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A Study of the Expression of Four Chemoresistance-related Genes in Human Primary and Metastatic Brain Tumours

Mireille Mousseau, Christiane Chauvin, Marie-France Nissou, Max Chaffanet, Dominique Plantaz, Basile Pasquier, René Schaerer and Alim Benabid

We investigated four mechanisms of intrinsic chemoresistance in a series of 67 human brain tumours including 31 gliomas (one grade I ganglioglioma, nine grade II and 10 grade III astrocytomas, 11 glioblastomas), 13 cerebral metastases, one medulloblastoma, one malignant teratoma, three ependymomas and 18 meningiomas. We studied four genes by northern blotting: multidrug-resistance (MDR 1), glutathione-s transferase (GSTπ), dihydrofolate reductase (DHFR), and topoisomerase II (Topo II). The Topo II gene was absent in the normal adult brain (100%) and in 64% of the tumour samples tested. A second gene, GSTπ, was found to be overexpressed in 38% of brain tumours. The two other chemoresistance-related genes were occasionally overexpressed in brain tumours (2% for MDR1, 9% for DHFR). Our results provide evidence that chemoresistance is intrinsic to the brain tissue and seems likely to be a multifactorial process.
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INTRODUCTION

BRAIN GLIOMA patients have an especially low 5 year survival rate, ranging from 0% to 38%, with a mean survival time of less than 1 year [1]. These tumours, because of their volume and/or their localisation, are often not accessible to locoregional treatments such as surgery or radiotherapy. Thus, numerous chemotherapy trials have been undertaken, with only low efficiency [1]. These disappointing results have been frequently attributed to the lack of efficient drug penetration across the blood–brain barrier [2]. This concept is discussed because the tumours disrupt the normal blood–brain barrier [2]. However, preclinical and clinical pharmacology data indicate that the drug concentration is often low in brain tumours but lower in the apparently normal brain tissue surrounding the tumour, which may contain infiltrating cancer cells [2]. These dispersed cells may represent the "stem cells" that can repopulate the tumour and that are the most chemoresistant cells [3]. We report here the results of our investigations on the mechanism of chemoresistance of brain tumour cells.

In this report, four main mechanisms of tumour cell resistance

are described: the overexpression of the multidrug resistance gene (MDR1) [4], of glutathione S-transferase (GSTπ) [5, 6], of dihydrofolate reductase (DHFR) [7] and the loss of topoisomerase II (Topo II) activity [8]. The mechanisms of tumour cell resistance are different according to the drug family.

The overexpression of the MDR1 gene is associated with a multidrug resistance phenotype to plant alkaloids and some antitumour antibiotics [4]. Another type of resistance, constituting an important cellular detoxifying process is represented by the overexpression of GSTπ [5, 6] which induces a resistance to alkylating drugs and antitumour antibiotics. A more specific resistance to methotrexate (MTX) is due to the overexpression of DHFR which can be the consequence of cell exposure to a variety of agents unrelated to MTX (hypoxia, alkylating agents, ultraviolet irradiation, phorbol esters, cisplatinum, doxorubicin, and 5-fluoro-deoxyuridine) [9].

In contrast the loss of topoisomerase II activity induces resistance to intercalating agents (anthracyclines, ellipticines, dactinomycin) or non-intercalating agents (epipodophyllotoxins) [8].

To date in the case of gliomas, the overexpression of the MDR1 gene has been observed in two human glioma cell lines [10] and an overexpression has occasionally been observed in human surgical glioma specimens [11]. The expression of GSTπ in gliomas is enhanced along with the degree of malignancy [12], but nothing is known about the two other mechanisms of chemoresistance (DHFR, Topo II) in these tumours.

We therefore investigated these four genes in a wide range of human brain tissues in order to document their involvement in intrinsic chemoresistance.

