Distribution and functional activity of P-glycoprotein and multidrug resistance-associated proteins in human brain microvascular endothelial cells in hippocampal sclerosis

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Abstract

Multidrug resistance protein, also referred as P-glycoprotein (P-gp, MDR1, ABCB1) and multidrug resistance-associated protein (MRP) 1 (ABCC1) and 2 (ABCC2) are, thus far, candidates to cause antiepileptic drug (AED) resistance epilepsy. In this study, we investigated P-gp, MRP1 and MRP2 expression, localization and functional activity on cryosections and isolated human brain-derived microvascular endothelial cells (HBMEC) from epileptic patients (HBMEC-EPI) with hippocampal sclerosis (HS), as compared with HBMEC isolated from normal brain cortex (HBMEC-CTR). We examined the expression and distribution of three transporters, P-gp, MRP1 and MRP2 on two major parts of the resected tissue, the hippocampus and the parahippocampal gyrus (Gph). P-gp showed diffuse expression not only in endothelium but also by parenchymal cells in both the hippocampus and the Gph. MRP1 labeling was observed in parenchymal cells in the Gph. By contrast, MRP2 was mainly found in endothelium of the hippocampus. P-gp and MRP1 expression in the Gph was relatively high in the patient with long-term seizure history. Quantitative RT-PCR analysis of HBMEC revealed that MDR1, MRP1 as well as MRP5 (ABCC5) and MRP6 (ABCC6) were overexpressed in HBMEC-EPI from epileptic patients (HBMEC-EPI) as compared with HBMEC-CTR. Accordingly, it is of particular interest that MRP functional activities were observed in HBMEC-EPI, but not in HBMEC-CTR. Our results suggest that complex MDR expression changes not only in the hippocampus but in the Gph may play a role in AED pharmacoresistance in intractable epilepsy patients with mesial temporal lobe epilepsy (MTLE) by altering the permeability of AEDs across the blood–brain barrier (BBB).

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1. Introduction

Mesial temporal lobe epilepsy (MTLE) associated with hippocampal sclerosis (HS) is the most frequent medically intractable epilepsy. HS consists of gliosis, neuronal loss and cell dispersion. The long-term follow-up after selective amygdalohippocampectomy (sAHE) in the presence of severe HS showed a greater than 80% of patients becoming seizure free (Wieser et al., 2003). This finding raises the question why the pharmacological treatment with antiepileptic drugs (AEDs) is not effective in MTLE–HS despite of good results after resection of the sclerotic hippocampal formation. Among various reasons for the pharmacoresistance of these disorders, it has been suggested that inadequate intraparenchymal AED concentration represents a possible mechanism of resistance to AEDs.

Penetration through the blood–brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) is necessary if a drug is to achieve the required concentration for a desired pharmacological effect. Efflux transport systems at the BBB and BCSFB provide a protective barrier function by removing drugs from the brain or cerebrospinal fluid and transferring them to the systemic circulation, respectively. Multiple transporters have been identified in this efflux mechanism at the BBB and BCSF (for review see Kushnara and Sugiyama, 2001; Mizuno et al., 2003).

Recent studies have shown that P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRPs), which belong to a superfamily of the ATP-binding cassette (ABC) transporters, play important roles in the efflux of AEDs out of the cells in epileptic brain tissue, resulting in lower intracellular concentration of AEDs (Tishler et al., 1995; Dombrowski et al., 2001; Loscher and Potschka, 2002; Sisodiya et al., 2002; Aronica et al., 2004). In epileptogenic lesions, these transporters appear not only in endothelial cells but also in astrocytes and neurons. Notably, P-gp usually shows widespread distribution in the epileptogenic lesion, while MRPs are expressed non-homogeneously (Hegmann et al., 1992; Sisodiya et al., 2002; Aronica et al., 2004). However, in detail the heterogeneous expression of these transporters has not been studied in the totality of the surgically resected tissue. Thus, the first aim of this study was to investigate distribution of P-gp, MRPs 1 and 2 in the specimens resected by sAHE.

Isolated endothelial cells from epileptogenic lesion might have different properties in comparison to normal ones, because MDR1, MRPs and MRP5 are overexpressed at mRNA level in epilepsy (Dombrowski et al., 2001). Therefore, the second aim of this study was to examine the expression, intracellular localization and functional activity of multidrug resistance-related proteins in human brain-derived microvascular endothelial cells (HBMEC) of epileptic patients as compared with HBMEC of control patients. We used quantitative real-time polymerase chain reaction (RT-PCR) to measure MDR1 and MRPs 1–6. At the protein level we focused on P-gp, MRPs 1 and 2 expression of HBMEC. Especially, the comparison of P-gp and MRPs functional activity between HBMEC of epileptic patients and control HBMEC has not yet been studied before. The P-gp inhibitor verapamil is widely used in experimental activity between HBMEC of epileptic patients and control HBMEC has not yet been studied before. The P-gp inhibitor verapamil also has been already used in a patient with intractable epilepsy, resulted in a prolonged seizure free time interval (Summers et al., 2004). Because of the lack of MRP specific inhibitors, probenecid and indomethacine were frequently used as organic anion transport inhibitors in previous studies (Huai-Yun et al., 1998; Regina et al., 1999; Declevès et al., 2000; Zhang et al., 2000; Dallas et al., 2003; Potschka et al., 2003). Together, the second aim of this study was to examine the expression,
intracellular localization and functional activity of multidrug resistance-related proteins in HBMEC-EPI as compared with HBMEC-CTR.

