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P-glycoprotein of blood brain barrier: cross-reactivity of MAb C219 with a 190 kDa protein in bovine and rat isolated brain capillaries

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Abstract

P-glycoprotein (P-gp), an active efflux pump of antitumor drugs, is strongly expressed in endothelial cells of the blood brain barrier (BBB). Two proteins (155 and 190 kDa) were detected by Western blot analysis of beef and rat capillaries with the monoclonal antibody (MAb) C219. In order to characterize the nature of these proteins, their profile of solubilization by different detergents was established and compared with that of P-gp from the CH^RC5 tumoral cell line. The 155 kDa protein (p155) of capillaries and the P-gp of CH^RC5 cells were well solubilized by deoxycholate and Elugent, whereas the 190 kDa protein (p190) was only solubilized by sodium dodecylsulfate (SDS). Both proteins have different patterns of extraction by Triton X-114, p155 partitioning as a membrane protein, while p190 was insoluble. Deglycosylation of capillary proteins resulted in a 27–28 kDa decrease in the apparent molecular weight of p155, similar to that observed for the P-gp of CH^RC5 cells, but a decrease of only 7–8 for p190. Only p155 was immunoprecipitated by MAb C219. These results suggest that only p155 is the P-gp in BBB and that MAb C219 cross-reacts with a 190 kDa MDR-unrelated glycosylated protein. Consequently, the use of this antibody, which is frequently used to detect P-gp in tumors, could be a pitfall of immunohistochemistry screening for cancer tissues and lead to false positive in the diagnosis of MDR.

Keywords: P-glycoprotein; Glycoprotein; Brain capillary; Membrane; Cancer

1. Introduction

Multidrug resistance (MDR) constitutes a major obstacle in the clinical treatment of cancer [1]. The overexpression of a membrane glycoprotein of 150–170 kDa [2–4], P-glycoprotein (P-gp), is considered to be the cause of this resistance to multiple chemotherapeutic drugs [5]. P-gp has been cloned from different species including mouse and hamster [6] which possess three gene classes (I, II and III) and from human who has two classes (I and III) [5,7,8]. A model based on its amino acid sequence suggests that P-gp is an integral membrane protein with 12 transmembrane domains. Hydrophilic domains include an external glycosylation site and two internal ATP-binding sites [5,9].

The presence of P-gp has been demonstrated in normal tissues such as adrenal medulla, liver, kidney, colon [10,11] and endothelial cells of brain capillaries [11–13] where it is strongly expressed. Brain capillaries have a unique structure and constitute a selective barrier between blood and brain [14–16]. A recent report showed that P-gp has a pharmacological effect in brain, it limits the amount of drug accumulating [17]. Viable mice with a disruption of the mdr1a P-glycoprotein gene have been generated. These chimeric mice presented extreme sensitivity to ivermectin, which was associated with high accumulation of ivermectin in the brain. The absence of mdr1a P-gp increased the accumulation of vinblastine in the brain, where it was eliminated much more slowly [17].

Among the various antibodies available to detect P-gp in human tumors and in animal studies, MAb C219 is by far the most widely used. MAb C219 recognizes the

Abbreviations: P-gp, P-glycoprotein; BBB, blood brain barrier; MAb, monoclonal antibody; p155, the 155-kDa protein; p190, the 190 kDa protein; MDR, multidrug resistance; PB, physiological buffer; HM, homogenate medium; TBS-T, Tris-buffered saline-Tween 20; PVDF, polyvinylidene difluoride.

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epitope VQEALD, which is a conserved carboxy-terminal cytoplasmic sequence. The extensive use of this antibody is justified by its high affinity for the different classes of P-gp isoforms identified so far in most species where it was studied, including human, rat, mouse, beef, hamster and cell lines of various origins [2,18]. However, cross-reactivity of MAb C219 have already been reported with a protein of 65 kDa by Western blot and with a muscular protein by immunohistochemistry [19,20]. In this paper, we present a biochemical characterization of P-gp in purified BBB endothelial cells. A tumoral cell line from chinese hamster ovaries (CH^RC5) which expresses the MDR phenotype was used as a reference [7,9,21]. Two proteins were immunodetected with MAb C219 in beef and rat brain capillaries. Different physico-chemical treatments were performed to compare these two proteins and determine whether they are related to P-gp. Our results suggest that p155 is P-gp while p190 is MDR-unrelated.

