ORIGINAL ARTICLE

Evidence that the choroids plexus in female sheep express P-glycoprotein

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Abstract

OBJECTIVES: Previous studies in the female sheep have shown that photoperiod modulates the passage of sex steroids between the blood and the cerebrospinal fluid (CSF) and have suggested the involvement of efflux transport. The objective of the present work was to assess the presence of P-glycoprotein (Pgp), which could be responsible for this transport at the level of the choroid plexuses (CP).
METHODS: We used flow cytometry and the UIC2 monoclonal antibody against Pgp, to demonstrate the presence of Pgp in the epithelial cell fraction isolated from the sheep choroid plexuses (CPEC). Thanks to the size of the brain structures in sheep, we analyse separately the CP from 4th ventricle (4V) and the CP from the lateral ventricle (LV).
RESULTS: In the whole population isolated from the CP, the 4V contained a higher percentage of living, epithelial cells than the LV. The immunoreactive cells to the UIC2 antibody i.e. bearing activated form of Pgp, represented 26.8% of the CPEC in the 4V, and 39.3% in the LV ($p<0.05$).
CONCLUSION: Pgp is expressed in the CPEC from the sheep. Differences in the expression of Pgp between CPEC from LV and 4V might suggest *in vivo* functional differences between LV and 4V CP in sheep.

INTRODUCTION

In the female sheep, a seasonal breeder, an increased negative feedback of estradiol (E2) on the hypothalamic structures during spring and summer, i.e. long days is responsible for the seasonal anoestrous [7, 10]. Several data from the literature show that exchange of the sex steroids between the blood stream and the brain could be under active regulation, despite their lipophilic nature implicating their passive diffusion across cellular membranes [12, 14, 19, 20]. Indeed, in the female sheep, we have previously shown a higher concentration of progesterone (P4) and estradiol (E2) in the cerebrospinal fluid (CSF) during long days than during short days, suggesting a photoperiodic modulation of the barriers of the brain probably through efflux mechanism [21, 22]. In this study however, a sharp increase in the concentration of P4 in the CSF was observed after intracarotid injection of a bolus containing this steroid. This suggests that P4 enters CSF directly from the blood via the blood-CSF-Barrier (BCSFB) located in the choroid plexuses (CP). Indeed, if P4 had entered

indirectly firstly in the brain interstitial fluid (ISF) via the blood-brain barrier (BBB) and then reaching the CSF by the slow current of the ISF bulk flow a much smoother increase in CSF P4 concentration would have been expected. The CP contain epithelial cells (CPEC) having tight junctions as anatomical support of the barrier and express several active transporters [17]. Among them, the P-glycoprotein (Pgp, an efflux ABC-transporter) could play an important role since it is present under the apical side of the CP in rat [15]. Most importantly, in mice, knockout of the mdr1 genes coding for the Pgp results in increased permeability of the brain to steroids [8, 25]. This led us to hypothesize that Pgp might participate in the exchange of steroids across the BCSFB in the female sheep. To support this hypothesis, we assessed the presence of the active form of Pgp in the CPEC, which form the BCSFB, by flow cytometry using UIC2 antibody. Apart from their localizations, LV and 4V have a distinct macroscopic aspect which could be linked with functional difference (Strazielle et al, 2005). We thus took advantage of the size of these organs in our animal model for comparing the expression of the Pgp between the PC originating from the lateral ventricle (LV) and from the fourth ventricle (4V).

MATERIALS AND METHODS

The experiments were conducted in accordance with Authorization N°37801 for Animal Experimentation and Surgery from the French Ministry of Agriculture, following the European Community Council Directive 86/609/EEC. Animals were killed by a licensed butcher (European Communities agreement n° 37175-01) in a certified slaughtering house (Iso9001/2000version, July 2003).

Isolation of cells

After decapitation, the skull was opened, the brain removed, and choroid plexuses from LV and 4V were extracted and rinsed in a warm (37°C) PBS (SIGMA Saint-Quentin Fallavier, France). Plexuses were cut in small pieces with scissors and the pieces were rinsed 3–4 times in PBS (without Ca^{2+} and Mg^{2+}) and thereafter incubated in filtered PBS containing 2mg/ml dispase (GIBCO Invitrogen Cergy Pontoise, France) for 45 minutes at 37°C. Pre-digested CP, after adding 12.5 µg/ml DNase (ROCHE Meylan, France) in warm PBS, were dissociated with a 1 ml pipette tip to release small clusters of CPEC, which were suspended and filtrated though 0.1 mm nylon mesh to eliminate tissue debris. After centrifugation (500 rpm for 5 min) the supernatant containing mostly single non-epithelial cells was discarded, and the pellet was resuspended in warm PBS containing 12.5 µg/ml DNase. The enriched epithelial cells fraction was pelleted by centrifugation (900 rpm for 5 min), the pellet resuspended in final culture media and filtered again through the 0.04 mm nylon mesh to eliminate large clusters.

