

Research Article

Characterization of the Gene Expression Profile of Human Hippocampus in Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis

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One of the main putative causes of therapy refractory epilepsy in mesial temporal lobe epilepsy (MTLE) with hippocampal sclerosis is the overexpression of multidrug transporters (MDTs) at the blood-brain barrier (BBB). It steps up the removal of antiepileptic drugs (AEDs) out of the brain cells across the BBB resulting in a low concentration of AEDs within the target cells. Some of the mechanisms of AED resistance are most likely to be genetically determined. To obtain more information about the underlying pathophysiology of intractability in epilepsy, we compared the global gene expression profile of human hippocampus and hippocampal-derived microvascular endothelial cells from MTLE with HS patients and controls. At the level of MDT, a significant up-regulation was found for ABCB1 (P-gp), ABCB2, ABCB3, and ABCB4, which was mainly related to endothelial cells. The data on the MDT were validated and extended by quantitative RT-PCR. Surprisingly, inflammatory factors such as interleukins (IL-1 α , IL-1 β , IL-6, and IL-18) and cytokines (TNF- α and TGF- β 1) were found to be up-regulated in hippocampal parenchyma. The overexpression of P-gp, IL-1 β , and IL-6 was also confirmed by immunohistochemistry (IHC). Our results suggest that complex expression changes of ABC-transporters may play a decisive role in pharmacoresistance in MTLE. Further studies on the new and unexpected overexpression of inflammatory cytokines may unlock hitherto undiscovered pathways of the underlying pathophysiology of human MTLE.

1. Introduction

Mesial temporal lobe epilepsy (MTLE) with hippocampal sclerosis (HS) is the most common type of epilepsy in adults [1]. HS consists of gliosis, neuronal loss and cell dispersion. As many as 75% of the patients with MTLE are considered to be resistant to drug therapy with AEDs. Pharmacoresistance is a common problem in neurological and psychiatric disorders which leads to a big distress among patients and family members. The long-term

followup after selective amygdalohippocampectomy (sAHE) in the presence of severe HS showed a greater than 80% of patients becoming seizure free [2]. The causes and mechanisms underlying drug resistance in MTLE are still badly understood, which hampers the development of new drugs and thus the availability of better treatment options [3–6]. The main factors which might contribute to the development of medically intractable MTLE are aetiology of epilepsy, the progression of seizure activity under drug therapy, changes in the targets of AEDs, abnormalities in

the neuronal networks owing to damage in the epileptogenic focus during epileptogenesis, and changes in the drug-uptake across the BBB [7]. Genetic factors may explain why patients with the same type of epilepsy respond differently to the same drug therapy. In recent years, several putative mechanisms underlying drug resistance in epilepsy have been identified. One theory that has received considerable attention is the removal of AEDs from the epileptogenic tissue through overexpression of multidrug transporters at the BBB. This theory is biologically feasible and can partly explain drug-resistance. Although MTLE is usually not regarded as a neuroimmunological disease, proinflammatory cytokines and chemokines may play a role in seizure onset [8, 9]. It has also been reported in experimental models of limbic seizures that there is a rapid and transient increase of IL-1 β , IL-6, and TNF- α mRNA in the hippocampus, and that intrahippocampal injection of IL-1 β has a detrimental effect on seizures [10].

Furthermore, a polymorphism in the IL-1 β gene has been associated with MTLE-HS compared to patients without sclerosis and nonepileptic controls [11, 12]. Febrile seizures, particularly complex febrile seizures and status epilepticus during early childhood, have been associated with hippocampal sclerosis and overexpression of IL-1 β [13]. The relevance of sclerotic hippocampus in seizure maintenance and therapy refractoriness has led to us to investigate the mRNA expression profile of the sclerotic hippocampus in order to obtain a comprehensive view of this particular pathology at the molecular level. Accordingly, we used a DNA microarray approach. The main aim of this study was to identify those genes which may be involved in the pathogenesis of MTLE-HS. A separate comparison was performed for mRNAs extracted from the entire tissue and from MVECs prepared from the resected tissue.

In the interpretation and discussion of the data, we focused on the multidrug-transporter overexpression theory and on the genes which were found to be among the most overexpressed in sclerotic hippocampus.