2. Materials and methods

2.1. Chemicals

Dextran T-70 and protein A sepharose 4 fast flow were purchased from Pharmacia LKB Biotechnology (Montréal, Quebec). Electrophoresis reagents were from Bio-Rad (Mississauga, Ontario). MAb C219 was a product of ID Labs (London, Ontario). Horseradish peroxidase-conjugated rabbit anti-mouse IgG and enhanced chemiluminescence reagents were obtained from Amersham (Oakville, Ontario). Endoglycosidase F/*N*-glycosidase F and Hepes were from Boehringer-Mannheim (Laval, Quebec). Zwittergent 3-14, Hecameg and Elugent were products of Calbiochem (La Jolla, CA), Triton X-100 and X-114 and Tween 20 were from Bio-Rad and SDS was from Pierce (Rockford, IL). All the other reagents were purchased from Sigma (St. Louis, MO).

2.2. Purification of brain capillaries

Fresh bovine brains were obtained from a local abattoir and transported to the laboratory in ice-cold physiological buffer (PB) composed of 147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose and 15 mM Hepes/Tris, pH 7.4. Beef brain capillaries were then purified with the procedure of Dallaire et al. [22]. Brain capillaries were also isolated from Sprague-Dawley rats. For both species, the pia mater was removed, and the cerebral cortex was homogenized in 5 volumes of PB with a polytron (Brinkman Instruments, Rexdale, Ontario). The homogenates were mixed with an equal volume of 26 or 31% (w/v) Dextran T-70 in PB for bovine and rat cortex, respectively. The final pellets were resuspended in PB and stored at −180°C.

2.3. Isolation of membranes from CH^RC5 cells

The CH^RC5 cells were resuspended in homogenate medium (HM) composed of 50 mM mannitol and 5 mM Hepes/Tris, pH 7.5, homogenized 1 min with a polytron and centrifuged at $30\,000 \times g$ for 20 min at 4°C. The final pellets were resuspended in MH and stored at −80°C.

2.4. Immunodetection of P-gp

P-gp was detected by Western blot analysis using MAb C219. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Mini-Protean II apparatus (Bio-Rad) according to the method of Laemmli [23]. Brain capillaries or CH^RC5 membrane preparations were resuspended in sample buffer composed of 62.5 mM Tris/HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.00625% bromophenol blue to a final protein concentration of 0.5 mg/ml. The samples were agitated 30 min at 25°C or heated 3 min at 85°C and loaded on 6.25% acrylamide/bisacrylamide (29.1:0.9) gels. Electrophoresis was carried out at a constant voltage of 120 V. Proteins were transferred electrophoretically to a 0.45 μm pore size polyvinylidene difluoride (PVDF) membrane using a Milli-Blot Graphite Electroblotter I apparatus (Millipore, Bedford, MA). The transfer buffer contained 96 mM glycine, 10 mM Tris and 10% methanol. The transfer was carried out for 1 h at a constant amperage of 1 mA/cm^2. Hydrophobic or non-specific sites were blocked overnight at 4°C with 5% powdered skimmed milk in Tris-buffered saline (50 mM Tris and 150 mM NaCl) containing 0.3% Tween 20 (TBS-T). Membranes were washed three times for 15 min in TBS-T. The PVDF membranes were incubated with MAb C219 (200 ng/ml) in TBS-T, 1% bovine serum albumin (BSA) and 0.05% NaN_3 for 2 h at 37°C. Membranes were washed four times for 15 min and incubated 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:2000) in TBS-T containing 2% milk powder. PVDF membranes were washed four times for 10 min in TBS-T, P-gp was detected using chemiluminescence reagents (ECL). Molecular weights were determined with a calibration curve made with Bio-Rad standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumine (66 kDa) and ovalbumine (45 kDa).