MATERIALS AND METHODS

Materials

Sixty seven human primary and metastatic brain tumour specimens were obtained during surgery, immediately frozen in liquid nitrogen and stored in sterile conditions at −80°C. The following tumours were collected (Table 1):

49 malignant tumours: 31 gliomas (one grade I ganglioglioma, nine grade II astrocytomas, 10 grade III astrocytomas, 11 glioblastomas), 13 cerebral metastases (from three epidermoid lung carcinomas, eight adenocarcinomas from the breast (3 cases), the lung (2 cases), an unknown primary (2 cases) and the colon (1 case), one Ewing's sarcoma, one poorly differentiated lung carcinoma), one medulloblastoma, one malignant teratoma, three ependymomas and 18 benign tumours (meningiomas). The majority of these patients (64/67) had received neither radiotherapy nor chemotherapy prior to the surgical sampling of their tumour. Two samples were obtained after prior radiotherapy and one sample (one ependymoma) was obtained after prior chemotherapy. Control specimens included 12 normal human brain tissues obtained from cortectomy performed for the surgical treatment of epilepsy. These normal specimens include four white, four grey, four mixed brain tissues. Other control specimens include two peritumoral brain tissues and 13 cell lines.

Correspondence to M. Mousseau.
M. Mousseau and R. Schaerer are at the Unité d'Oncologie Médicale, B. Pasquier is at the Service d'Anatomo-pathologie, C.H.U.R.G., BP 217, 38043 Grenoble cedex 9; and C. Chauvin, M.F. Nissou, M. Chaffanet, D. Plantaz and A. Benabid are at the INSERM U 318, Neurobiologie préclinique, Université Joseph Fourier, CHRU Pavillon B, BP 217, 38043 Grenoble cedex 9, France.
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We selected KB lines for their known chemosensitivity or chemoresistance phenotype and we decided to study other cell lines currently available in our laboratory. Two cell lines, KB_{3-1} and KB_{8-5} were obtained from J. Benard (Institut Gustave Roussy, Villejuif, France) and from I. Pastan and M. M. Gottesman (NIH, Bethesda, Maryland). KB_{3-1} is the drug-sensitive parental KB (HeLa) cell line. KB_{8-5}, which is resistant to doxorubicin and vinblastine, was derived from KB_{3-1}; the KB_{8-5} cells have an increase in MDR_1 mRNA without gene amplification. The other nine cell lines were obtained from the American Type Tissue Collection. We had six human cell lines: KB (derived from an epidermoid carcinoma), A_{431} (vulvar epidermoid carcinoma), $CaKi_2$ (renal carcinoma), IMR_{32} (neuroblastoma), EJ (bladder carcinoma), HL_{60} (promyelocytic leukaemia). We also used three rodent cell lines: C_6 (malignant glial tumour), NS_{20} (neuroblastoma), Y1 (adrenal tumour) and we tested two other cell lines established in our laboratory from a grade III astrocytoma (A line, HBT 38) and from a grade IV glioma (D line).

Methods

RNA extraction and electrophoresis. Total RNA was prepared from tumours according to the method of Chirgwin [13]. The tissues were homogenised in 4 mol/l guanidinium isothiocyanate containing 0.1 mol/l β-mercaptoethanol and 0.5% sarcosyl in 5 mmol/l sodium citrate, followed by centrifugation through a 5.7 mol/l CsCl cushion. The RNA pellet was extracted with phenol and chloroform/isoamyl-alcohol, precipitated with 2.5 volumes of ethanol and dissolved in water.

The RNA was electrophoresed in 1% agarose–6% formaldehyde gels. Twenty micrograms of total RNA were loaded per lane. Only samples in which the ribosomal RNA appeared undegraded (ethidium bromide control) were analysed.

Chemoresistant and actin gene probes. These were removed from the appropriate plasmids by restriction enzyme digestion, and labelled with α-^{32}P dCTP (29.6 TBq/mmol) by random priming. The human MDR1 probe was a 1.2 kb *Eco*R 1 3' end fragment of cDNA cloned in pHU (a generous gift of W.L. McGuire, San Antonio, Texas). The topoisomerase II probe was a 1.8 kb *Eco*R 1 fragment of cDNA cloned in SK (+)/pC_{15}, given by L.F. Liu (Baltimore, Maryland). The dihydrofolate reductase probe was a 1.8 kb *Eco*R 1 fragment and upstream sequences cloned in $pCHB_{213}$ given by A. Murphy and R.T. Schimke (Stanford, California). The glutathione-S-transferase π probe was a 0.8 kb *Eco*R 1 fragment of cDNA cloned in $PGEM_4$, given by K.H. Cowan (NCl, Bethesda, Maryland). The β actin probe was a 1.2 kb *Pst*I restriction fragment from plasmid PBR 322, as control for the equal amount of mRNA.