2. Methods

2.1. Surgical specimens

Thirteen clinical specimens were obtained from patients with chronic pharmacoresistant MTLE, who underwent surgical treatment at the Department of Neurosurgery, University Hospital Zurich (Table 1). Surgical removal of the hippocampus was clinically indicated in every case. The specimens were obtained by sAHE, in which parts of the amygdala, the hippocampus and the anterior portion of the parahippocampal gyrus were selectively removed. All tissues were diagnosed by two pathologists at the Department of Neuropathology, University Hospital Zurich. The hippocampus and the parahippocampal gyrus (Gph) were examined and separately rated for the presence and severity of HS. The severity of gliosis and neuronal loss was defined by three degrees (mild, marked and severe) and quantified by the Wyler grading system (Wyler et al., 1992). All hippocampal regions revealed HS with various severities of gliosis and neuronal loss. The parahippocampal gyrus resected along with the hippocampus displayed some pathological features except in case 9. The parahippocampal gyrus of case 9 exhibited no abnormalities and was used as a “normal” tissue. For limitation of the specimen size it was difficult to perform both immunohistochemistry (patients #8, 9 and 11–13) and isolation of HBMEC (patients #1–10) at once. In two cases (patients #8 and 9), the resected tissue was divided into anterior and posterior parts to use for immunohistochemistry and cell culture, respectively. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Canton Zurich. Informed written consent was obtained from all patients.

2.2. Isolation of human brain-derived microvascular endothelial cells

HBMEC-CTR were purchased from Cell Systems Inc. (Kirkland, WA) and used between passage 3 and 7. The cells were isolated by elutriation technique of dispase dissociated normal brain frontal cortex of 24- and 37-year-old patients, who died by accidents without prior history of epilepsy, cancer or circulatory diseases requiring any medication. Isolation and culture of HBMEC-EPI were performed as described (Wachtel et al., 2001). Briefly, the tissue samples were cleaned of large vessels under a microscope and homogenized in DMEM (Gibco, Invitrogen AG, Basel, Switzerland) containing 5% FCS (Gibco) with a 5 ml syringe, digested with 0.25 mg/ml collagenase (Roche Applied Science, Rotkreuz, Switzerland) for 30 min at 37°C in a stirring water bath, cooled on ice and centrifuged (250 × g, 10 min, 4°C). The pellet was resuspended in DMEM/5% FCS, triturated with a fire-polished pipette, and then layered on 25% (w/v) bovine serum albumin (BSA) (Sigma, Fluka AG, Buchs, Switzerland) in HBSS. After centrifugation (800 × g, 20 min, 4°C), the pellet was resuspended in DMEM/5% FCS containing 1 mg/ml collagenase/dispase (Roche) and 0.05 mg/ml DNase I (Sigma) and stirred another 30 min at 37°C. The cells were pelleted (380 × g, 10 min, 4°C), washed with DMEM/5% FCS and resuspended in phenol-red free culture medium (DMEM/F12, Gibco) containing 15 mM HEPES (Gibco), 10% FCS (Gibco), 10% horse serum (Gibco), 50 μg/ml endothelial cell growth supplement (Serva, Heidelberg, Germany), 1.2 mg/ml bovine brain extract (Cambrex, BioConcept, Allschwil, Switzerland), 0.1 ng/ml recombinant human (rh) epidermal growth factor (PeproTech, PeproTech EC Ltd., London, UK), 1 ng/ml rh acidic fibroblast growth factor (PeproTech), 10 pg/ml hydrocortisone (Sigma), 2 mM N-acetyl-l-alanyl-l-glutamine (Biochrom, Berlin, Germany), 50 μg/ml gentamycin (Gibco), 16 U/ml heparin (Sigma), seeded into collagen type I-coated culture dishes (BD Falcon, BD Biosciences, Basel, Switzerland) and incubated in a humidified atmosphere at 37°C in 5% CO2 in air. Medium was changed every 2–3 days and at 70–90% confluence, HBMEC were either trypsinized (0.025% trypsin: 0.27 mmol/l EDTA, Gibco) and subcultured at a split ratio of 1:3 on collagen type I-coated dishes (BD Falcon) or used for RNA isolation and cell extracts. Because the cell morphology was altered at high passage number, HBMEC-EPI was used between the first and fifth passage and HBMEC-CTR between passage 3 and 7. The purity of the endothelial cultures was checked using polyclonal rabbit anti-human von Wille-
Table 1
Clinical history and pathological diagnosis of 13 patients who underwent selective amygdalohippocampectomy

