greatest range in sensitivity occurred between P497 and P540 for the cell cycle specific agents VDS and 5-FU where the ID₅₀'s varied between 23.1 and 56.0 fold respectively.

The cell lines were ranked in order of sensitivity (ID₅₀) from least to most sensitive for each drug and shown in table 3. No single cell line was consistently most or least sensitive to all of the nine drugs. However, the cell line considered to be the morphologically least differentiated, P497, tends to be less sensitive to more drugs than either of the other two lines. It has equal or higher ID₅₀'s to all nine drugs when compared with the line of intermediate differentiation, P540. This trend is continued when P497 is compared with P560 which displays the greatest degree of astrocytic differentiation, although P560 is significantly more resistant to VCR than either of the other two lines. No clear pattern of ranking with regard to chemosensitivity emerges between cell lines P560 and P540.

Discussion

The ³⁵S-methionine uptake assay has been used extensively in our laboratory for in vitro chemosensitivity studies on a large number of human glioma short term cultures. A range of sensitivity for 120 cultures has been established for PCB, VCR and CCNU.¹² The mean ID₅₀ of the three VM/Dk cell lines for these three drugs falls within the range determined for human glioma cultures. There is therefore some correlation, at least in vitro, in therapeutic sensitivity between the VM/Dk cell lines and human glioma cultures.

Differential chemosensitivity of cell lines derived from a single neoplasm has been described previously in both human^{13 14} and animal tumours.¹⁵ The data presented in this paper demonstrate that sensitivity to a given agent is also expressed differentially by the three VM/Dk cell lines. We can at present only spec-

ulate on the reasons for the differences in chemosensitivity. Various mechanisms have been proposed to explain differential drug sensitivities between tumour cells. Differences in cell membrane properties may alter the transport of drugs into or out of cells.¹⁶ Intracellular drug metabolism may also differ such that there is variability in the conversion of anticancer drugs to active or inactive forms.¹³ The presence of alternative biochemical pathways may allow the cell to bypass the drug inhibited step.¹⁷ The ability of the cell to repair drug induced DNA damage is also an important factor for the survival of cells damaged by chemotherapy.¹⁸ For each agent any one of these mechanisms may be responsible for the pattern of chemosensitivity which has emerged for the three VM/Dk cell lines.

Patterns of cross-resistance and collateral sensitivity to chemotherapeutic agents have been investigated in a number of different tumour systems. Many inconsistencies have arisen between studies making interpretation of data difficult. It is of interest, however, that for the agents for which P497 is not the least sensitive, that is, ADM, VCR and C-PLAT, previously reported patterns of cross-resistance and collateral sensitivity exist. Cell line P560 has the highest ID₅₀ to ADM and VCR. Cross-resistance between these two drugs has been reported frequently.^{19 20} In

Table 3 Relative sensitivity of three VM/DK SMA derived sublines

Drug	Ranking*
PCB	P497 > P560 > P540
VCR	P560 > P540 = P497
CCNU	P497 = P540 = P560
BCNU	P497 > P540 = P560
VDS	P497 = P560 > P540
ADM	P560 = P497 = P540
C-PLAT	P540 = P497 > P560
BLM	P497 > P560 = P540
5-Fu	P497 > P560 > P540

*Least to most sensitive

†Significant difference (p < 0.05)

resistant tumour sublines, these agents can be shown to enter the cell but are then actively transported out. This results in a relatively low intracellular level of drug and thus to low cytotoxicity.^{21 22} A common cellular efflux process could explain cross-resistance between the two drugs. P540 is the least sensitive to only one drug, C-PLAT, but is considerably more sensitive than the other two cell lines to the vinca alkaloid VDS. An impressive collateral sensitivity for VDS has been reported *in vivo* in a subline of the Ehrlich ascites tumour selected for resistance to C-PLAT.²³ The mechanism of this collateral sensitivity remains to be explained and indeed it was not found in one of the few other studies of these two drugs. McMillan and coworkers²⁴ reported cross-resistance between C-PLAT and VDS in a C-PLAT resistant murine mammary carcinoma. Clearly more data are required about these two drugs from different tumour systems before firm conclusions can be made, although some encouraging results have been obtained with a combination of VDS and C-PLAT in clinical chemotherapy.²³