Hybridisation methods. Northern blots. Filters were incubated for 24 h at 42°C in 50% formamide, 5 × SSPE, 5 × Denhart's solution, 0.5% sodium dodecyl sulphate (SDS) and 200 μg/ml denatured herring sperm DNA, with 12.5 ng/ml of probe. After hybridisation, filters were washed at high stringency (to 0.1 × SSPE/0.1% SDS for 1 h at 42°C) and exposed to Agfa (Curix RP1) film for 1 to 10 days at −80°C using intensifying screens.

Quantification. The intensity of mRNA bands in various types of tumours selected after ethidium bromide control was calculated as a gene/β-actin ratio measured by serial dilutions in dot blot hybridisation and is expressed in arbitrary units relative to the expression in normal brain and in cell lines with known

Table 1. Summary of human primary and metastatic brain tumours

Type	Human brain tumour no.	Age/sex	Histology WHO classification	Survival (months)
Grade I glioma	HBT127	—/F	Ganglioglioma	—
Grade II gliomas	HBT3	35/M	Oligo astrocytoma	D(36)
	HBT21	36/F	Oligodendroglioma	D(25)
	HBT34	33/F	Astrocytoma	D(84)
	HBT51	32/M	Astrocytoma	—
	HBT93	26/F	Astrocytoma	A(62)
	HBT94	36/M	Astrocytoma	A(59)
	HBT95	14/M	Astrocytoma	A(8)
	HBT96	13/F	Astrocytoma	A(10)
	HBT113	0.6/F	Astrocytoma	A(5)
Grade III gliomas	HBT7*	27/M	Astrocytoma	D(36)
	HBT14	40/M	Astrocytoma	—
	HBT15	66/F	Astrocytoma	D(5)
	HBT19*	30/F	Astrocytoma	D(78)
	HBT35	48/M	Astrocytoma	D(8)
	HBT38	63/M	Astrocytoma	D(4)
	HBT49	44/F	Astrocytoma	D(11)
	HBT65	50/F	Astrocytoma	D(7)
	HBT89	64/M	Astrocytoma	D(3)
	HBT90	53/F	Astrocytoma	D(20)
Grade IV gliomas	HBT5	73/M	Glioblastoma	—
	HBT13	55/F	Glioblastoma	D(12)
	HBT16	32/M	Glioblastoma	—
	HBT20	55/M	Glioblastoma	D(30)
	HBT40	41/M	Glioblastoma	D(1)
	HBT43	69/F	Glioblastoma	—
	HBT44	69/M	Glioblastoma	D(4)
	HBT48	60/F	Glioblastoma	D(14)
	HBT97	—/F	Glioblastoma	D(7)
	HBT98	64/F	Glioblastoma	D(5)
	HBT108	—/M	Glioblastoma	D(4)
Cerebral metastases	HBT1	65/M	Epidermoid carcinoma (lung)	D(60)
	HBT23	50/M	Adenocarcinoma (lung)	—
	HBT41	62/F	Adenocarcinoma (breast)	D(21)
	HBT63	59/F	Adenocarcinoma (unknown origin)	D(15)
	HBT76	42/M	Epidermoid carcinoma (lung)	—
	HBT88	42/F	Adenocarcinoma (breast)	D(6)
	HBT100	35/M	Adenocarcinoma (colon)	—
	HBT101	54/F	Poorly differentiated carcinoma (lung)	A(16)
	HBT102	49/M	Adenocarcinoma (unknown origin)	—
	HBT103	29/M	Ewing sarcoma	D(9)
	HBT104	63/M	Epidermoid carcinoma (lung)	—
	HBT105	63/F	Breast adenocarcinoma	—
	HBT110	50/M	Adenocarcinoma (lung)	D(22)
Medulloblastoma	HBT123	10/M	Medulloblastoma	A(17)
Malignant teratoma	HBT82	7/M	Teratoma	—
Ependymomas	HBT91	—/F	Anaplastic ependymoma	—
	HBT122	—/M	Ependymoma prechemotherapy	A(35)
	HBT128	—/M	Ependymoma postchemotherapy	A(35)
Meningiomas	HBT31	72/F	Meningotheliomatous	—
	HBT33	74/F	Psammomatous	D(12)
	HBT45	70/F	Fibroblastic	—
	HBT55	76/F	Transitional	—
	HBT56	74/F	Fibroblastic	—
	HBT57	56/F	Transitional	—
	HBT58	29/F	Meningotheliomatous	—
	HBT59	56/F	Meningotheliomatous	D(0,75)
	HBT60	48/M	Angioblastic hemangiopericytic	—
	HBT64	33/F	Meningotheliomatous	—
	HBT66	59/M	Meningotheliomatous	—
	HBT67	69/M	Transitional	—
	HBT69	53/M	Meningotheliomatous	A(28)
	HBT72	48/F	Fibroblastic	A(30)
	HBT74	37/F	Transitional	A(11)
	HBT77	66/M	Meningotheliomatous	A(3)
	HBT92	37/M	Meningotheliomatous	A(25)
	HBT114	59/F	Transitional	A(17)