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (years), sex</th>
<th>Pathological diagnosis</th>
<th>AED medication</th>
<th>Type of seizures</th>
<th>Years with epilepsy</th>
<th>Frequency of seizures (seizure days/month)</th>
<th>Total number of seizure days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42, M</td>
<td>Severe gliosis (W4)</td>
<td>CBZ, LEV</td>
<td>CPS</td>
<td>11</td>
<td>0.5</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>38, M</td>
<td>Marked gliosis, focal marked neuronal loss (W4)</td>
<td>CBZ, LEV, LTG</td>
<td>SPS, CPS</td>
<td>31</td>
<td>7</td>
<td>2604</td>
</tr>
<tr>
<td>3</td>
<td>21, F</td>
<td>Marked gliosis, diffuse severe neuronal loss (W4)</td>
<td>n.a.</td>
<td>PHT, TPM</td>
<td>17</td>
<td>20</td>
<td>4060</td>
</tr>
<tr>
<td>4</td>
<td>9, M</td>
<td>Marked gliosis, diffuse mild neuronal loss (W3)</td>
<td>Marked gliosis</td>
<td>PHT, GP, LTG</td>
<td>8</td>
<td>8</td>
<td>768</td>
</tr>
<tr>
<td>5</td>
<td>33, F</td>
<td>Mild gliosis (W1)</td>
<td>PR, OXC</td>
<td>SPS, CPS, SGCP</td>
<td>22</td>
<td>4</td>
<td>1056</td>
</tr>
<tr>
<td>6</td>
<td>36, F</td>
<td>Mild gliosis, focal mild neuronal loss (W2)</td>
<td>Mild gliosis</td>
<td>CBZ, LEV</td>
<td>n.a.</td>
<td>5</td>
<td>n.a.</td>
</tr>
<tr>
<td>7</td>
<td>35, F</td>
<td>Marked gliosis (W2)</td>
<td>PR, LEV</td>
<td>CPS</td>
<td>41</td>
<td>4</td>
<td>1968</td>
</tr>
<tr>
<td>8</td>
<td>31, M</td>
<td>Mild gliosis (W1)</td>
<td>OXC</td>
<td>SPS, CPS, SGCP</td>
<td>22</td>
<td>20</td>
<td>5258</td>
</tr>
<tr>
<td>9</td>
<td>25, F</td>
<td>Mild gliosis, focal mild neuronal loss (W2)</td>
<td>No pathological findings</td>
<td>CBZ, LEV, LTG</td>
<td>13</td>
<td>18</td>
<td>2080</td>
</tr>
<tr>
<td>10</td>
<td>23, M</td>
<td>Mild gliosis (W1)</td>
<td>OXC, LEV</td>
<td>SPS, CPS, SGCP</td>
<td>13</td>
<td>3</td>
<td>468</td>
</tr>
<tr>
<td>11</td>
<td>16, F</td>
<td>Mild gliosis (W1)</td>
<td>CBZ</td>
<td>CPS</td>
<td>1</td>
<td>8</td>
<td>96</td>
</tr>
<tr>
<td>12</td>
<td>46, F</td>
<td>Marked gliosis, focal mild neuronal loss (W3)</td>
<td>Marked gliosis</td>
<td>LTG</td>
<td>41</td>
<td>30</td>
<td>14760</td>
</tr>
<tr>
<td>13</td>
<td>33, F</td>
<td>Severe gliosis (W4)</td>
<td>CBZ, VPA</td>
<td>n.a.</td>
<td>1</td>
<td>30</td>
<td>360</td>
</tr>
</tbody>
</table>

AEDs, antiepileptic drugs; CBZ, carbamazepine; LEV, levetiracetam; LTG, lamotrigine; PR, phenytoin; OXC, oxcarbazepine; VPA, valproic acid; SPS, simple partial seizures; CPS, complex partial seizures; SGCP, secondary generalized complex partial seizures. n.a., not available; CTR, control.

a Wyler grading system.
b Immunoblotting.
c P-gp functional assay.
d MRP functional assay.
e Laser scanning confocal microscopy.
f Quantitative real-time RT-PCR (TaqMan).
g Immunohistochemistry.

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brand factor (vWF) and anti-human glial fibrillary acidic protein (GFAP) antibodies (both Dako Diagnostics, Zug, Switzerland) and found to be greater than 90%.

2.3. Glioma cell lines

The human cell line T98G was obtained from the American Type Culture Collection (Rockville, MD) and LN-229 was kindly provided by Dr. N. de Tribolet (Geneva, Switzerland). They were maintained at 37 °C in a 92% air–8% CO₂ humidified atmosphere in DMEM (Gibco) containing 10% FCS, 2 mM N-acetyl-l-alanyl-l-glutamine (Biochrom), 1 mM sodium pyruvate (ICN Biomedicals, Birsfelden, Switzerland) and 10 µg/ml gentamycin (fabcoc).

2.4. Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from HBMEC at low passages (between 1 and 4) using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. First-strand cDNA synthesis was performed with the Reverse Transcription System from Promega. To a master mixture containing 5 mM MgCl₂, 1× reverse transcription buffer, 1 mM deoxynucleotide triphosphate mixture, 1 unit/µl recombinant RNasin® ribonuclease inhibitor, 0.75 U/µl AMV reverse transcriptase and 1 µg of random hexamer primers, we added 2 µg of total RNA and sterile H₂O to a final volume of 40 µl. The reaction mixture was incubated at 42 °C for 60 min, followed by heat inactivation of the enzyme at 95 °C for 5 min. After cooling on ice for 5 min, the cDNA was stored at −20 °C.

Primers and quantitative real-time reverse transcription-PCR (TaqMan) probes for the analysis of ABCB1 and ABCC1–ABCC6 were designed with PrimerExpress Software, Ver. 2.0 (Applied Biosystems) and have been described recently (Langmann et al., 2003). All primers and ABI fluorescence 6-carboxyfluorescein (FAM)-labeled probes were obtained from MWG-Biotech. For the normalization of our results, we used a VICTM-labeled 18S ribosomal RNA control reagent set (Applied Biosystems). Each of the probes was quenched by 6-carboxytetramethylrhodamine (TAMRA) at its 3’ end.

