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Identification of a Multidrug Resistance Associated Antigen (P-Glycoprotein) in Normal Human Tissues

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Abstract—*The multidrug resistance (MDR) phenotype describes a pattern of cross-resistance to unrelated compounds observed in mammalian cell lines selected* in vitro *for resistance to a single agent. Overexpression of a* 170 000 *dalton cell membrane glycoprotein (P-glycoprotein) is associated consistently with this phenotype in these cell lines. Recently, several human tumours have been shown to contain P-glycoprotein and expression was greatest in tumours exhibiting clinical drug resistance. To explore further the significance of P-glycoprotein, we examined normal human tissues obtained at autopsy by polyacrylamide gel electrophoresis and immunoblotting using a monoclonal antibody directed against P-glycoprotein. We showed expression of P-glycoprotein in normal liver and small bowel mucosa but not in other organs examined. This suggests there may be significant expression of P-glycoprotein in certain normal human tissues and any plan to exploit P-glycoprotein clinically must take these findings into account.*

INTRODUCTION

RAPID advances in the chemotherapy of various cancers that occurred during the 1960s and 1970s have slowed in the past 10 years as the supply of effective new antinoeplastic agents has dwindled. Moreover, tumour resistance to available agents has emerged as a major obstacle to further progress. The multidrug resistance (MDR) phenotype has aroused considerable interest as a possible explanation for pleiotropic resistance to chemotherapy in human tumours [1, 2]. Aspects of this MDR phenotype have been characterized in the colchicine-resistant Chinese hamster ovary (CHO) cell system. Mutants in this system are cross-resistant to a variety of unrelated antinoeplastic agents [3, 4]. Similar pleiotropic drug resistance is described in mammalian and human tumour systems [5].

Expression of a 170 000 dalton plasma membrane glycoprotein (P-glycoprotein) correlates with the degree of drug resistance in MDR cells and is a consistent marker in MDR cell lines from several mammalian species, including human [5, 6]. Reduced intracellular accumulation of drug also correlates with the degree of drug resistance [7, 8] and suggests P-glycoprotein may have some functional role in MDR cells. Recently, a monoclonal antibody to P-glycoprotein has been developed [9]. Using this monoclonal antibody, P-glycoprotein expression has been reported in human neoplasms including ovarian carcinoma [10], sarcoma [11] and leukaemia [12].

The range and level of expression of P-glycoprotein in normal human tissues needs to be explored for two reasons. First, it is a necessary step towards defining the clinical usefulness of P-glycoprotein expression. Second, some insight may be gained into the function of the protein.

We examined intra-abdominal tissues obtained at autopsy from patients without underlying malignancy to determine levels of P-glycoprotein expression in normal human tissues.

MATERIALS AND METHODS

1. *Sample preparation and storage*

Multiple intra-abdominal organ samples were collected from patients undergoing autopsy at this institution, in whom malignant disease was not identified and who had not received cytotoxic drugs *ante mortem*.

At least 3 g of each tissue was disaggregated using at Bellco Cellector (Bellco Glassware, Vineland, NJ 98360, U.S.A.) within hours of obtaining the material. Solid organs, such as liver, were disagggregated *in toto*, while only the mucosal lining was used from various parts of the alimentary tract. Tissues

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Table 1. Patients from whom samples were obtained

Patent no.	Sex	Age (yr)	Diagnosis
1	F	74	Congestive cardiac failure, chronic renal failure, chronic airways disease, hypertension
2	M	68	Septicaemia postgrafting aortic aneurysm
3	F	64	Myocardial infarct—ruptured ventricle
4	F	56	Diabetic ketoacidosis, myocardial infarct
5	M	72	Ischaemic heart disease, pneumonia
6	M	59	Myocardial infarct
7	F	60	Myocardial infarct
8	F	70	Myocardial infarct
9	M	68	Pulmonary embolism, ischaemic heart disease
10	M	71	Myocardial infarct
11	F	69	Myocardial infarct
12	M	64	Ruptured aortic aneurysm, myocardial infarct
13	M	75	Pneumonia, cerebrovascular accident
14	F	62	Myocardial infarct postcoronary artery bypass graft
15	F	66	Myocardial infact—ruptured ventricle

examined are listed in Table 1.

After disaggregation, the material was suspended in cold (4°C) phosphate-buffered saline (PBS) and centrifuged (100 ***g***, for 10 mins). The resulting pellet was suspended in alpha-modified Eagle's medium containing 10% v/v dimethylsulphoxide and 10% v/v fetal calf serum for storage at −70°C until further processing.

2. *Human lymphoid cell lines*

CCRF-CEM is a human lymphoid cell line originally derived from a child with drug-resistant leukaemia [13]. CEM/VLB_{100} is a vinblastine-resistant subline which grows in 100 ng/ml vinblastine [6]. This subline was cloned and made available for this work by Dr V. Ling (Toronto, Canada). CEM/VLB_{100} is multi-drug resistant and overexpresses P-glycoprotein [9].