2.5. Triton X-114 phase partitioning

Brain capillary proteins (5 mg/ml) were solubilized at 4°C for 30 min in PB containing 2% Triton X-114. The mixture was centrifuged at $100\,000 \times g$ for 30 min at 4°C and the pellet was resuspended in PB and 1% SDS. The supernatant was layered over a cushion composed of 1:10 volume of 250 mM sucrose and incubated for 10 min at 30°C. The phase separation was completed by centrifuging the turbid mixture at $2000 \times g$ 5 min at 30°C. The upper

detergent-poor phase was separated from the lower detergent-rich phase with a pasteur pipette.

2.6. Deglycosylation

Capillaries and crude membranes from CH^RC5 cells were resuspended in HM at a protein concentration of 6 mg/ml. Proteins were solubilized 10 min at 25°C in 0.5% SDS. The samples were diluted in HM containing 0.25% β-mercaptoethanol, 1% Triton X-100, 2 U/mg protein of endoglycosidase F/*N*-glycosidase F and proteinase inhibitors (2 μg/ml aprotinin, 10 μg/ml pepstatin A and 100 μg/ml bacitracin). Deglycosylation was carried out overnight at 25°C. Laemmli's sample buffer was added and Western blot analysis was performed as described above.

2.7. Peptide synthesis

The peptide VQEALD was synthesized with an Applied Biosystems synthesizer model 431A (Mississauga, Ontario) using Fmoc (9-fluoronylmethyloxycarbonyl) chemistry. Peptide purity was verified with a semipreparative HPLC column.

2.8. Immunoprecipitation

Brain capillaries and membranes from CH^RC5 cells were washed and resuspended in TBS (pH 7.6) at a protein concentration of 1 mg/ml. SDS (0.5%) was added and the solubilization was carried out for 30 min at 25°C. Samples were diluted (1:5) in TBS containing 1.25% Triton X-100, 0.625% deoxycholate and proteinase inhibitors, and centrifuged at 14000 × *g* for 10 min. MAb C219 (3 μg/ml) was added to the supernatants, and samples were incubated overnight at 4°C. Protein A sepharose equilibrated in TBS containing 1% Triton X-100, 0.5% deoxycholate and 0.1% SDS (RIPA) was added (120 μl/ml). The suspensions were incubated for 1 h at 25°C and centrifuged at 8000 × *g* for 4 min. Pellets were washed once with RIPA buffer, once with RIPA diluted 1:10 in TBS and twice with TBS. Sample buffer was added to the pellet for SDS-PAGE and Western blot analysis as described above.

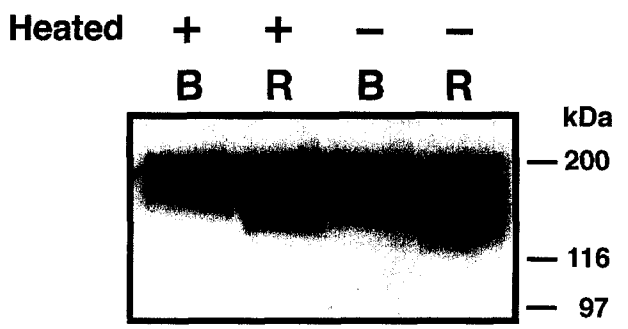

Fig. 1. Detection of P-gp from brain capillaries. Proteins (10 μg) from beef (B) and rat (R) were heated 3 min or solubilized 30 min at room temperature before SDS-PAGE. Proteins were transferred onto PVDF membranes and analyzed by Western blot with MAb C219 as described in Materials and methods.