Quantitation of selected transcripts was performed as recently described (Langmann et al., 2003). Briefly, we prepared 384-well plate containing a master mixture. Triplicates of cDNA templates equivalent to 50 ng of RNA were added to a final volume of 20 µl. The thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Measurements were performed in triplicate. Results were analyzed with an ABI sequence detector software Version 2.0 (Applied Biosystems) and quantified by the calibration curve method as described earlier (Langmann et al., 2003).

2.5. Immunohistochemistry

Frozen tissues were cut at the coronal slices of 4 µm thick by a cryostat. The slides were fixed with acetone for 10 min and incubated with the following antibodies with 2% human serum; anti-P-gp (clone JSB-1, 1:20; Alexis, Lausen, Switzerland, and clone C219, 1:20; Alexis), anti-MRP1 (clone MRPr1, 1:20; Alexis) and anti-MRP2 (clone M2 I-4, 1:20; Alexis). For P-gp staining, at least two antibodies were used for protein detection, as well as to confocal microscopy analysis below. Rabbit anti-mouse or rabbit anti-rat immunoglobulin (Dako) were incubated as secondary reagents, followed by mouse or rat APAAP (Dako). The signals were visualized using the Dako Fuchsine Substrate System (Dako), followed by counterstaining with hematoxylin.

2.6. Western blot analysis

Extracts of HBMEC (between passage 1 and 4) were prepared in 200 µl lysis buffer consisting of 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% TritonX-100 and 1× complete protease inhibitor mixture (Roche). Protein concentration of the lysate was measured by the Bradford assay (Bio-Rad, Munich, Germany). Thirty micrograms of proteins were loaded in 7.5% gels for 2 h and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). All blots were blocked with bloting buffer (5% skim milk in TBS containing 0.05% Tween-20) for 45 min at RT. The membranes were incubated with the following antibodies; anti-P-gp (clone C219, 1:500; Alexis), anti-MRP1 (clone MRPr1, 1:300) and anti-MRP2 (clone M2 I-4, 1:50) in blotting buffer at 1.5 h at...
RT, following incubation with horseradish peroxidase-conjugated secondary antibodies in blotting buffer for 45 min at RT. The membrane was washed by the stripping buffer (25 mM glycine–HCl, pH 2.1, 1% SDS) and reblotted with anti-α-tubulin (1:4000, Sigma, clone B-5-1-2) following detection with anti-mouse secondary antibody (1:2000). Bands on the membranes were detected using the ECL chemiluminescence reagent (Amersham Biosciences, Olteffingen, Switzerland).

2.7. Immunocytochemistry and laser scanning confocal microscopy

1 × 10^5 endothelial cells (between passage 2 and 4) were seeded on the upper part of collagen-coated inserts (Transwell chamber system, no. 3493, 12 mm diameter, 0.4 μm pore size, Costar, Cambridge, MA) to check for polarized localization of P-gp and MRPs on HBMEC according to the method of Anderle et al. (1998). Three individual cultures and stainings were performed using both types of HBMEC. After 4 days of culture, HBMEC formed a monolayer and the upper inserts were washed once in phosphate-buffered saline (PBS), followed by fixation with 4% paraformaldehyde (15 min at 4°C) and treatment with 0.1 M glycine (5 min at 4°C). The cells were permeabilized with 0.5% Triton-X/PBS for 1.5 min and washed three times in PBS. The membranes of upper inserts were cut carefully by a scalpel and put on SuperFrost slides (Menzel-Glaser, Germany), followed by drying for 2 h at room temperature. The cells on slides were counterstained with propidium iodide (PI) (1 g/ml) for 10 min, followed by incubation with the following antibodies; anti-P-gp (clone MRK16, 1:25, Alexis and JSB-1, 1:4), anti-MRP1 (clone MRPr1, 1:20, Alexis), anti-MRP2 (clone M2 I-4, 1:50, Alexis), anti-vWF (rabbit polyclonal, 1:200, Dako) and isotype antibodies at the appropriate concentration overnight at 4°C. Secondary antibodies conjugated with fluoresceine isothiocyanate (FITC) were diluted in 1:100 dilution for 1 h at 4°C. After washing, coverslips were drained and mounted in 70% glycerol, 5% α-propyl-gallate in 30 mM Tris–HCl (pH 9.5). The images were analyzed by laser scanning confocal microscopy mounted on a Leica inverted microscope. Data were analyzed by a 3D multi channel image processing software Imaris (Bitplane AG, Zurich, Switzerland).

2.8. P-gp functional assay

P-gp transport activities were determined by rhodamine-123 expulsion assay in the presence or absence of the P-gp inhibitor verapamil as previously described (Hegmann et al., 1992; Rieger et al., 2000). Cells (1 × 10^5) were trypsinized and incubated with 10 μM rhodamine-123 (Sigma) in 15 min. Thereafter, the cells were washed twice with serum-free medium, following incubation in absence or presence of 30 μM P-gp inhibitor verapamil (Tocris, Bristol, UK) at 37°C. After 1 h, the cells were washed twice and reussuspended in 500 μl fixation buffer (1% paraformaldehyde and 0.01% sodium azide in PBS) and analyzed (excitation, 488 nm; emission, 520 nm) for intracellular fluorescence by EPICS Altra flow cytometer (Beckman Coulter Inc., Fullerton, CA).