3. *Membrane preparation*

Samples were thawed, washed by centrifugation (1000 ***g***, for 10 min) three times in cold PBS and resuspended in lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.4 at 25°C and 2 mM phenylmethylsulphonylfluoride) for 10 min at 4°C. Cells were disrupted using a hand-held Dounce homogenizer (20 strokes) and the membrane-containing supernatant was collected by centrifugation (4000 ***g***, for 10 min). A plasma membrane-enriched microsomal pellet was harvested from this supernatant by high-speed centrifugation (35 000 ***g***, for 30 min) and this pellet was resuspended in PBS and stored at −70°C.

Protein assays were performed using the BioRad protein assay (BioRad, Australia) with bovine serum albumin (BSA) standards.

4. *Electrophoresis and immunoblotting*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting were performed as described previously [9]. Immunoblots were blocked overnight in 10% v/v BSA at 37°C and, after washing with cold PBS, were probed with C219, a murine monoclonal antibody against P-glycoprotein, made available for this work by Dr V. Ling (Toronto, Canada) [9]. After washing three

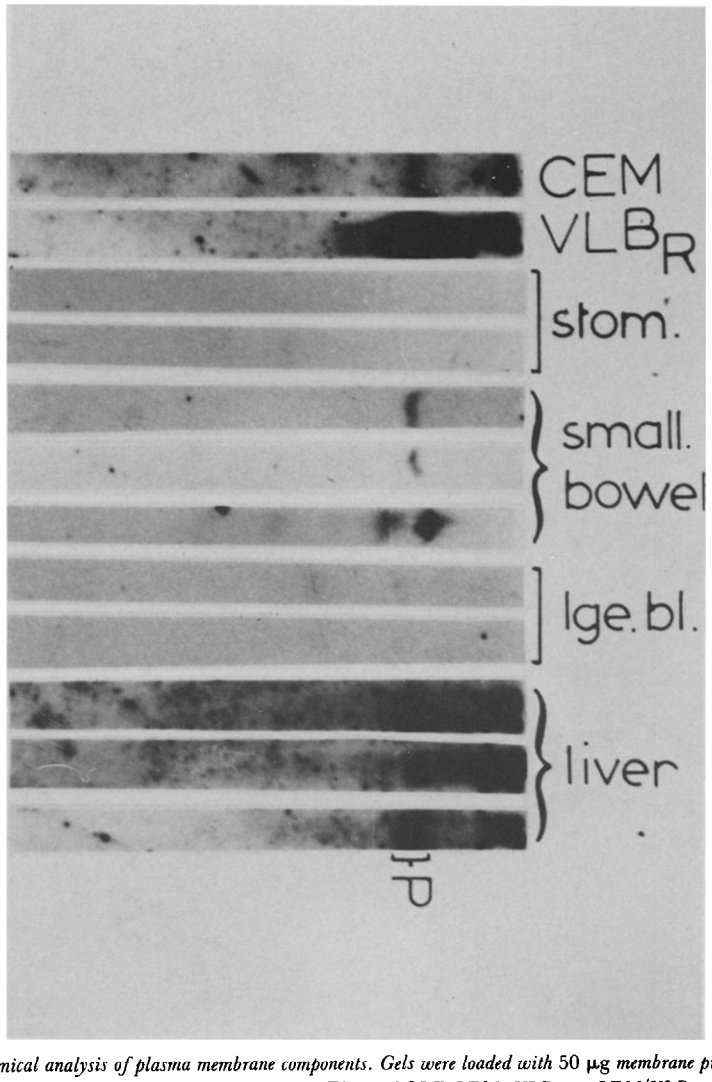

Fig. 1. *Immunochemical analysis of plasma membrane components. Gels were loaded with* 50 μg *membrane protein per lane. The figure represents a composite of several treated identically. CEM = CCRF-CEM; VLB$_R$ = CEM/VLB$_{100}$; stom. = stomach; lge.bl. = large bowel. P—indicates P-glycoprotein.*

Table 2. P-*glycoprotein expression in normal tissues*

Tissue	No.	No. P-glycoprotein positive
Liver	12	11
Small bowel	12	6
Spleen	12	0
Stomach	11	0
Pancreas	10	0
Kidney	12	0
Large bowel	11	0
Urinary bladder	6	0
Prostate	4	0
Uterus	5	0
Ovary	5	0

times in cold PBS, the immunoblots were overlaid with ^{125}I-labelled sheep antimouse IgG (Amersham Pty. Ltd., Australia) and binding was visualized by autoradiography.