*Recurrent tumours, previously treated by cranial irradiation.
A = alive; D = deceased; — = unknown.

overexpression. For sequential hybridisation RNA–DNA from the same filter with various probes, filters were stripped by incubation at 65°C in 5 mmol/l Tris–HCl pH 8.0, 2 mmol/l EDTA, 0.1 × Denhart's solution.

RESULTS

The expression of four chemoresistance related genes was studied in material from a series of 67 human brain tumours. The results are summarised in Table 2, where 59 tumours were screened for the MDR1 gene, 47 for the DHFR gene, 32 for the GSTπ gene and 42 for the topoisomerase II gene.

Overexpression study of GSTπ, MDR 1 and DHFR genes

In 12 normal brain specimens the GSTπ gene (0.7 kb mRNA) was expressed by the same band density.

The GSTπ gene was overexpressed in four tumours (two metastases of one epidermoid and one glandular lung carcinoma: 10-fold, one ependymoma: 10-fold, one grade I ganglioglioma: 5-fold) and was less overexpressed (3-fold) in three meningiomas, three grade II gliomas, one grade III glioma, one cerebral metastasis of a breast carcinoma (Fig. 1). The accumulation of GSTπ mRNA was only found in non-cerebral tumour cell lines: KB_{3-1} and EJ (3-fold), HL_{60} (5-fold), A_{431} (10-fold). GSTπ is found activated in 12/32 of human brain tumours (38%), more precisely in 6/18 glial brain tumours, in 3/8 metastases and in 3/6 meningiomas (Table 2).

The size of the MDR1 gene transcript was as expected (4.5 kb mRNA) in the 12 normal brain specimens. An accumulation of the MDR1 mRNA (Fig. 2) was observed in 1/59 tumour: one grade IV glioma (3-fold). MDR1 gene was not overexpressed in any of the cell lines studied, except KB_{8-5}.

The DHFR gene transcript size (4.8 kb mRNA) was as expected in each of the 12 normal brain specimens. The DHFR gene was found overexpressed (3-fold) in 4/47 (9%) tumours (one grade II and one grade III gliomas, one metastasis from an epidermoid lung carcinoma, and one meningioma). This gene was expressed normally in each of 13 tumour cell lines (Fig. 1).

Study of the absence of Topo II expressions

First of all, it is very important to note that the Topo II gene was not detected in our 12 samples of normal adult brain tissue.

Table 2. Expression of chemoresistant genes in human primary and metastatic brain tumours

Gene expression in HBT	Overexpression of MDR$_1$ 3-fold	DHFR 3-fold	GSTπ 3-fold	GSTπ ≥ 5-fold	Loss of topoisomerase II expression
Gliomas					
Grade I	—	—	0/1	1/1	0/1
Grade II	0/8	1/8	3/7	0/7	6/7
Grade III	0/10	1/8	1/1	0/1	2/4
Grade IV	1/11	0/9	0/4	0/4	6/7
Brain metastases	0/12	1/10	1/8	2/8	6/8
Medulloblastoma	—	—	0/1	0/1	1/1
Malignant teratoma	0/1	0/1	0/1	0/1	1/1
Ependymomas	—	—	0/3	1/3	1/3
Meningiomas	0/17	1/11	3/6	0/6	4/10
All (percentage)	1/59 (2%)	4/47 (9%)	8/32 (25%)	4/32 (13%) (38%)	27/42 (64%)