2.9. MRP functional assay

We investigated the functional activity of MRPs in HBMEC to check fluoresceine accumulation with or without the non-specific MRP inhibitors indomethacine and probenecid (Huai-Yun et al., 1998; Roller et al., 1999). Cells (2 × 10^4) were seeded in collagen type I-coated 96-well F-plates, cultured until confluency was reached, and then treated with various concentrations of indomethacine (Sigma) for 24 h. The cells were incubated with 100 μM fluoresceine (Sigma) and indomethacine for further 2 h. In an analogous manner, cells were cultured with 500 μM probenecid for 30 min, and consequently incubated with fluoresceine. Cells were washed in ice-cold PBS three times, lysed with Triton X-100 (0.5%) in 10 mM Tris–HCl/10 mM EDTA, and fluorescence read at 485 nm excitation and 530 nm emission in a CytoFluor 2300 plate reader (Millipore Corp., Bedford, MA).

3. Results

Quantitative RT-PCR was performed to test for the expression of mRNA of different drug resistance-related proteins in HBMEC-CTR and HBMEC-EPI. Intermediate expression of MDR1, MRP4, MRP5 and MRP6 was observed in HBMEC-CTR (threshold cycles (Cts) between 26 and 30) and up-regulated
Fig. 1. Multiple drug resistance gene expression in human brain-derived microvascular endothelial cells (HBMEC) of epileptic and control patients. The results of RT-PCR analysis were expressed as relative gene expression and normalized with 18S rRNA expression as reference gene. One to three cultures per patient were performed, and triplicate measurements were assessed. Data are presented as mean ± S.D. expression in HBMEC-EPI (MDR1: 3.8-fold, MRP4: 1.3-fold, MRP5: 3.3-fold and MRP6: 7.6-fold) (Fig. 1).

A strong expression level was found for MRP1 in HBMEC-CTR (Cts between 20 and 25), which was up-regulated 2.5-fold in HBMEC-EPI. In contrast, MRP2 and MRP3 were only weakly expressed in both types of HBMEC (Cts between 31 and 35).

In the present study, five specimens (patients #8, 9 and 11–13) were analyzed for the presence of P-gp, MRP1 and MRP2 protein by immunohistochemistry. P-gp was widely distributed not only in the hippocampus but also in the Gph (Fig. 2). Expression of P-gp was detectable using JSB-1 antibody on both endothelium and parenchymal cells, although their expression levels differed between specimens and within regions (Figs. 2 and 3A-G). We were not able to detect P-gp using the C219 antibody. No parenchymal P-gp immunoreactivity was found in the normal Gph of sample 9 (Fig. 3E and F). Interestingly, MRP1 immunoreactivity on parenchymal cells in the Gph was seen in four samples but not in sample 9 with normal Gph tissue (Fig. 3H and I), whereas no MRP 1 positive signals were obtained in the hippocampus. MRP1 staining was detectable on parenchymal cells around capillary endothelium, even though it was frequently difficult to localize the origin of MRP1 signals and to distinguish between endothelial cells and astrocyte foot processes or pericytes (Fig. 3H and I). One specimen (patient #8) showed strong expression of MRP2 on endothelium and some parenchymal cells in the hippocampus (Fig. 3J and K). The distribution of MRP2 positive cells was seen mainly in the hippocampus and the subiculum, whereas no immunoreactivity of MRP2 was observed in the Gph. Neither MRP1 nor MRP2 expression was observed in the normal Gph of sample 9. No immunoreactivity of P-gp, MRP1 and MRP2 was detected by using negative control isotype antibodies in the consecutive sections (Fig. 3L and data not shown).

In regard to the clinical features, there is no correlation between the degree of severity of gliosis in the hippocampus and the Gph, and transporter expression. Although the number of specimens analyzed were only five and among them one specimen (patient #11) showed gliomeatal hamartoma in the Gph, two patients (patients #8 and 12), who had high numbers of total days of seizure showed relatively strong expression of P-gp and MRP1 on parenchymal cells in the Gph compared to those with low numbers of seizure days (Fig. 2).

To investigate whether P-gp, MRP1 and MRP2 were expressed in isolated HBMEC, Western blot analysis was performed. P-gp expression sustained over all passages of the culture time of the HBMEC and at a similar level from both epileptic and normal patients (Fig. 4A). HBMEC-CTR showed relatively weak MRP1 bands compared with HBMEC-EPI. The positive controls, glioma cell line T98G and LN-229, showed strong P-gp and MRP1 signals, in which MRP1 expression was weaker in LN-229 than in T98G as shown previously (Roller et al., 1999) (Fig. 4A). In contrast to immunohistochemistry results, HBMEC-EPI were virtually devoid of MRP2 protein with the exception of two patient samples (patients #9 and 10) having a faint band. The 190kDa band was prominent observed on liver canaliclar membrane as a positive control (Fig. 4B). No correlation was found between P-gp and MRP1 expression in HBMEC-EPI and total number of seizures.
Cellular localization of the three transporters was investigated using 12-well Transwell chambers as shown in Section 2 (patients #5, 6 and 8). P-gp was expressed on both types of HBMEC, and was localized on the apical side. The signals obtained by the MRK16 antibody were stronger than JCB-1 (data not shown). MRP1 and MRP2 were seen in one specimen (patient #5) and in two specimens (patients #6 and 8), respectively. MRP1 was localized on the basolateral side of the endothelial cell, whereas MRP2 was localized on the apical side (Fig. 5). HBMEC-CTR showed no or only very weak MRP1 and no MRP2 expression. No signals were found by the mouse and rat IgG isotype controls (data not shown).