Immunolocalisation of Pgp using flow cytometry

After cells isolation, pellets were re-suspended in PBS before being aliquoted into 300,000 cell-containing tubes. Taking into account the procedure of isolation, these cells represented enriched rather than pure CPEC fraction. Quantification of Pgp [9] was obtained with R-phycoerythrin-conjugated monoclonal anti-Pgp antibodies (UIC2 clone, Immunotech, Marseille, France). The UIC2 antibody is characterized by its binding to the active form of Pgp [11]. Cells were pretreated with medium (DMEM-F12 supplemented with Hepes, gentamycin, antibiotics cocktail, 5% of fetal calf serum treated with charcoal to eliminate steroids and BSA (2 mg/ml) for 10 min, thereafter centrifugated at 900 rpm for 5 min. The supernatants were discarded and the cells were incubated for 90 min at room temperature by adding 25 µl of pure UIC2 mAbs (15 µg/ml). They were washed with 3 ml medium and suspended in 0.5 ml medium before analysis. To assess specificity, CPEC from the same batch (control) were similarly treated with isotypic IgG2a mAbs, conjugated with R-phycoerythrin (U7.27 clone, Immunotech). In order to distinguish between living and dead cells, 7-Amino-actinomycin D (7AAD, 6 µl/ml for 5 min) was added to the medium [26]. The two light-scatter parameters, forward and side scatter, enabled cells to be identified from residual debris. Epithelial identity of cells was assessed by immunoreactivity [5] using an FITC anti-cytokeratin mAbs (clone J1B3 IgG1, Immunotech), with the use of the isotypic IgG2a mAbs as control. This allowed further delimitation of the two light-scatter parameters of CPEC. The individual fluorescence intensities of cells were estimated with a FACStar $^{Plus\ TM}$ cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA), using an argon ion laser with the excitation line set to 488 nm and the light power adjusted to 80 mW. The orange fluorescence corresponding to the R-phycoerythrin conjugated anti-Pgp mAb emission was measured through a 575/26 nm band-pass filter, and 7AAD with a 675/20 nm band pass filter. Instrument settings were selected such that 15,000 cells were analysed per sample. Three replicates per isolate and per condition of treatment were performed.

Mathematical treatment

All values are given as Mean ± SEM. Student t test was used for the comparison of means in the immunological labelling between populations of cell from flow cytometry.

RESULTS

Immunolocalisation of Pgp using flow cytometry

Cells harvested from the 4V CP contained significantly more CPEC (73%) than the cells harvested from the LV CP (56.25%), as identified by immunoreactivity to the cytokeratin FITC antibodies. Among them, the use of 7AAD enabled us to identify 62.8% and 61.8% of living