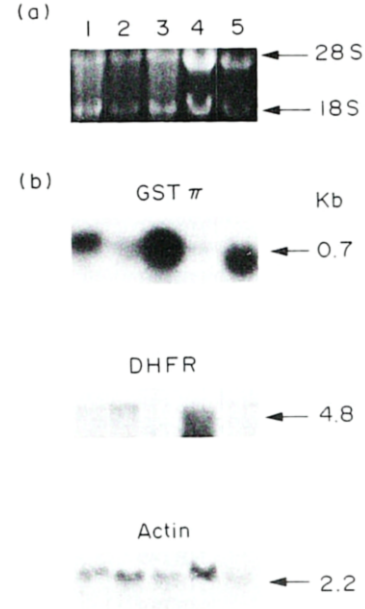

Fig. 1. Chemoresistance related gene expression in tumoral cell lines. Northern blot analysis of total RNA. (a) Ethidium bromide stained gel. (b) Successive hybridisations of the same blot with GSTπ, DHFR and actin probes. KB 3-1 (lane 1); KB 8-5 (lane 2); A 431 (lane 3); C6 (lane 4); HL 60 (lane 5). Exposure was for 5 days. Kb kilobase. Three cell lines (KB 3-1, A 431, HL60) with a GSTπ overexpression.

However, we observed one copy of Topo II expressed in the peritumoral tissue, surrounding one ependymoma, which itself lacks Topo II gene expression. Therefore, as normal brain tissue expression of Topo II could not be used as a reference, we chose the EJ cell line in which Topo II gene expression (6.6 kb, 4.5 kb, 1.8 kb mRNA) is observed. In our study, we found an absence of Topo II expression in 27/42 human brain tumours (64%) (Fig. 3): first in 23/32 malignant tumours (72%) (including 6/8 grade I and II gliomas, 8/11 grade III and IV gliomas, 6/8 brain metastases (three breast, one lung and one colon adenocarcinomas and one poorly differentiated lung carcinoma), 1/1 medulloblastoma, 1/1 malignant teratoma, 1/3 ependymomas. The nine remaining malignant specimens (28%) expressed the Topo

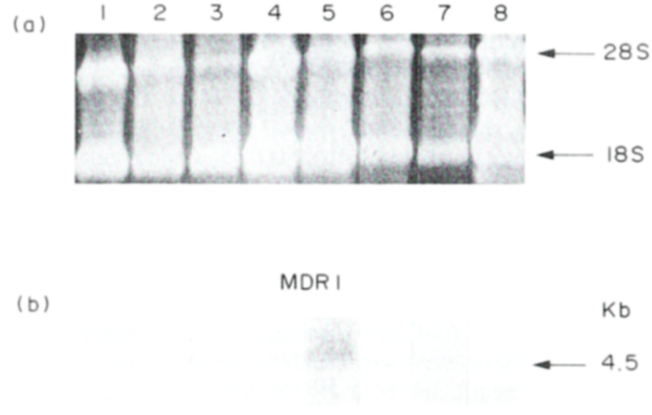

Fig. 2. MDR1 gene expression in human brain tumours. Northern blot analysis of total RNA. (a) Ethidium bromide stained gel. (b) Hybridisation with MDR1 probe. A431 (lane 1); peritumoral tissue (lane 2); gliomas: grade IV, HBT 5 (lane 3); grade IV, HBT 13 (lane 4); grade III, HBT 38 (lane 5); grade IV, HBT 40 (lane 6); grade IV, HBT 48 (lane 7); grade III, HBT 15 (lane 8). Exposure was for 7 days. Kb, kilobase. Only one grade IV glioma (HBT 13) overexpresses 3-fold MDR1.

