Comparisons of *P*-glycoprotein expression in isolated rat brain microvessels and in primary cultures of endothelial cells derived from microvasculature of rat brain, epididymal fat pad and from aorta

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Abstract In vivo expression of *P*-glycoprotein in isolated rat brain microvessels is compared with that in vitro in primary cultures of brain endothelial cells. More *P*-glycoprotein is detected by Western immunoblotting in microvessels than in cultured endothelium. RT-PCR with isoform-specific primers and immunoblotting with a mdr1b-specific antibody reveals only mdr1a in vivo but both mdr1a and mdr1b in vitro. Thus mdr1a decreases whereas mdr1b increases during culture. *P*-Glycoprotein activity is evident in vitro, with resistance modulators, e.g. verapamil, producing increases in intracellular [^{3}H]vincristine accumulation. Endothelial cells cultured from epididymal fat pad microvasculature and aorta contain little or no *P*-glycoprotein. Here, resistance modulators are less effective.

Key words: *P*-Glycoprotein isoforms; Rat brain microvessel endothelium; Primary cell culture; Blood–brain barrier; Vincristine transport

1. Introduction

P-Glycoprotein is a drug efflux pump important in expelling a range of cytotoxic agents from tumour cells thus rendering them resistant to anticancer treatment, i.e. multidrug resistant (MDR) [1]. This protein is also present in a number of normal tissues including vascular endothelial cells lining the capillaries that form part of the blood-brain barrier [2,3,4]. Recent evidence suggests that it may be important in this situation for preventing entry of toxic substances to the brain [5].

Several different in vitro systems of brain endothelial cell culture have been developed to investigate various aspects of blood–brain barrier function [6,7,8] including likely access of compounds to the brain. It has been shown both in primary cultures [9,10,11] and in immortalised cell lines [12,13,14] that endothelial cells derived from brain microvessels can express *P*-glycoprotein. However, it has been reported that brain endothelial cells in culture may lose many of their characteristics [15]. Clearly therefore it is important to determine how closely structural and functional expression of *P*-glycoprotein in brain endothelial cells during culture mirrors that seen in vivo in the brain microvessels.

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Abbreviations: BSA, bovine serum albumin; Dil-Ac-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-labelled acetylated low density lipoprotein; DMSO, dimethyl sulfoxide; MDR, multidrug resistant; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulphate.

The purpose of this study was therefore two-fold: (i) to compare the amount and nature of *P*-glycoprotein expressed in isolated brain microvessels with that seen in endothelial cells derived from these microvessels and maintained in primary culture; and (ii) to establish the extent of *P*-glycoprotein activity in the cultured endothelial cells. We have also investigated expression in endothelial cells derived from rat epididymal fat pad microvasculature and from rat aorta where *P*-glycoprotein has not been reported in vivo [2,3,9]. We demonstrate here that there is a correlation in these cultured endothelial cells between the level of *P*-glycoprotein expression and its functional activity as judged by chemosensitivity to vincristine and doxorubicin, ability to efflux vincristine and sensitivity of this process to various resistance modulators.

2. Materials and methods

2.1. Cell isolation and culture

Each primary culture of brain endothelial cells was grown from capillary fragments isolated from the cortical grey matter from the brains of 4–5 male Wistar rats 180–200 g in weight according to a method previously described [8]. This method includes a preliminary digestion of cortical grey matter dissected free of meninges, removal of myelin by centrifugation through 22% BSA followed by more prolonged digestion, then separation of individual microvessels from contaminating cells by centrifugation through a Percoll gradient. Microvasculature from the epididymal fat pad was isolated by a similar method using tissue dissected at the same time. Aortic endothelial cells were cultured from aortic rings taken from the same animals. Cells were grown in collagen coated culture flasks or multiwell plates in Hams F10 medium containing 20% plasma derived serum and 75 μg/ml endothelial cell growth supplement (both from Adv anced Protein Products, West Midlands, UK), 80 μg/ml heparin, 0.5 μg/ml vitamin C, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. 1–3 days after isolation of brain microvessels, spindle-shaped cells grow out from the original endothelial cell clusters. Other cell types [8] may be found in this initial culture, but following the first passage at day 7, few if any non-endothelial cells remain. Endothelial cells derived from the epididymal fat pad microvasculature and from the aorta are less spindle-shaped. Cells were used for drug accumulation studies or for RNA and protein analysis after 1–5 passages. To confirm their endothelial nature, cells were examined for their ability to take up fluorescence-labelled Dil-Ac-LDL (Biogenesis, Bournemouth, UK) following a 4 h exposure to a 20 μg/ml solution.