RESULTS

Specimens from a number of normal intra-abdominal organs obtained from patients who died from non-malignant causes were examined. The group consisted of seven males and eight females with a median age of 68 years (range 56–75 years) at the time of death. Most deaths were due to cardiovascular disease, with 10 out of 15 (66%) related directly to recent myocardial infarction (Table 1).

P-glycoprotein was clearly observed in the multi-drug-resistant human lymphoid line, CEM/VLB$_{100}$ (Fig. 1), as previously reported [10]. This cell line is approximately 270 times more resistant to vinblastine than its parent line, CCRF-CEM. CCRF-CEM also expressed P-glycoprotein, although at a greatly reduced level. As this cell line was established from a child with clinically drug-resistant leukaemia [13], it may have a low inherent level of MDR which would account for the low level of P-glycoprotein expression. This level of MDR and P-glycoprotein expression may be clinically significant.

Definite expression of P-glycoprotein was noted in 11 out of 12 samples of liver (Table 2) with staining intensity less than that observed for CEM/VLB$_{100}$ (Fig. 1). The 11 positive liver samples all stained with a similar intensity. P-glycoprotein was also demonstrated in 6 out of 12 samples of small bowel mucosa with a similar intensity to that in CCRF-CEM cells (Fig. 1). P-glycoprotein was not detected in the other intra-abdominal organs surveyed.

DISCUSSION

This is the first report of P-glycoprotein expression in normal human tissues. The normal tissues in this study were evaluated using the same methodology which has been used previously to detect P-glycoprotein in multidrug-resistant cell lines [9] and human tumour samples [10, 11]. This immunoblotting technique has two major limitations. First, it may not be sensitive enough to detect low levels of P-glycoprotein expression which nevertheless have clinical importance. Second, the C219 monoclonal antibody may cross-react with a protein in human tissues of similar antigenicity to P-glycoprotein. C219 antibody binds to a protein of MW 170 000–180 000 present in membrane fractions from drug-resistant CHO and CCRF-CEM cells [9]. Until the amino acid sequence for P-glycoprotein is defined, cross-reaction of the monoclonal antibody with other proteins cannot be excluded.

The interval between death and autopsy varied from 12 to 72 hours in our patients. Although *post mortem* autolysis could potentially make P-glycoprotein more difficult to detect, there was no significant variation in P-glycoprotein expression within the positive liver samples or within the positive small bowel mucosa samples. This suggests that during the time period in which our samples were obtained autolysis was not significantly affecting the results. In addition cross-reactivity of the antibody with proteins from gut organisms may have lead to a positive result in small bowel, but this is difficult to reconcile with the absence of P-glycoprotein expression in large bowel specimens.

Despite these limitations immunoblotting using a monoclonal antibody to P-glycoprotein has been the most widely used method of detection of this protein. Other detection techniques, such as immunocytochemical methods, as we have reported recently [12], may improve sensitivity and address the problem of cellular heterogenity. We are currently evaluating such techniques for use in *post mortem* tissues. Northern blot analysis or *in situ* hydridization techniques for mRNA utilizing cDNA probes for the P-glycoprotein gene may also have some potential, but mRNA instability may make these techniques less useful in evaluating *post mortem* tissue.

Characteristics of the MDR phenotype so far described suggest a membrane-related phenomenon causing reduced intracellular accumulation of drug, probably due to active efflux of antineoplastic agents from such cells [7, 8]. Whether P-glycoprotein in drug-resistant cell membranes is the cause of, or just a marker for, this phenomenon is not known [8]. The demonstration of P-glycoprotein expression in normal human tissues is important in the elucidation of the function and significance of the protein.

Recently, Gerlach *et al.* (1986) described amino acid sequence homology beteen P-glycoprotein and hly B, the alpha haemolysin export protein of *E.*

coli [14]. They postulate that these proteins share similar evolution and function. Studies *in vitro* revealed an energy-dependent efflux pump in the membranes of both *E. coli* and MDR cells [14]. By blocking this pump using inhibitors of energy production (e.g. cyanide) or local anaesthetics, increased intracellular accumulation of haemolysin occurred in *E. coli*, and of antineoplastic agent in MDR cells. This supports a functional as well as structural homology between the two systems.

A functional transport role for P-glycoprotein would provide a logical explanation for the detection of significant levels of this protein in human liver and small bowel mucosa. Transportation of substances across membranes is a prominent feature of these tissues. Another well-described human cell membrane transport protein, the transferrin-receptor protein, is strongly expressed in liver [15].

Clearly, other normal tissues must be examined to define fully the expression of P-glycoprotein, but our findings do indicate a need for caution in the interpretation of P-glycoprotein identified in tumour samples. In particular, the potential for targeting drug-resistant tumour cells using a monoclonal antibody to P-glycoprotein requires accurate definition of its role in normal tissues. Further examination of normal tissues with careful clinical correlation is a necessary part of the elucidation of the role of P-glycoprotein in human tumour biology.

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