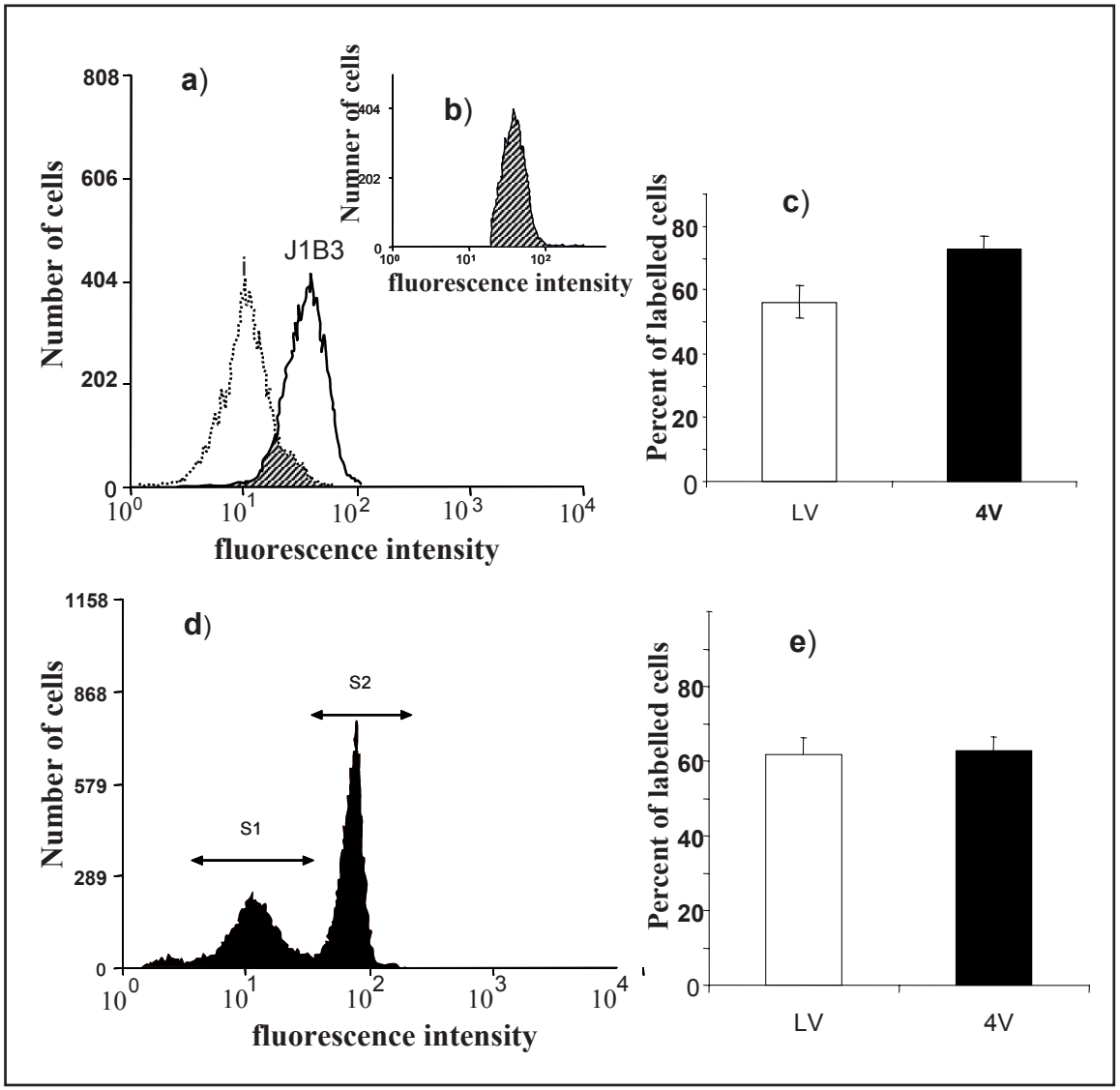

Figure 1: Identification of epithelial cells (CPEC) from the whole population isolated from PC. **a)** The black histogram on the right side (J1B3) represents the number of cells labeled by the J1B3 anti-cytokeratin mAbs, identifying CPEC, and the dotted histogram on the left side (i) represents the number of cells labeled by the isotypic antibody. **b)** This histogram is obtained by the subtraction of the values from the two histograms shown as hatched area in (a) and represents the population specifically labeled by the J1B3. **c)** Percentage of CPEC in the population isolated from PC originating from the LV (56.2%) or from the 4V (73%). **d)** Identification of living and dead cells from the population isolated from PC, using 7-Amino-actinomycin D. S1 is the population of living cells and S2 the population of dead cells. **e)** Percentage of living CPEC in the population isolated from PC originating from the LV (61.7%) or from the 4V (62.8%).

cells respectively for the cells of the 4V and the LV (Figure 1). Within this latter population, immunoreactive cells to the UIC2 antibody i.e. bearing activated form of Pgp (Figure 2), represented 26.8% and 39.3% from the LV and 4V cells, respectively ($p<0.05$).

DISCUSSION

This study revealed for the first time the presence of the active form of Pgp in the CPEC in the female sheep and showed quantitative difference in its expression between the LV and 4V. The identification of Pgp in the CP in sheep is consistent with the localisation of the transporter observed in rat, mouse and human [15, 17], and in the immortalized cells from the CP in sheep [12]. The choice of UIC2 and flow cytometry for identification of Pgp deserves explanation. Among others, C219 antibody is one of the most frequently used, and we have performed trials for quantification of Pgp with its use by Western Blotting, without success. Conversely, UIC2 associated with flow cytometry, and its assessment with the use of the isotypic mAbs, revealed to be a powerful antibody labelling external epitopes of "activated" Pgp. It has been used to identify the transporter in various cancer cells [13, 6] as well as in the eggshell from parasite nematode [9]. Furthermore, in our ex-