P-gp and MRP activities of HBMEC were studied by measuring intracellular rhodamine-123 and fluoresceine accumulation in the absence or presence of P-gp and MRP inhibitors, respectively. As expected, the P-gp inhibitor verapamil promoted rhodamine-123 accumulation in HBMEC from both patient groups (Table 2). The glioma cell lines T98G and LN-229 as positive controls showed strong P-gp activity (Rieger et al., 2000). An increased MRP activity was only seen in HBMEC-EPI, whereas HBMEC-CTR did not show any MRP activity (Table 2). The increased MRP activity with indomethacine was statistically significant ($p<0.05$). Probenecid did not show statistical difference between control and epileptic HBMEC, but a tendency was seen in the epileptic samples to inhibit MRP activity. In addition, the fluoresceine retention of HBMEC-EPI was dose-dependently increased by indomethacine and probenecid (data not shown). The functional MRP activities were not correlated to the degree of severity of gliosis nor seizure frequency.
4. Discussion

sAHE is one of the surgical treatment options for medically intractable MTLE. The advantage of this approach is the selective removal of seizure generating tissue rendering approximately 80% of previously drug resistant epileptic patients seizure free, if HS is diagnosed in the resected tissue (Wieser et al., 2003). Since MTL E with HS is very often drug-resistant, the investigation of transporters involved in multidrug resistance in the seizure generating tissue is important.

With regard to immunohistochemistry, we investigated the resected tissue as two main regions, the hippocampus and the Gph. P-gp showed a widespread and MRP1 and MRP2 a heterogenous distribution in the removed specimens including the hippocampus.
Fig. 4. P-gp and MRP1 expression in endothelial cells from epilepsy and control patients. Endothelial P-gp and MRP1 protein expression in three epilepsy patients was compared with endothelium isolated from control tissue (A). Comparably similar levels were seen in the epilepsy and control samples. Two glioma cell lines LN-229 and T98G served as a positive controls. Endothelial samples of four epilepsy patients were virtually devoid of MRP2 protein with the exception of patient 8 having a faint band (B). Liver specimen showed a clear signal at the corresponding band of MRP2.

Table 2
Endothelial P-gp and MRP activities of epileptic and control patients

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Passage number</th>
<th>The ratio of probe retention (+P-gp or MRP inhibitor/-inhibitor)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P-gp activity</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>HBMEC-CTR</td>
<td>5–7</td>
<td>1.37 ± 0.21 (n = 3)</td>
</tr>
<tr>
<td>HBMEC-EPI</td>
<td>1–5</td>
<td>1.20 ± 0.38 (n = 4)</td>
</tr>
<tr>
<td>LN-229</td>
<td>1.31 ± 0.14 (n = 3)</td>
<td>1.18 ± 0.04 (n = 3)</td>
</tr>
<tr>
<td>T98G</td>
<td>1.45 ± 0.32 (n = 3)</td>
<td>2.23 ± 0.31 (n = 3)</td>
</tr>
</tbody>
</table>

P-gp assay, rhodamine-123 expulsion assay in the presence or absence of the P-gp inhibitor verapamil. MRP assay, MRP substrate fluoresceine accumulation following exposure to various concentrations of the MRP inhibitor indomethacine or probenicid.

* Mean ± S.D. of 3–11 separate experiments.

** Rhodamine-123 (10 μM) was used as a substrate.

Fluoresceine (100 μM) was used as a substrate.

Significantly higher intracellular fluoresceine retention (p < 0.05, Bonferroni/Dunn) than for HBMEC-CTR.
the subiculum and the anterior portion of the Gph. We used several anti-P-gp antibodies for immunostaining, since the detection capacity of each antibody is different (Schinkel, 1999). According to distribution of P-gp positive cells from a recent study (Aronica et al., 2004), P-gp labeling was observed diffusely in endothelium and glial cells in the hippocampus in all 12 HS samples and one-fourth of the samples showed the expression of P-gp in the subiculum. The distribution and expression of P-gp in our study is in agreement with their results. In contrast, another immunohistochemistry study showed that P-gp was distributed in reactive astrocytes in the hippocampus, but not in hippocampal endothelium, and in astrocytes in the adjacent subiculum (Sisodiya et al., 2002). This staining discrepancy is attributed to various factors, such as tissue preparation, fixation method and antibodies (Toth et al., 1994; Sisodiya et al., 2002; Aronica et al., 2004; Volk et al., 2005). P-gp expression in endothelium was found in our human cryostat preparations due to acetone fixation, by which P-gp immunoreactivity in astrocytes might rather be masked (Volk et al., 2005). No immunoreactivity was found using C219 antibody in concordance with previous studies (Sisodiya et al., 2002; Aronica et al., 2004).