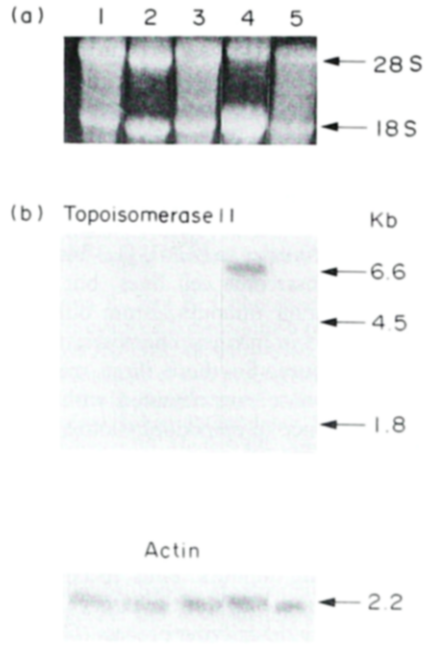

Fig. 3. A study of the Topoisomerase II gene expression in human brain tumours. (a) Ethidium bromide stained gel. (b) Successive hybridisations of the same blot with Topoisomerase II and actin probes. Gliomas: grade I, HBT 127 (lane 1); grade III, HBT 49 (lane 2); meningioma: HBT 60 (lane 3); HBT 57 (lane 4); grade III astrocytoma (HBT 7) (lane 5). Exposure was for 7 days. Kb, kilobase.

II gene: seven tumours exhibited the expression of one copy of the gene, two specimens (one pre and one post-chemotherapy) obtained from one grade III ependymoma expressed more than one copy of the gene. In this late case the gene was found expressing 5-fold the base line value before any radio- or chemotherapy. The patient then received six courses of etoposide (VP16, 150 mg/m^2/day × 3 days), Etoposide was associated alternatively every other month with either carboplatin (200 mg/m^2/day × 3 days) or cyclophosphamide (1000 mg/m^2/day × 3 days). Three weeks after the last course, there was no tumour regression and the tumour was surgically removed; pathology disclosed the same pattern. By northern-blot the mRNA of Topo II was found to be expressed 3-fold more than the EJ base line which, of course, is not significantly decreased as compared to the pre-chemotherapy expression level.

Second, out of 10 meningiomas the gene of Topo II was not detected in 4 cases, 1-fold expressed in 4 cases and more than 5-fold in 2 cases (Fig. 3, lane 4).

Lastly, among the tumoral cell lines, only the two derived from grade III and grade IV human gliomas (A and D) lacked Topo II expression; by contrast, seven human cell lines (KB, KB_{3-1}, KB_{8-5}, A_{431}, $CaKi_2$, IMR_{32}, HL_{60}) expressed three to five copies of Topo II gene and the three rodent cell lines (C_6–NS_{20}–Y_1) expressed one copy of the Topo II gene.

DISCUSSION

Chemotherapy cannot cure malignant human brain tumours [1]. The delivery of chemotherapy could be limited by the blood–brain barrier, although its role remains uncertain [2]. A more important problem than insufficient delivery of drugs in these tumours might be their intrinsic chemoresistance. Drug resistance can be due to a decreased uptake of molecules or an enhanced DNA-repair of drug damage [14]. Other mechanisms of chemoresistance, such as active drug removal mediated by P-glycoprotein [4], drug detoxification related to increased glutathione S-transferase activity [5, 6], alteration in drug target specificity [8] or gene amplification followed by an overexpression of the target molecule [7] may play an important role; we have therefore investigated these four mechanisms, by mRNA measurements. However, the expressed mRNA may not necessarily be translated. Therefore, these results should be treated with caution.

Topoisomerases are nuclear enzymes that are involved in cell growth and division [15]. There are two groups of topoisomerases: type I creates breaks in single stranded DNA and type II in double stranded DNA. These enzymes are the target of different antitumour drugs and reduced levels of these enzymes are associated with drug resistance. Topoisomerase I is inhibited by camptothecin and its analogues; topoisomerase II is the target of DNA intercalaters (anthracyclines) and of non-intercalating drugs (epipodophyllotoxins) [8]. The resistance to a topoisomerase I inhibitor seems to confer a collateral sensitivity to inhibitors of topoisomerase II and vice versa [16]. In this study, we have only investigated the expression of topoisomerase II. We have detected the non-expression of topoisomerase II in 64% of our samples. This phenomenon could be related to the growth kinetics of these tumours which are sometimes detected and then surgically removed at the time they have already reached a plateau-phase [17]. Indeed, the topoisomerase II activity is lower in plateau-phase CHO cells compared to log-phase cells [18]. These data could explain our observation of a loss of topoisomerase II expression in human brain tumours as well as in normal adult human brain. Nevertheless, it remains paradoxical that in highly malignant tumours like glioblastomas Topo II cannot be detected. This defect of Topo II could be a consequence of the necrosis and the high proportion of quiescent cells, which is a common feature of high grade gliomas [1]. In contrast, our finding in meningiomas is surprising: 6/10 expressed the Topo II gene, whereas meningiomas are slowly growing benign tumours. All the original human and rodent cell lines (never exposed to drugs) that we studied except EJ, expressed the topoisomerase II gene. This expression is known to disappear when these cell lines are exposed to drugs [19]. In cerebral metastases, no expression of Topo II was found in six out of eight samples. In the same way, Riou *et al.* [20] observed a loss of Topo II expression in a highly metastasising transplantable rabbit tumour.