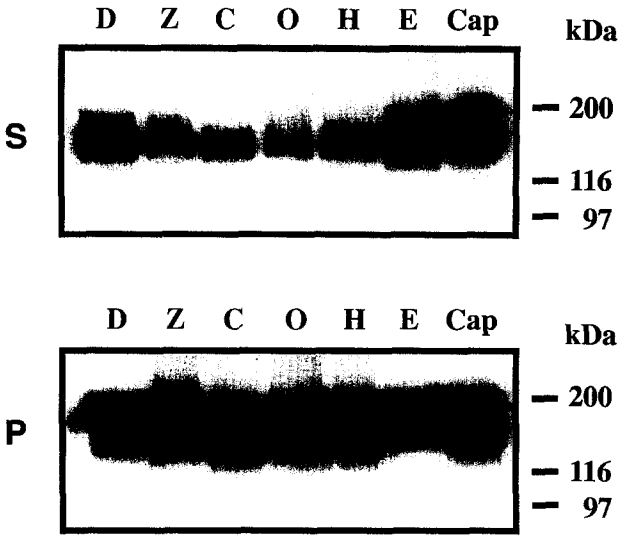

Fig. 2. Solubilization of P-gp from brain capillaries. Brain capillary proteins (0.5 mg/ml) from rat were solubilized 30 min at 25°C in homogenate medium with the following detergents: 0.4% deoxycholate (D), 0.1% Zwittergent 3-14 (Z), 1.2% CHAPS (C), 1.4% *n*-octylglucoside (O), 1.3% Hecameg (H) and 1.5% Elugent (E). Proteins (10 μg) from supernatants (S), pellets (P) and untreated capillaries (Cap) were resolved by SDS-PAGE and detected by Western blot analysis.

3. Results

Two proteins with apparent molecular mass of 155 and 190 kDa from beef and rat brain capillaries were immunodetected with the MAb C219. The detection of p155 was significantly reduced by heating the samples from both species (Fig. 1). However, p190 was always present with the same intensity, whether the samples were heated or not before electrophoresis.

The two proteins revealed by MAb C219 possess different patterns of solubilization by various detergents. At twice their critical micellar concentrations (CMC), several detergents solubilized very well p155 from brain capillaries (Fig. 2). Deoxycholate, an anionic detergent, and elugent, a mixture of non-ionic detergents, were the most effective in solubilizing p155 (Fig. 2). These detergents also efficiently solubilized P-gp from CH^RC5 cells (Table 1). In contrast, p190 was extracted only by SDS (Table 1) and by urea (results not shown).

Triton X-114 phase partitioning is a method used to separate the proteins according to their solubilization by this detergent [24] and it allows to evaluate the degree of hydrophobicity of proteins [25]. Fractionation of beef and rats brain capillary proteins with Triton X-114 showed that the two proteins detected by MAb C219 partitioned in different phases (Fig. 3). P155 was extracted by Triton

Table 1
Solubilization of P-gp from the membrane of CH^RC5 cells and proteins from brain capillaries detected with MAb C219

Detergent (concentration)	% Solubilization		
	P-gp from CH^RC5 cells	p155	p190
SDS (0.5%)	95	80	48
Hecameg (1.3%)	84	31	0
Octylglucoside (1.4%)	82	15	0
Elugent (1.5%)	81	90	0
Zwittergent 3-14 (0.1%)	81	34	0
Deoxycholate (0.4%)	78	88	0
CHAPS (1.2%)	75	30	0

Proteins from rat brain capillaries (1 mg/ml) and CH^RC5 cells were solubilized with different detergents 60 min at 25°C and immunodetected with MAb C219 as described in Materials and methods. Autoradiograms were scanned with an LKB Ultroscan XL enhanced laser densitometer and the relative area under each peak was calculated. Data shown represent the percentage of solubilization compared to the control.

X-114 and partitioned in the detergent-rich phase, while p190 was completely insoluble in Triton X-114 and remained in the first pellet. The fractionation profile of p155 and p190 was identical for beef and rat capillaries.

Both brain capillary proteins detected with MAb C219 are glycoproteins since both proteins had a different profile of migration after endoglycosidase F/*N*-glycosidase-F treatment (Fig. 4). The difference in migration rate is greater for p155 (27–28 kDa) than for p190 (7–8 kDa). The shift in the apparent molecular weight of p155 from capillaries was similar to that obtained for the P-gp of CH^RC5 cells after the same deglycosylation treatment.