MDR and parent cell lines used as positive and negative controls were cultured as described [16]. These included mouse mammary tumour cell lines, EMT6/P and the MDR variant, EMT6/AR1.0, which is maintained in 1.0 μg/ml doxorubicin and is known to contain only the *P*-glycoprotein isoform, mdr1a [16]. Membrane protein of the mouse fibrosarcoma line, L0.5, which contains the *P*-glycoprotein isoform, mdr1b (Holmes, personal communication) was supplied by Ms. Julie Holmes of MRC Clinical Oncology Unit in Cambridge.

2.2. Western blot analysis

Membranes were prepared from isolated brain capillaries or from

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cultured cells by centrifugation (60,000 × *g* for 1 h) following cell lysis in 1 mM Tris buffer, pH 7.4 containing protease inhibitors (2 μg/ml aprotinin, 5 μg/ml leupeptin and 0.08 μg/ml pepstatin). Some samples were further subjected to glycosidase digestion, i.e. 25 μg aliquots of protein, solubilised in 0.5% SDS at room temperature for 10 min, diluted five-fold with 50 mM mannitol, 5 mM HEPES, 10 mM Tris, 1% Triton, 0.25% β-mercaptoethanol at pH 7.4 in the presence of protease inhibitors as above were subdivided and incubated for 18 h at room temperature with or without endoglycosidase *F*/*N*-glycosidase F mixture (Sigma; E 8762) at 2 U/mg protein. Proteins were resolved by SDS gel electrophoresis in 7.5% polyacrylamide, electroblotted onto nitrocellulose, the filters probed with anti-*P*-glycoprotein antibodies, C219 (CIS UK Ltd., Bucks) or 265/F4 (originally a gift from McGuire [16]), both at 100 ng/ml in Tris-buffered saline containing 2.5% bovine serum albumin and *P*-glycoprotein visualised using an ECL western blotting analysis system (Amersham Int.).

2.3. RT-PCR analysis

Total cellular RNA was prepared from PBS washed cells by guanidine hydrochloride lysis, phenol/chloroform extraction and ethanol precipitation. Single-strand cDNA was made from 1 μg of this RNA by reverse transcription using a random hexamer following which fragments specific to rat mdr1a, mdr1b and mdr2 genes were amplified through 20 or 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min using oligonucleotide primers appropriate to the particular isoform, i.e. sense sequence common to all three isoforms, ACAGAAACAGAGGATCGC (bases 1599–1617) and antisense sequences for mdr1a, CGTCTTGATCATGTGGCC (bases 2032–2015), for mdr1b, AGAGGCACCAGTGTCACT (bases 1951–1934) and for mdr2, ATGCGTGCTTTCCAGCCA (bases 1981–1964) [17]. As an internal control, a sample of each first-strand cDNA was also treated with primers able to amplify a 200 base-pair fragment of the rat β_2-μ-globulin gene [18]. In some experiments, sets of primers able to amplify either a 200 base pair fragment and or a 400 base pair fragment of the rat β-actin gene [19] were used. The PCR products were separated by electrophoresis in 2% agarose and visualised by UV in the presence of ethidium bromide.

2.4. Chemosensitivity assay

The assay used was a modification [20] of that originally described by Mosmann. Briefly cells plated in 96-well plates at 10^3 cells per well were grown for 5 days with or without cytotoxic drug. They were then incubated for 5 h with 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium (MTT) to allow an insoluble red precipitate to form in cells possessing viable mitochondria. The bulk of the medium was then removed and the precipitate dissolved by shaking for 10 min in 200μl DMSO. The optical densities were read at 540 nm on a Titertek Multiskan plate reader and the results expressed as a fraction of absorbance in drug free wells.