Little is known about differences in therapeutic sensitivities between human gliomas of different grades. Low grade gliomas are only rarely treated with chemotherapy and therefore clinical correlations are difficult. Indirect evidence from Burger and Vollmer²⁵ suggests that high grade gliomas should be more responsive to adjuvant therapy. These authors estimated the cellularity of gliomas and correlated this with patient survival. They found a positive correlation between cellularity and survival in a group of patients treated with postoperative chemotherapy and radiotherapy. It was concluded that this was consistent with the idea that more primitive cells are more susceptible to these treatments. Animal models of glioma, which have been used extensively for therapeutic studies²⁶ have provided little information on the relationship of tumour differentiation and chemosensitivity. The ependymoblastoma model of glioma comprises a group of four tumours said to be histologically similar.²⁷ One of these tumours Ependymoblastoma A is a mutant subline of the original ependymoblastoma and is considered to be more malignant than the parent line.²⁷ These two tumours, however, show similar sensitivity in drug treated tumour-bearing mice.²⁸ Human gliomas of various grades have been transplanted intracranially into nude mice.²⁹ Generally, grade III and IV gliomas take and grow in a more predictable fashion than lower grade tumours²⁹ and to date therapeutic experiments have not been reported on low grade human glioma xenografts. High grade gliomas growing in nude mice respond to clinically effective agents such as the nitrosoureas. However, different tumours of similar histological grade do not respond in the same way

to any one particular agent.^{30 31} Kornblith and coworkers³² have also found that pathological grading of glioma is not strictly related to chemosensitivity. They studied the sensitivity of 42 gliomas of grade III and IV to BCNU using an *in vitro* microcytotoxicity assay. A wide variation in sensitivity existed between tumours of the same grade.

We have found that the pattern of chemosensitivity in relation to the degree of astrocytic differentiation is complex. Morphologically P497 is the least differentiated of the three cell lines.⁶ It is only weakly GFAP positive and contains 10 nm filaments in only a small percentage of cells. The presence of 10 nm filaments within cells is thought to be a reliable guide to the degree of astrocytic differentiation in experimental brain tumours.³³ P497 has a tendency to be the least chemosensitive of the three cell lines, although the differences in ID₅₀ for some of the agents is small. There is a theoretical basis for relative chemoresistance in less differentiated tumour cells. One mechanism of repair of DNA damage is associated with the degree of cellular differentiation. This involves the repair enzyme poly-ADP-ribose polymerase which is activated by DNA strand breaks.³⁴ Higher poly-ADP-ribose polymerase activity is generally found in less differentiated, more rapidly growing cells.¹⁸ Less differentiated cells, such as P497, may have a higher capacity for the repair of DNA damage and therefore a greater ability to survive the effects of chemotherapeutic agents. Secondly gene amplification, which appears to be of considerable importance in developing drug resistance can easily be induced in undifferentiated cells whereas tumour cells showing some degree of differentiation undergo gene amplification only with difficulty.³⁵

Although a number of animal brain tumour models have been developed for use in chemosensitivity studies²⁶ none has been based upon a tumour which has arisen spontaneously. Of the animal models in use the 9L gliosarcoma in the rat is the only one to date which has been refined for both *in vitro* and *in vivo* chemotherapy studies.^{36 37} The tumour is biologically unstable, however, and whether its current sarcomatous nature detracts from clinical applications is unclear. The three cell lines derived from the VM/Dk spontaneous astrocytoma offer the potential for the development of an animal brain tumour model which fulfills many of the criteria of Wilson.² In addition it allows the relationship between chemosensitivity and astrocytic differentiation to be explored both *in vitro* and *in vivo*. The present study forms the basis for further development of this model in which *in vivo* chemosensitivity of the three cell lines will be determined. Whether the *in vitro* pattern of differential sensitivity observed in this study will be reflected in the animal model remains to be seen.

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