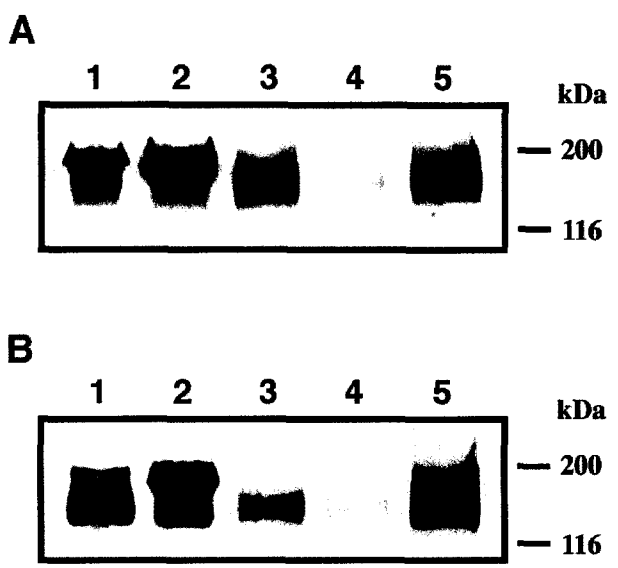

Fig. 3. Phase partitioning of brain capillary proteins with Triton X-114. Brain capillary proteins (lane 1) from beef (A) and rats (B) were solubilized in 2% (w/v) Triton X-114. After a 30 min centrifugation at 100000× *g*, these were separated into pellet (lane 2) and supernatant (lane 3) fractions. Phase separation of the latter gave a detergent-poor (lane 4) and a detergent-rich phase (lane 5). The proteins from each fraction were separated by SDS-PAGE and detected by Western blot analysis.

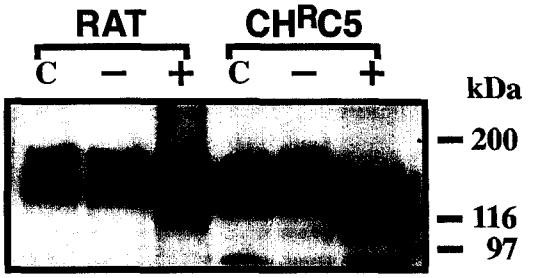

Fig. 4. Deglycosylation of brain capillary and CH^RC5 membrane proteins. Solubilized proteins from rat (RAT) and CH^RC5 crude membranes (CH^RC5) were incubated with (+) and without (−) endoglycosidase F/*N*-glycosidase F as described in Materials and methods. Electrophoresis and immunodetection with MAb C219 were described as mentioned previously. Untreated capillary or CH^RC5 cells are also shown (C).

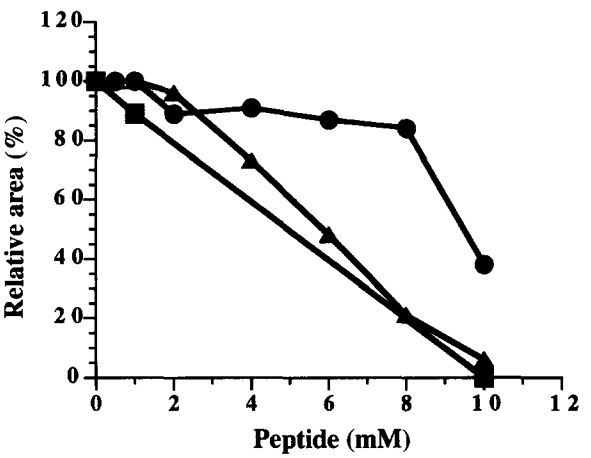

Fig. 5. Inhibition of MAb C219 binding by peptide VQEALD. Western blot analysis of the rat brain capillary proteins p155 (▲), p190 (●) and CH^RC5 membranes (■) were performed as described in Materials and methods with the addition of peptide to the primary antibody incubation medium. Autoradiograms were scanned with an LKB Ultroscan XL enhanced laser densitometer and the relative area under each peak was calculated. Data shown represent the percentage of solubilization compared to the control.

Competition studies with the VQEALD peptide have shown that the detection, with MAb C219, of p155 and P-gp from CH^RC5 cells is displaced at a lower peptide concentration than that of p190 kDa (Fig. 5). The binding of MAb C219 to the P-gp of CH^RC5 cells and to p155 is completely inhibited by 10 mM peptide. At the same peptide concentration, however, binding of MAb C219 to p190 was inhibited only by 45%.