2.5. Drug accumulation studies

Cells were plated into 12-well plates (for efflux) or 24-well plates (for uptake) at a density of 5×10^4 cells/well and left for 5 days to reach near confluence. For uptake, cells were pretreated for 30–60 min at 37°C with Ham's F10 containing 5 mg/ml BSA and 0.5μg/ml vitamin C with or without the resistance modifying drugs, verapamil, quinidine, tamoxifen, cyclosporin A (gift from Dr P.R. Twentyman), progesterone, forskolin and dideoxyforskolin, before incubation in the presence of 30 nM [^{3}H]vincristine (5–10 Ci/mmol)(Amersham Int., Bucks., UK) with or without drugs. Following 3 washings with ice-cold PBS, the cells were lysed with 400 μl of 0.1% SDS and the amount of [^{3}H]vincristine accumulated assessed by liquid scintillation counting. Preliminary time course experiments established that vincristine accumulation had reached equilibrium by 90 min. This single time interval was used in subsequent experiments. For efflux, cells were loaded by incubation for 2 h at 37°C in the Hams/BSA/vitamin C medium containing 30 nM [^{3}H]vincristine and 0.33 μM verapamil. After 3 washings with ice-cold PBS, cells were then incubated for 90 min in 2 ml of medium alone with or without the resistance modifying drugs. The amount of intracellular [^{3}H]vincristine remaining at this time was assessed following removal of efflux medium and lysis of the cells in 400 μl of 0.1% SDS by liquid scintillation counting.

To determine the effect of energy depletion on drug accumulation, uptake and efflux of [^{3}H]vincristine was investigated using buffer of similar ionic composition and pH to Ham's medium with 10 mM glucose excluded and 6 mM deoxy-glucose and 10 mM sodium azide added to the test wells to reduce ATP levels.

Unless otherwise stated, all compounds were either standard laboratory reagents of analytical grade or from Sigma (Poole, Dorset, UK).