In the parahippocampal region, P-gp was widely detectable on endothelial cells and parenchymal cells in the present study. Drug resistance transporters expression in Gph, to our knowledge, was not well investigated before. In general, normal hippocampus shows P-gp expression only in endothelium (Cordon-Cardo et al., 1998; Tatsumi et al., 1992; Regina et al., 1998; Schinkel, 1999; Aronica et al., 2004). However, P-gp appears on astrocytes and neurons in an epileptogenic lesion including the hippocampus and the subiculum (Toshier et al., 1995; Seetharaman et al., 1998; Sisodiya et al., 2002; Aronica et al., 2004). It is suggested that peripheral temporal lobe structures, such as the amygdala, subiculum, limbic system, temporopolar cortex and parahippocampal gyrus showed histological changes, resulting in a participation to compose of epileptogenic zone in intractable epilepsy. Because there is a close connection between the hippocampus and the Gph, stimuli in the hippocampus showed anatomical and functional effects on the peripheral temporal structures including the Gph (Bernasconi et al., 2003; Bartolomei et al., 2005; Urbach et al., 2005; Chabardes et al., 2005). Despite the limited number of patients investigated in our study, a relative higher expression of P-gp on parenchymal cells in the Gph was observed in patients with higher number of lifetime seizures. Seizure activity is known to give an influence to transporter expression (Seegers et al., 2002; van Vilet et al., 2004). Particularly, in a chronic epileptic rat model, P-gp was up-regulated prominently in glial cells around endothelium in the Gph in addition to P-gp positive gial cells in the dentate gyrus.
Vilet et al., 2004). Clinically, neuroradiological abnormal changes in the Gph were more frequent in patients with over 20 years of seizures (Ferreira et al., 2003). It may be conceivable that not only the anatomical morphology but expression of drug resistance transporters in the Gph are affected during progression of epilepsy.

Distribution of MRP1 and MRP2 was heterogeneous within the resected region compared with a widespread P-gp expression. Of all four HS samples, MRP1 is distributed mainly on parenchymal cells in the Gph, but not in the hippocampus. By contrast, in one case (patient #8) out of five, MRP2 was present in endothelium rather than parenchymal cells in the hippocampus. Although MRP expression in the Gph has not been examined previously, in the hippocampus, 50–62.5% of HS cases showed MRP1 expression in reactive astrocytes, and 92% of HS revealed MRP2 staining in endothelium and astrocytes (Sisodiya et al., 2002; Aronica et al., 2004). The fact that we could not detect any MRP1 signal in the hippocampus in the present study and the different rate of MRP expression from the previous studies, may depend on staining method as described above or sample’s bias, since there is a discrepancy of MRP1 expression in the subiculum in the literature (Sisodiya et al., 2002; Aronica et al., 2004). Neither MRP1 nor MRP2 was found in the normal Gph in case 9 as well as in the hippocampus (Kool et al., 1997; Sisodiya et al., 2002; Aronica et al., 2004; Berezowski et al., 2004; Nies et al., 2004). High expression of MRP1 in the Gph in patients with long-life seizures was more prominent than P-gp. The mechanism to induce these transporters expression is suggested that a variety of environmental stress or xenobiotic sensors, such as orphan nuclear receptors (Loscher and Potschka, 2005). However, it remains to be investigated whether seizures can induce MRP expression similar to P-gp.

Concerning cultured endothelial cells in our study, HBMEC-CTR were dissociated from normal brain frontal cortex and as HBMEC from the normal hippocampus cannot be evaluated due to ethical reasons, a direct comparison between normal and pathological hippocampal regions is not possible. However, the substantial comparison of transporters expression in cells forming microvasculature in the brain could be evaluated, even though the isolation procedure and isolated region are different from those used for HBMEC-EPI.

First we performed the characterization of these two types of HBMEC with regard to transporter expression, localization and functional activity. At the protein level, both HBMEC-EPI and HBMEC-CTR displayed the same intensity signal and apical localization of P-gp. In contrast to the protein level, increased P-gp mRNA was seen in HBMEC-EPI in concordance with a previous study (Dombrowski et al., 2001). Although the reason for discrepancy between mRNA and protein P-gp expression level is unknown, same P-gp expression level of HBMEC-CTR and HBMEC-EPI suggested that P-gp plays an important role at the BBB. Notably, it is intriguing that in our study HBMEC-EPI showed an up-regulation on the protein level for MRP1 and MRP2 compared to HBMEC-CTR. MRP1 expression is variable during cell culture, in which the expression of MRP1 was stronger in HBMEC-EPI than in HBMEC-CTR at protein and mRNA levels. MRP1 seems to be up-regulated in culture condition, but relatively high expression of MRP1 in HBMEC-EPI may indicate MRP1 up-regulation in epilepsy. It is, however, true that changes of endothelial cell property in cultured condition is not ignored, when compared to the in vivo milieu, where astrocytes, microglial cells, pericytes, neurons and other cell types might also have an impact on transporter expression of endothelial cells (Berezowski et al., 2004). Furthermore, as described above, the isolation method, species differences, detection methods and antibodies are affecting the transporter expression in cultured cells (Hirrlinger et al., 2002; Regina et al., 1998; Nies et al., 2004). It has been suggested that MRP1 is not expressed in normal endothelial cells because MRP1 expression was absent in isolated capillary microvessels, and is appearing during cell culture (Pardridge et al., 1997; Seetharaman et al., 1998; Declèves et al., 2000; Hirrlinger et al., 2002). On the contrary, one recent study using immunofluorescence microscopy (Nies et al., 2004) revealed that weak MRP1 labeling was present in the apical side of human brain endothelium. Although further studies are required to disclose MRP1 expression and localization in endothelial cells in epilepsy, basolateral MRP1 expression, shown in our confocal microscopy analysis, indicate no involvement in drug resistance because of its efflux direction from apical to basolateral. In contrast, MRP2 expression level at BBB is still an open question. No MRP2 mRNA was, on the one hand, detected in bovine brain microvascular endothel-
lial cells (Zhang et al., 2000) and, on the other hand, MRP2 mRNA expression was observed in endothelial cells from epileptic patients and intracranial aneurysm (Dombrowski et al., 2001). In this study the level was increased in epileptic endothelial cells compared to controls obtained from aneurysm domes and umbilical venas. This is in contrast to our findings and might reflect differences in the applied technique (gene array versus quantitative RT-PCR) and origin of cellular source. At the protein level, MRP2 is detectable by immunoblotting in isolated capillary endothelial cells from rat (Fellner et al., 2002) and porcine (Fricke et al., 2002), however, bovine capillary endothelium showed no MRP2 expression (Zhang et al., 2000). In the present study, confocal microscopy revealed MRP2 expression in the apical side on HBMEC-EPI in two out of three specimens. However, we could not detect MRP2 by immunoblotting, although the bands in 80% of HBMEC-EPI at the size of 170 kDa might be most likely corresponding to degradation products of MRP2. Taken together, although MRP2 expression in normal brain more or less depends on the detection methods, up-regulation of MRP2 was observed in HBMEC-EPI by immunohistochemistry as previously shown (Dombrowski et al., 2001; Aronica et al., 2004).