2. Methods

2.1. Surgical Specimens. Clinical specimens were obtained from ten caucasian patients with chronic pharmaco-resistant 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 specimen was obtained by selective amygdalohippocampectomy (sAHE). With this surgical approach, parts of the amygdala, the hippocampus, and the anterior portion of the parahippocampal gyrus (Gph) are selectively removed. All tissues were diagnosed by two pathologists at the Department of Neuropathology, University Hospital Zurich. The hippocampus and the Gph were examined and separately rated for the presence and severity of hippocampal sclerosis (HS) based on the extent of gliosis and neuronal loss (mild, marked, and severe). All hippocampal regions revealed HS with various degrees of gliosis and neuronal loss. All analyses were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee

of the Canton of Zurich. Informed written consent was obtained from all patients. Control human brain hippocampus total RNA were commercially purchased by Ambion (Ambion, Inc. Texas, USA). The autopsy controls ($n = 3$; 45, 5 ± 10 years old; 2 female, 1 male; Caucasians) had no history of brain-related disease and suffered sudden death without associated brain damage. Autopsy was rapidly performed with a short postmortem delay. Autopsy hippocampi were dissected and immediately collected in RNA-Later tubes and frozen until processing. Total RNA was isolated using the modified version of Ambion's RNA KIT for RNA-Isolation, and RNA was stored in 1mM sodium citrate (pH 6.4) at -70° .

2.2. Isolation of Human Brain-Derived Microvascular Endothelial Cells (MVECs). MVECs were isolated as described by [14, 15]. Briefly, ependym, large vessels and pia membrane were dissected away with the help of a microscope, and the remaining tissue minced and homogenized with a 10 mL-syringe in 10 mL MCDB 131 medium (Gibco, Invitrogen, Basel, Switzerland), containing 2% FBS (PAA Laboratories, Linz, Austria), 16 U/mL heparin (Sigma, Fluka Chemie, AG Buchs, Switzerland), 20 μ L BBE, 5 μ L hEGF, 5 μ L hydrocortisone, 5 μ L FGF (all from Cell System Inc. Kirkland, WA), 50 μ L ECGS (Serva, Heidelberg, Germany), 500 μ L gentamicin (Sigma). The tissue was dissociated in a glass flask in dispase II solution (Roche, Rotkreuz, Switzerland) at a final concentration of 0.096 U/mL and the solution was placed in a water bath at 37°C and stirred for approximately 1 hour. The homogenate was centrifuged ($1,000 \times g$, 10 min, 4°C) and the supernatant was removed. The pellet was dissolved in 2 mL MCDB 131 medium, containing 20% dextran, (Sigma) and gently homogenized with a fire-polished pipette and centrifuged ($1,700 \times g$, 10 min, 4°C). The top layer containing most of the myelin was discarded. The pellet was resuspended in MCDB 131, containing 2% FBS medium, layered on 2 mL 13% dextran, and again centrifuged ($1,700 \times g$, 10 min, 4°C) to remove the remaining myelin. The pellet was solubilized in 1 mg/mL collagenase/dispase (Roche) and 0.05 mg/mL DNase I (Sigma) and stirred for 1 hour. The cells were pelleted ($380 \times g$, 10 min, 4°C) and washed with MCDB 131 containing 2% FBS medium, and the pellet was finally placed in 1 mL QIAzol lysis reagent (Qiagen, Basel, Switzerland) frozen and kept at -70°C until further processing.

2.3. Processing of Hippocampal Tissues. Immediately after surgery hippocampal specimen from epileptic patients were stored in RNAlater stabilization reagents (Qiagen) in order to prevent degradation of RNA. The samples were stored at 4°C overnight and subsequently frozen and kept at -70°C until further processing.