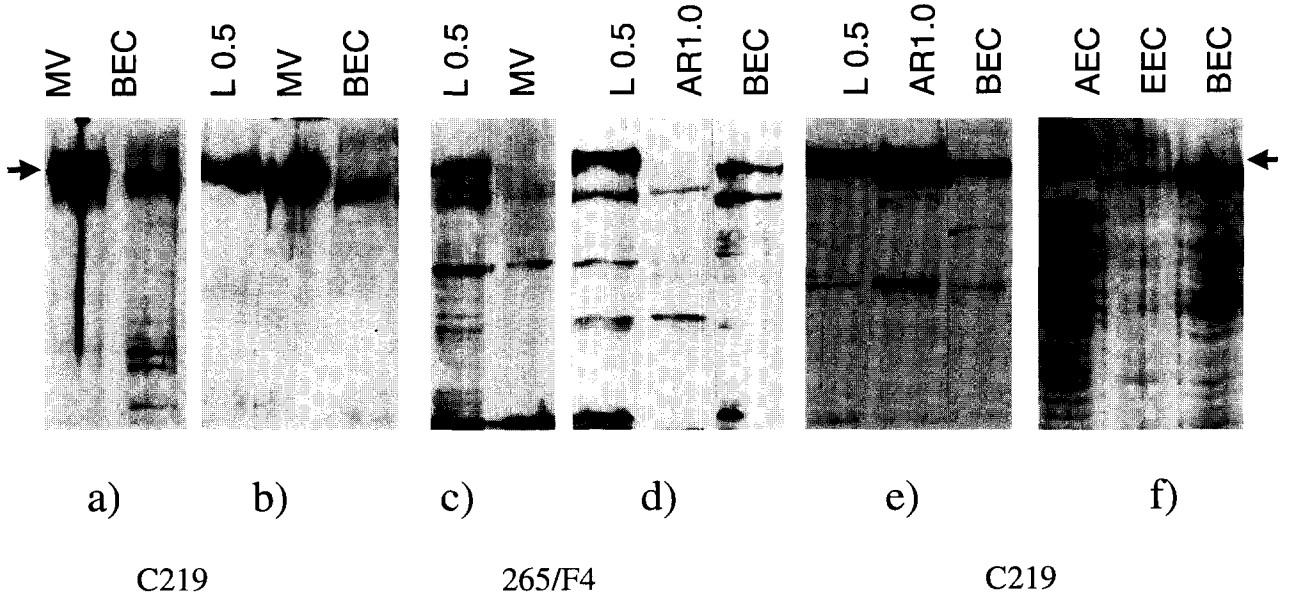

Fig. 1. Immunodetection of *P*-glycoprotein on Western blots of membrane protein from isolated brain microvessels (MV), primary cultured brain endothelial cells (BEC), endothelial cells cultured from epididymal fat pad microvasculature (EEC) and cultured aortic endothelial cells (AEC) and also from multidrug resistant mouse tumour cell lines, AR1.0 and L 0.5. 20 μg aliquots of protein were loaded per well in (a), (f) and BEC in (d) and (e); 10 μg were loaded in (c); 5 μg were loaded in (b) and L 0.5 and AR1.0 in (d) and (e). Filters were probed with C219 in (a), (b), (e) and (f) and 265/F4 in (c) and (d). Each section, i.e. (a) to (f) is from a separate gel. Arrow denotes position of 170 kDa proteins including glycosylated *P*-glycoproteins.

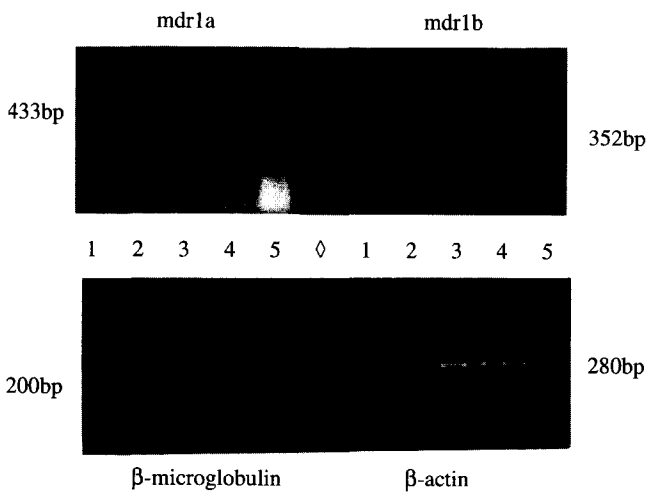

Fig. 2. RT-PCR analysis of mdr1a, mdr1b (upper gel), β-microglobulin and β-actin (lower gel) expression in RNA samples prepared from isolated brain microvessels (lanes 1 and 2), cultured brain endothelial cells (lane 3), endothelial cells cultured from epididymal fat pad microvasculature (lane 4) and cultured aortic endothelial cells (lane 5). PCR products stained with ethidium bromide are visualised under UV. DNA size markers from *Hae*III-digested ΦX174-RF DNA are shown in lane ◇.

3. Results

P-Glycoprotein expression in membranes prepared from isolated brain microvessels and from cultured brain endothelial cells was analysed on Western blots using monoclonal antibody, C219. With this antibody, P-glycoprotein was detected in membranes of the isolated microvessels (MV) (Fig. 1a and b) in amounts comparable to those found in MDR mouse tumour cell lines, EMT6/AR1.0 [16] and L 0.5. A moderate amount of *P*-glycoprotein was observed in membranes of endothelial cells cultured from the brain microvessels (BEC) (Fig. 1a, b, e and f) and this was maintained through several passages (not shown). The monoclonal antibody, 265/F4, that detects the protein product of the mouse mdr1b gene but not that of the mdr1a gene [16] did not recognise the *P*-glycoprotein in the brain microvessel membranes (MV) (Fig. 1c) or in the AR1.0 cells (Fig. 1d) but did recognise the *P*-glycoprotein present in the primary cultured brain endothelial cells and in the L 0.5 cells (Fig 1c and d). Low amounts of *P*-glycoprotein were found in endothelial cells cultured from the epididymal fat pad microvasculature (EEC) (Fig. 1f) and little if any in endothelial cells cultured from the aorta (AEC) (Fig. 1f).

The *P*-glycoprotein in the cultured brain endothelial cells was of slightly lower molecular weight (150–160 kDa) than that found in the isolated brain microvessels (170 kDa) (Fig. 1a and b). Endoglycosidase treatment reduced the apparent molecular weights in both cell preparations to around 130–140 kDa (gels not shown).