Immunoprecipitation of brain capillary proteins with MAb C219 revealed that only p155, but not p190, is immunoprecipitated (Fig. 6).

4. Discussion

Two proteins bands of comparable intensity were detected by Western blot analysis of brain capillaries using MAb C219. The physicochemical characteristics of these

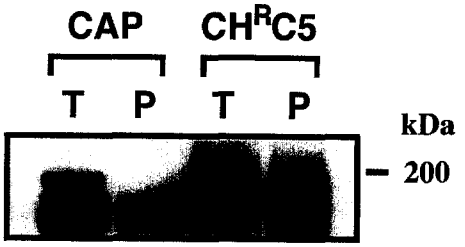

Fig. 6. Immunoprecipitation of brain capillary proteins and P-gp from CH^RC5 cells with MAb C219. Solubilized proteins from rat brain capillaries (CAP) and CH^RC5 cells (CH^RC5) were immunoprecipitated with MAb C219 as described in Materials and methods. Total (T), and the immunoprecipitated proteins (P) were separated by SDS-PAGE and Western blot analysis performed as described previously.

two proteins (190 and 155 kDa) were strikingly different. First, in contrast with p190, p155 from BBB presented a high sensitivity towards heat treatment. P-gp from CH^RC5 cells was also sensitive to heat treatment [26]. Second, extraction of brain capillary proteins with a variety of detergents indicated that the two proteins detected by MAb C219 showed different patterns of solubilization. The detergents that solubilized efficiently p155 from brain capillaries also solubilized P-gp from CH^RC5 cells. P190 behaved very differently since SDS was the only detergent that solubilized it. Urea (8 M) was the only other agent able to extract p190. Third, comparison of the hydrophobic properties by phase partitioning of the two proteins showed a strong difference. P155 partitioned in the detergent-rich phase and behaved like a membrane protein. In contrast, p190 was insoluble in Triton X-114. It is possible that p190 is a cytoskeleton associated protein since it was only solubilized by SDS and urea. The cytoskeleton is often experimentally defined as the insoluble residue remaining when cells are extracted with a non-ionic detergent [27]. Fourth, p155 and p190 reacted very differently to the *N*-deglycosylation treatment. P155 was more strongly affected in its migration pattern than p190. Fifth, lower concentration of the VQEALD peptide were required to abolish the binding of MAb C219 to p155 and to P-gp from CH^RC5 cells than to p190. Finally, among brain capillary proteins, only p155 was immunoprecipitated by MAb C219.

A cross-reactivity of MAb C219 to other membrane protein was recently reported. [19]. A protein of approx. 65 kDa was immunodetected by Mab C219 in the drug resistant cell lines, P388/ADR and P388/VCR-600. This protein seemed MDR-related and was called mini-P-glycoprotein. However, contrarily to P-gp, $P\text{-}gp_{mini}$ was not *N*-glycosylated neither phosphorylated at serine or threonine residues [19]. MAb C219 was also found to react immunohistochemically with striations in fast-twitch fibers in skeletal muscle [20].

A multidrug resistance associated membrane protein of 190 kDa (MRP) gene is overexpressed in adriamycin resistant HL60 cells. However, the mdr1 gene is not overexpressed in these cells and the level of P-gp is not detectable [28]. MRP, which binds ATP, presents a very small sequence homology with P-gp, since cross-reactivity of some antibodies against MRP is observed with P-gp [29]. However, the epitope recognized by MAb C219 is absent from the deduced amino acid sequence of MRP. These results suggest that p190 detected by MAb C219 in brain capillaries is not MRP.

In the present study, two proteins (p155 and p190) were revealed by Western blot analysis of brain capillaries with MAb C219. The results suggest that p155 is P-gp. However, cross reactivity was observed between MAb C219 and a glycoprotein of 190 kDa, probably unrelated to MDR. So, MAb C219, which is frequently used to demonstrate the presence of P-gp in tumor samples, also reacts with a distinct protein which could lead to false positive in the diagnosis of MDR in tumors.

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