2.4. Isolation and Analysis of RNA. Total RNA from isolated MVEC was extracted by using QIAzol lysis reagent according to the manufacturer's protocol. The isolated MVECs were placed in 1 mL QIAzol reagent, frozen and kept at -70°C . After thawing, 0.2 mL dichloromethane was added and the samples were centrifuged ($12,000 g$, 15 min, 4°C) for phase

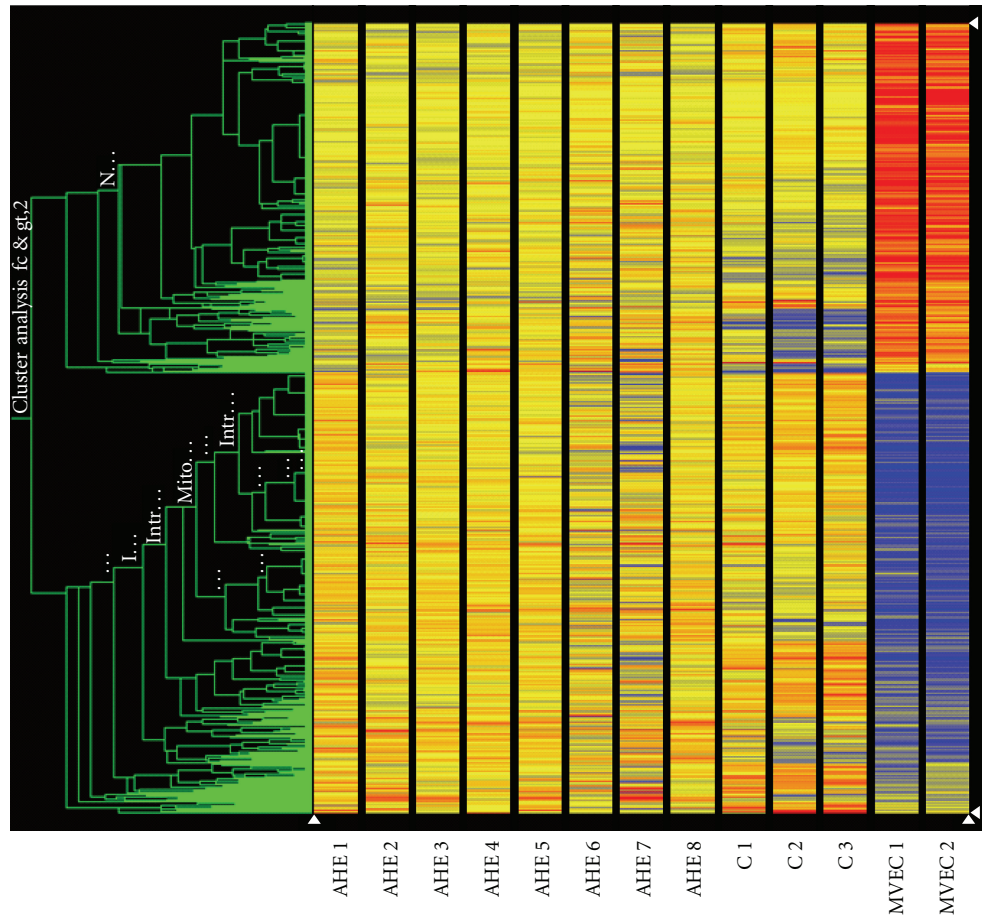


FIGURE 1: Cluster analysis of the gene expression patterns of isolated MVECs ($n = 2$) and AHEs ($n = 8$) from patients with MTLE after selective amygdalohippocampectomy (sAHE) and control hippocampal tissue specimens ($n = 3$). The analysis was performed using GeneSpring software. The analysis included all differentially expressed genes expressed in oligonucleotide microarray analysis (Affymetrix, HG-U133Plus2.0). Each horizontal colour bar denotes the intensity of expression of a particular gene in a distinct sample relative to the mean expression value of all samples. Yellow denotes little or no change, red denotes increased expression, and blue denotes decreased expression. AHE: amygdalohippocampectomy, MVEC: microvascular endothelial cells, and C: control.

separation. The RNA from the upper aqueous phase was precipitated by mixing with 0.5 mL isopropyl alcohol. After 10 min incubation the samples were centrifuged (12,000 \times g, 10 min, 4°C) and the RNA pellet was washed with 1 mL 75% ethanol. At the end of the procedure, the RNA was briefly dried and then dissolved in RNase-free water. The hippocampal biopsies were thawed, placed in 1 mL QIAzol reagent for RNA isolation, and processed according to the protocol RNeasy Lipid Tissue Mini Handbook (Qiagen). The quality of the isolated RNA was determined by NanoDrop ND 1000 (NanoDrop Technologies, Delaware, USA) and a Bionalyser 2100 (Agilent Technologies, Basel, Switzerland). Only those samples with a 260 nm/280 nm ratio between 1.8–2.1 and a 28S/18S ratio within 1.5–2 were processed further and investigated. Control human hippocampal RNA was commercially purchased (Ambion Ltd., Huntingdon, UK).