RT-PCR analysis was undertaken to determine the isoform of *P*-glycoprotein expressed. With primers specific for the mdr1a gene (Fig. 2, upper gel), a band corresponding to the expected size (433 bp) was obtained from RNA prepared from isolated brain microvessels (lanes 1 and 2) and from endothelial cells cultured from these microvessels (lane 3). Whether amplification was done through 20 or 30 cycles, there was no obvious difference in the level of expression between the different RNA preparations. With primers specific for the mdr1b gene (Fig. 2, upper gel), a fragment corresponding to the expected size (352 bp) was amplified from RNA prepared from cultured cells of brain endothelium and epididymal fat pad microvasculature (lane 4) but not from isolated brain microvessels or from aortic endothelial cells (lane 5). There was little evidence of mdr2 expression in any of the samples analysed (gels not shown). To check the integrity of the RNA and efficiency of the RT-PCR, primers specific for rat β-μ-globulin were used as internal controls on samples of first strand cDNA. A band of the expected size (200 bp) was obtained with RNA from isolated brain microvessels and from cultured cells of brain endothelium and epididymal fat pad microvasculature but not from aortic endothelial cells (Fig. 2, lower gel). Rat β-actin primers used as an alternative control produced fragments of the expected sizes in all samples of RNA from each endothelial cell type (Fig. 2, lower gel). Thus aortic endothelial cells apparently do not express the β-μ-globulin gene during culture.

The effect of the cytotoxic agents, vincristine and doxorubicin, on viability of the endothelial cells in culture was examined. Endothelial cells from the brain microvessels (BEC) were less susceptible to cytotoxic drug treatment than those from the epididymal fat pad microvasculature (EEC) with EC_{50} values (mean ± S.E.M. from 4–6 experiments) in mg/ml for vincristine of 0.27 ± 0.027 (BEC) vs. 0.037 ± 0.008 (EEC) and for doxorubicin of 0.44 ± 0.051 (BEC) vs. 0.095 (EEC). The resistance modifying agent, verapamil, increased the sensitivity of the brain endothelial cells to vincristine, the +isomer being equally as effective as the racemic mixture with regard to its chemosensitising action (shift in EC_{50} from 0.22 μg/ml to 0.03, 0.013 and 0.005 μg/ml with 0.3, 1 and 10 μM racemic verapamil and to 0.04, 0.014 and 0.006 μg/ml with 0.3, 1 and 10 μM dex-verapamil, values being the mean of results from two separate experiments).

Movement of [^{3}H]vincristine into and out of the cultured endothelial cells was monitored as uptake during exposure to 30 nM vincristine and as efflux after preloading of cells by exposure to 30 nM vincristine. More drug was taken up and less drug effluxed in 90 min from endothelial cells derived from the epididymal fat pad microvasculature (EEC) and from the aorta (AEC) than in cells derived from the brain microvessels (BEC): uptake values in fmol/10^5 cells (mean of results from 4–6 experiments ± S.E.M.) of 32.5 ± 8.5 (AEC), 18.6 ± 3.1 (EEC) and 9.9 ± 1.2 (BEC) and efflux values in terms of proportions remaining after 90 min (mean ± S.E.M.) of 0.93 ± 0.05 (AEC), 0.29 ± 0.02 (EEC) and 0.18 ± 0.02 (BEC).

Both uptake and efflux were also investigated in the presence of various resistance modifiers including verapamil (10 μM), quinidine (10 μM), tamoxifen (50 μM) and cyclosporin A (2.5 μg/ml). At the concentrations given, these modifying agents increased drug accumulation very little in aortic endothelial cells (AEC) (Fig. 3c), to a small extent in epididymal endothelial cells (EEC) (Fig. 3b) but to a significant degree in brain endothelial cells (BEC) (Fig. 3a). Similar increases in brain endothelial cell drug accumulation were observed in the presence of forskolin (40 μM), dideoxyforskolin (40 μM) and progesterone (50 μM) (Fig. 3a). The effects of verapamil and progesterone were dose dependent (Fig. 3d).