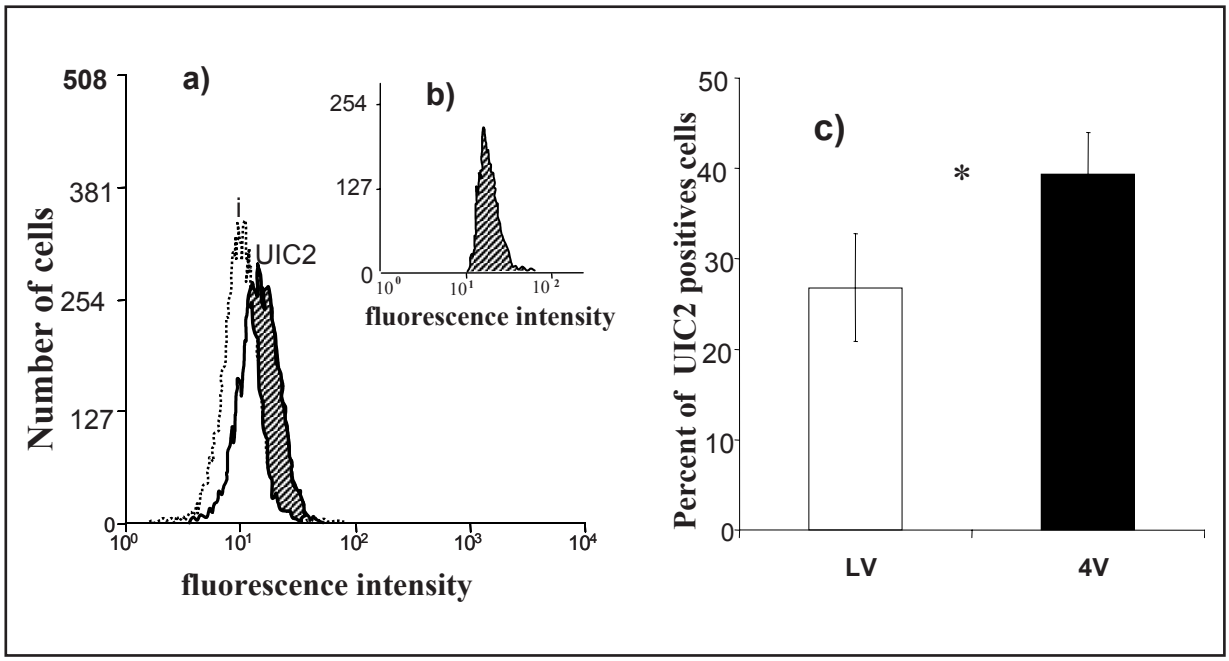

Figure 2: Identification of CPEC expressing Pgp and comparison between CPEC from the LV and those from the 4V. **a)** The black histogram on the right side (UIC2) represents the number of cells labeled by the UIC2 antibody, identifying Pgp, and the dotted histogram on the left side (i) represents the number of cells labeled by the isotypic antibody. **b)** This histogram is obtained by the subtraction of the values from the two histograms shown as hatched area in (a) and represents the population specifically labeled by the UIC2. c) Difference between the populations of CPEC expressing Pgp originating from the LV (26.8%) or from the 4V (39.3%), $p<0.05$.

periment, the flow cytometry allowed us to identify the epithelial nature of the labelled cells among the population of CP containing blood and endothelial cells and allowed comparison of number of cells expressing Pgp of the CP originating from different ventricles.

Obviously, other ABC-transporters like the MRP family could be responsible for this transport, since MRP1 is involved in the efflux transport of E217βG across the blood brain barrier [19], and MRP8 transports the steroid sulphates [2, 3]. Also, the gluco-conjugated form of E2 crosses the brain barriers actively in the rat using transporters of organic anion (oat3) for efflux [12], suggesting a complex regulatory mechanism of the concentration of steroids within the CSF.

Our study revealed different expression of Pgp between the CPEC enriched fraction originating from LV when compared to that originating from the 4V. Interestingly in dog, some molecules from the CSF were shown to be absorbed in the tissue more efficiently at the level of the 4V than in the rest of the ventricular system [1]. The 4V shows a higher proportion of epithelial cells, among which a larger proportion expresses Pgp. Apart from anatomical differences [18], it is possible that *in vivo* functional differences could be responsible for the variations in the characteristics of the CP from the 4V and from the LV. Physiologically, several candidates could be involved in the difference between cells from the LV and 4V. Among them, the involvement of melatonin deserves attention. CP *in vivo* are soaked with the CSF, where melatonin concentration in sheep has been shown to follow both circadian and seasonal changes, with concentrations up to 100 to 1,000 times higher than in blood plasma [16]. Furthermore, other results from our laboratory suggest large variations in the melatonin concentration between the sites of the ventricular system [24]. In the golden hamster, another photoperiodic species, the density of microvillies from the apical side of the CP has been shown to be different between the 4V and LV, a difference abolished by *in vivo* melatonin treatment. Such treatment also results in a 50% increase both in the total number and size of the cells coming from the CP of the LV but not from the 4V [4]. Then, we can suggest that differences in the expression of Pgp between the cells originating from the two types of ventricles in sheep could be explained by a local difference in the melatonin content.

In conclusion, our results demonstrate the presence of Pgp in the cells from the CP in sheep compatible with an active efflux of sex steroids from the CSF to the blood, but the actual involvement of the transporter remains to be established. The differences in permeability of the cultured CP coming from the LV and the 4V suggest a possible functional specificity of CP *in vivo*.

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