Obviously to explore further why 64% of brain tumours do not have Topo II expression and why 36% do express the gene, at least three investigations should be undertaken:

- a complementary study at the cell level either of the gene by *in situ* hybridisation, or of the enzyme by immunohistochemistry;
- a highly quantitative assay based on the polymerase chain reaction (PCR), which could detect low levels of Topo II mRNA expression;
- a study of the correlation of the expression of the gene with the proliferation index in the tumour tissue by the K_{i67} antigen;

Yet our results provide evidence that normal brain and a high proportion of primary and secondary brain tumours do not express the Topo II gene, but that benign tumours like meningiomas may express the gene.

Glutathione S-transferases (GST) constitute an important multigene (including 11 subunits) family whose main function is cellular detoxification [6, 21]. The tissue distribution of GST is highly variable [21, 22]. In human brain tissue, immunohisto-

chemical methods show a weak reaction with GSTπ in normal glial cells, and in low grade astrocytomas; the intensity of immunostaining is stronger in anaplastic gliomas, especially in the gemistocytes and giant cells [12]. With northern blotting, we have observed a significant overexpression of GSTπ in four out of 32 tumours of different types including one ependymoma, one grade I glioma and two cerebral metastases and a lower overexpression of GSTπ in 8/32 cases including malignant and benign tumours as well (Table 2). According to our study, it is not possible to establish any correlation between GSTπ expression and the intensity of dedifferentiation of the gliomas. An increased activity of GST has been described in a series of experimental animal tumours expressing resistance to nitrogen mustards [23] after X-ray exposure [24]. We have shown here, however, that the GSTπ expression occurs also in previously non-treated tumours. Then, this spontaneous GSTπ expression could constitute a natural detoxification pathway for many environmental toxins [6, 21], and a potential intrinsic chemoresistance mechanism for some brain tumours. Although the elevated levels of GSTπ could constitute a mechanism of resistance to nitrosoureas for approximately one third of our brain tumours, two other mechanisms could account for nitrosourea resistant features: glutathione transferase μ [5] and O^6 alkylguanine DNA alkyl-transferase [25]. These two activities are a major contributor to cellular resistance to nitrosoureas in animal cell lines and could have the same importance in human brain tumours.

This defence system of GSTπ overexpression has been reported to be more widely observed than enhanced cellular efflux of toxins by P-glycoprotein in other human tumours [26] and it may contribute to the classic MDR phenotype [26]; therefore, there could exist a common regulatory control for these two genes [24].

P-glycoprotein, which is the product of the MDR1 gene, induces an increased drug efflux resulting in a decreased drug accumulation in cells. This phenomenon induces a cross-resistance to many natural cytotoxic drugs [4]. In normal human brain, there is a very low level of MDR1 expression [27]. This expression of P-glycoprotein was found only in endothelial cells of capillary blood vessels in the brain [28, 29]. Similarly, in human brain tumours, MDR1 expression is detected in only 2/25 specimens [29]. Moreover, the immunostaining for P-glycoprotein is positive for more than 20% of cells in 3/18 gliomas [11] and is negative for 12 different malignant brain tumours [28]. In 59 human brain tumours, we observed an overexpression of MDR1 gene in only one non-pre-treated grade IV glioma. The metastatic tumours did not, surprisingly, show elevated levels of MDR1 mRNA, but this gene is often associated with some types of tumour such as colon cancer. Thus, the overexpression of the MDR1 gene cannot be the main mechanism of chemoresistance of human brain tumours. Indeed, the gene could constitute a blood–brain barrier and regulate the entry of cytotoxic drugs into the central nervous system by its expression in peri-tumoral endothelial cells [28]. An immunohistochemical analysis of our specimens could add some proof to this hypothesis.