Following energy depletion, there was a significant increase

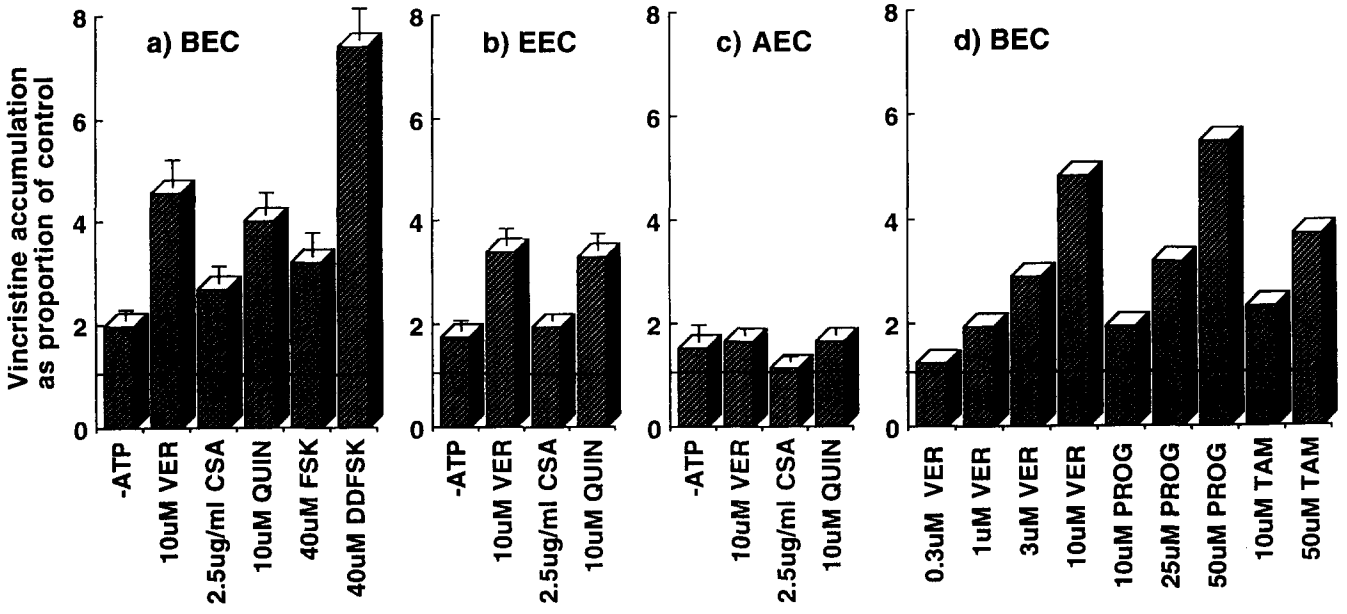

Fig. 3. Effects of energy depletion (−ATP) and of resistance modifiers, 10 μM verapamil (VER), 10 μM quinidine (QUIN), 10 μM tamoxifen (TAM) and 2.5 μg/ml cyclosporin A (CSA) on drug accumulation in (a) brain endothelial cells (BEC), (b) endothelial cells of epididymal fat pad microvasculature (EEC) and (c) aortic endothelial cells (AEC) following 90 min exposure to 30 nM [^{3}H]vincristine. (d) Effects of different concentrations of verapamil and progesterone on accumulation in brain endothelial cells. Values shown are the mean ± S.E.M. of results from 3–4 separate experiments, triplicate determinations being obtained from each.

in drug accumulation in the brain endothelial cells (BEC) (Fig. 3a). A small but insignificant increase also was evident in the aortic endothelial cells (AEC) in the uptake but not the efflux experiments.

4. Discussion

The present study compares the amount and isoform of *P*-glycoprotein expressed in brain microvessels in vivo with that in primary cultures of cells grown in vitro from these microvessels. The levels in the isolated vessels are comparable with those detectable in the mouse MDR cell lines, L 0.5, maintained in 0.5 μg/ml vindesine and EMT6/AR1.0 maintained in 1 μg/ml doxorubicin [16]. Lower levels are present in the endothelial cells cultured from the microvessels. There is thus significant loss of expression during cell culture.