From the other four members of the MRP subfamily investigated in the present study, an intermediate expression was observed for MRP4, MRP5 and MRP6 in HBMEC-CTR which was up-regulated in HBMEC-EPI 1.3-, 3.3- and 7.6-fold, respectively. In contrast, MRP3 was only weakly expressed in both types of HBMEC. These findings are in accordance with recent studies that showed expression of MRP3, MRP4, MRP5 (Nies et al., 2004) and MRP6 mRNA (Beck et al., 2005) by quantitative RT-PCR in normal human brain. On the protein level MRP4 and MRP5 appear not only to be the major MRP isoforms synthesized in human brain but being clearly localized to the luminal side of capillary endothelial cells by confocal laser scanning microscopy (Nies et al., 2004). An additional localization of MRP4 in the basolateral (abluminal) membrane, as observed for the bovine capillary endothelial cells (Zhang et al., 2004), was not found in human brain capillary endothelial cells and may be due to species differences. Notably, MRP4 and MRP5 proteins were also detected in astrocytes of the subcortical white matter and MRP5 in pyramidal neurons (Nies et al., 2004).

Cultured HBMEC-EPI did not show any correlation between its expression level of transporters and total number of seizure days. This is because the occurrence of seizures might affect parenchymal cells rather than endothelial cells. This is supported by a recent investigation (van Vilet et al., 2004) suggesting that glia-like cells expressed P-gp in the Gph at the chronic stage. Consequently, we checked whether the P-gp/MRP activities were different between HBMEC-CTR and HBMEC-EPI, since one strong point of isolated endothelial cells is its availability for functional assays. Several studies showed that P-gp in endothelial cells is functionally active in different species including humans (Tatsuta et al., 1992; Seetharaman et al., 1998; Regina et al., 1999; Rieger et al., 2000). As HBMEC-CTR and HBMEC-EPI showed P-gp expression at the same level in immunoblotting, P-gp activity did not display a significant difference between the two cell types in rhodamine-123 retention assay. As discussed before, a direct comparison of HBMEC function between normal and pathological hippocampus could not be addressed in this study. Endothelial cells in normal frontal cortex is well expressed P-gp (Cordon-Cardo et al., 1989), suggesting that P-gp function is mandatory for a physiological barrier in microvasculature, and that more number of P-gp positive cells might be associated with stronger drug resistance in BBB. Furthermore, the MRP inhibitors indomethacine and probenecid revealed MRP activity in HBMEC-EPI, but not in HBMEC-CTR. In addition to MRP, probenecid is found to inhibit organic anion transporting polypeptide (OATP) including well investigated Oatp2 and OATP-A, which are present in BBB (Loscher and Potschka, 2005). Unfortunately, so far, MRP- or OATP-specific inhibitors are not available to dissect the various transporter activities involved. Because of its apical localization in endothelial cells, MRP2 might be a probably putative organic anion transporter in HBMEC-EPI. From the in vivo study using MRPI knockout mice, MRPI functional activity was limited in BBB due to the fact that MRPI substrates fluoresceine accumulation in the brain showed no significant differences between MRPI knockout and wild-type mice (Sun et al., 2001). In contrast to MRPI, Potschka et al. (2003) demonstrated for MRPI in a rat model and by in vivo microdialysis technique, a contribution to BBB function and drug resistance for AEDs. Capillary vessels isolated from porcine brain revealed MRPI involve-
ment in drug delivery (Fricker et al., 2002). At the moment an involvement of OATPs in multidrug resistance in epilepsy is still unknown.

Taken together, we demonstrated the distribution of P-gp, MRPI and MRP2 not only in the hippocampus but also in the Gph of patients with multidrug resistant epilepsy. Chronic seizures might induce high P-gp and MRP expression in the Gph. Moreover, cultured HMEC-CTR have different properties of MRP expression and functional activity in comparison to HMEC-CTR. Elucidation of area-specific transporter expression and transporter-specific functional activity may lead to enhanced understanding of the mechanisms underlying drug resistance in epilepsy.

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