The pattern of cross-resistance to both methotrexate and drugs of the multidrug resistance family has been described for MTX-selected CCRF-CEM sublines [30]. We have studied the DHFR gene in human brain tumours, where we found its overexpression in 4/47 cases, including one grade II and one grade III glioma, one cerebral metastasis from a lung carcinoma and one meningioma. Thus, although elevated levels of DHFR have been observed in childhood malignancies and acute leukaemia [7], such a mechanism cannot belie the intrinsic chemoresistance of human brain tumours.

Nitrosourea drugs remain the chemotherapeutic agent of reference for the treatment of human brain tumours [1] and have been used in association with epipodophyllotoxins [1]. The mechanisms of resistance to nitrosoureas are complex. Indeed, GSTμ [5] and elevated alkyl transferase activity [25] may be involved in resistance to BCNU [1,3-bis (2-chloroethyl)-l-nitrosourea] for gliosarcoma cell lines, but this is still not proven for human brain tumours. From our results, GSTπ could also be involved in intrinsic chemoresistance of 38% the different brain tumours. So these three mechanisms could explain the low response rate obtained with nitrosoureas in clinical trials. Resistance to epipodophyllotoxines is associated with overexpression of MDR1 and with loss of topoisomerase II activity [8]. Although we did not observe overexpression of MDR1, we noted an absence of Topo II expression in 72% of malignant brain tumours. This could explain the low efficacy of this drug family in brain tumours. Thus, in a high proportion of human brain tumours, the lack of the target (Topo II) and the increase in the cellular detoxifying process (GSTπ) for related drugs could be the main mechanism of intrinsic chemoresistance, which is unrelated to MDR1 and DHFR gene overexpression. In consequence, our results suggest that drug resistance is likely to be a multifactorial process in human brain tumours. Whether this is because of the presence of several different mechanisms acting within a single cell or a reflection of the characteristic cellular heterogeneity of these tumours remains to be determined. Thus, when nitrosoureas and epipodophyllotoxins are used as a treatment, not only would the response be low, but the treatment might even amplify resistance.

However, despite these limitations of our work due to the particular characteristics of clinical material, the conclusions of our study would seem to provide some information towards a better understanding of the molecular basis of drug treatment failure in human brain tumours.

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Isolation of pMGT1: a Gene that is Repressed by Oestrogen and Increased by Antioestrogens and Antiprogestins

D.L. Manning and R.I. Nicholson

In order to isolate additional markers of oestrogen responsiveness in breast cancer and to study the mechanisms associated with the development of endocrine resistance, we have searched for oestrogen regulated genes. Differential hybridisation analysis of a cDNA library prepared from oestrogen-stimulated T-47D cells has led to the isolation of a sequence (pMGT1) whose expression is repressed (up to 8-fold) by oestrogen (10^{-9} mol/l) and represents the first down-regulated gene to be identified by this methodology. Further studies of pMGT1 expression in MCF-7 cells has revealed that the pure antioestrogens, ICI164384 (10^{-7} mol/l) and ICI182780 (10^{-7} mol/l) and the antiprogestin Ru38486 (10^{-7} mol/l), increase pMGT1 mRNA levels by approximately 40–50-fold relative to the value seen in cells exposed to oestrogens. Under the same conditions, pS2(pLIV2), a gene which is positively regulated by oestradiol, was almost undectable. Significantly, both tamoxifen (10^{-7} mol/l), and 4-hydroxytamoxifen (10^{-7} mol/l), failed to increase pMGT1 mRNA levels. Since cell culture studies have indicated that ICI164384 and ICI182780 are more effective than tamoxifen and 4-hydroxytamoxifen at inhibiting the growth of MCF-7 cells by mechanisms that lower their viability and sensitivity to growth factors, it is feasible that pMGT1 plays a central role in mediating these events and instigating pathways associated with cell death.
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INTRODUCTION

DIFFERENTIAL HYBRIDISATION strategies have proved extremely useful in the isolation of oestrogen regulated genes in breast cancer cells. The technique usually involves the screening of cDNA libraries, prepared from oestrogen-stimulated cells, with two cDNA probes; one prepared from the mRNA of hormone-stimulated cells and the other from hormone withdrawn cells. Its sensitivity is limited however, since oestrogen withdrawal procedures have been inadequate. Consequently, the differential expression of responsive genes is not maximised.