Three isoforms of *P*-glycoprotein have been identified in rodent tissues, mdr1a, mdr1b and mdr2 [21]. The antibody, C219, used in this study recognises a highly conserved region of *P*-glycoprotein [22] so it cannot distinguish between the different isoforms. The other antibody, 265/F4, recognises only the mdr1b isoform [16] and so can detect differences in isoform expression. This antibody revealed that mdr1b is not present in vivo in the brain microvessels but appears in the brain endothelial cells during culture. This was confirmed by RT-PCR analysis using primers specific for the mdr1a and mdr1b genes. These observations are in agreement with previous reports in both rat and mouse that mdr1a is the only isoform found in vivo in brain capillaries [23]. Indeed, ablation of the mdr1a gene alone in mice leads to altered pharmacokinetics of certain cytotoxic drugs, allowing much higher levels to enter the brain [5].

An additional protein, 190 kDa in size has been detected by others using C219 in membranes prepared from rat brain capillaries [24]. No such protein was observed in this present study. The C219 antibody used in that investigation was, however, obtained from a source different from that used here and it is possible (Beaulieu, personal communication) that it detects epitopes in addition to the highly conserved sequence already mapped [22].

Thus mdr1a decreases whereas mdr1b increases during culture of rat brain endothelial cells. Similar results have been obtained by other workers (Bergmann and Roux, personal communication). Various factors including hormones and adverse environmental stresses, as may occur during cell culture, can affect mdr gene expression. Isolated rat hepatocytes for example show dramatic increases in *P*-glycoprotein expression over the course of 2–4 days in culture [25]. This expression which appears to be negatively regulated by a protein factor [26] involves upregulation of the mdr1b isoform with concomitant down-regulation of the mdr1a isoform [27]. Culture conditions including the presence of serum and the nature of the substratum seem to be important in altering expression in hepatocytes and may well play a part in modifying *P*-glycoprotein expression in brain endothelial cells.

There was no detectable expression of mdr2 RNA in any of the endothelial cell preparations. This isoform, expressed primarily on bile canalicular membranes of hepatocytes [28], is associated with normal bile secretion but is not involved with decreased accumulation of cytotoxic drugs and increased drug resistance [29].

In addition to changes in isoform of the *P*-glycoprotein expressed during culture, there appeared to be alterations also in glycosylation with fewer carbohydrate moieties linked to the protein in vitro. There are known to be variations in glycosylation of *P*-glycoproteins derived from different mouse tissues [21] but whether these reflect differences in functional activity is unclear. Though complete *N*-glycosylation of the human MDR1 gene product is not thought essential for drug binding

[30] or transport, *N*-glycosylation may affect drug transport indirectly by influencing routing and stability of *P*-glycoprotein [31].

Expression of *P*-glycoprotein is not confined to microvessels in the brain but has been observed also on microvasculature in other tissues, e.g. lung and testis [3]. In the present study, a small amount of *P*-glycoprotein was detected in endothelial cells grown from the microvasculature of the epididymal fat pad. Too few cells could be harvested during the initial isolation to provide sufficient material for analysis so it is not clear whether *P*-glycoprotein is normally expressed here in vivo or whether it arises during in vitro culture.

These cells were originally cultured as putative *P*-glycoprotein-negative endothelial cell controls for the functional studies. The endothelial cells derived from the aorta proved to be more appropriate for this purpose since these cells lack detectable *P*-glycoprotein.

Functional activity of *P*-glycoprotein as assessed by drug sensitivity, drug accumulation and the sensitivity of these parameters to resistance modulators was not tested in the isolated brain microvessels owing to the small amount of material available but was investigated in the cultured endothelial cells. In these cells, there was a clear correlation between the degree of functional activity and the level of *P*-glycoprotein expression with significant activity in the brain endothelium and none in the aortic endothelium.

The alterations seen in this study in the isoform of *P*-glycoprotein expressed during cell culture may be of significance with regard to the transport capability of the protein in vitro. The protein products of the mdr1a and mdr1b genes have overlapping but not identical capacities for transport of different drugs [32] and their sensitivities to modulators are also different. Phosphorylation is an additional factor that may regulate *P*-glycoprotein activity and requires investigation in both in vivo and in vitro situations. The possibility of changes occuring to *P*-glycoprotein during cell culture should be born in mind when in vitro models are used to assess the capability of various lipophilic agents to cross the blood–brain barrier.

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