2.5. cRNA Preparation. 3 μ g and 100 ng of total RNA per sample were reverse-transcribed into double-stranded cDNA

with one-cycle and two cycle cDNA synthesis kit (Affymetrix Inc., P/N 900431, Santa Clara, CA), respectively. The double-stranded cDNA was purified using a Sample Cleanup Module (Affymetrix Inc.). The purified double-stranded cDNA was in vitro transcribed (IVT) in the presence of biotin-labeled nucleotide using an IVT Labeling Kit (Affymetrix Inc.) and the biotinylated cRNA was purified using a Sample Cleanup Module (Affymetrix Inc., P/N 900371). The quality and quantity of biotin-labeled cRNA were determined using NanoDrop ND 1000 and Bioanalyzer 2100.

2.6. Array Hybridization. 15 μ g of biotin-labeled cRNA was fragmented randomly to 35–200 bp at 94°C in fragmentation buffer (Affymetrix Inc., P/N 900371) and were mixed in 300 μ L of hybridization buffer containing a hybridization control cRNA and control Oligo B2 control (Affymetrix Inc., P/N 900454), 0.1 mg/mL herring sperm DNA, and 0.5 mg/mL acetylated bovine serum albumin in 2-(4-morpholino)-ethane sulfonic acid (MES) buffer, pH 6.7, before hybridization to GeneChip Human Genome

TABLE 1: Clinical history, MRI findings, pathological diagnosis, and postoperative outcome of 10 patients who underwent selective amygdalohippocampectomy.

Case no.	Age/sex	MRI findings	Pathological diagnosis of hippocampus	AED medication	Type of seizures	Years with epilepsy	No. seizures/month
1 ^{a,b,c}	21/M	MTS, left	mild gliosis, focal neuronal loss	LTG, LEV	SPS, CPS	19	30
2 ^{a,b,c}	26/F	MTS, right	mild gliosis, severe neuronal loss	CBZ, GP, LEV	CPS, SGS	10	8
3 ^{a,b,c}	28/F	MTS, left	marked gliosis, severe neuronal loss (Wyler II) ^f	OXC, LTG, PHT	SPS, CPS, SGS	26	2
4 ^{a,b,c}	27/M	MTS, right	marked gliosis, severe neuronal loss (Wyler II)	LTG, LEV	CPS, SGS	25	4
5 ^{a,b,c}	34/F	MTS, left	marked gliosis, severe neuronal loss (Wyler III)	LEV, PB	SPS, CPS, SGS	32	6
6 ^{b,c}	40/F	MTS, left	marked gliosis, focal mild neuronal loss	VPA, LEV	CPS, SGS	18	12
7 ^{b,c}	68/F	MTS, right	marked gliosis, severe neuronal loss	LTG, LEV	SPS, CPS	43	4
8 ^{b,c}	22/M	MTS, right	marked gliosis, severe neuronal loss (Wyler IV)	CBZ, GP, LEV	SPS, CPS, SGS	18	4
9 ^{d,e}	41/M	MTS, right	marked gliosis, severe neuronal loss (Wyler IV)	CBZ, VPA, TPM	SPS, CPS, SGS	6	2
10 ^{d,e}	41/F	MTS, left	marked gliosis, severe neuronal loss	CBZ, LEV, PHT	SPS, CPS, SGS	20	2

AEDs: antiepileptic drugs; CBZ: carbamazepine; LEV: levetiracetam; LTG: lamotrigine; PHT: phenytoin; TPM: topiramate; GP: gabapentin; PB: phenobarbital; OXC: oxcarbazepine; VPA: valproic acid.

SPS: simple partial seizures; CPS: complex partial seizures; SGS: secondary generalized seizures.

^aImmunohistochemistry.

^bOne-cycle target labelling gene array.

^cQuantitative real-time RT-PCR (TaqMan).

^dBrain-derived microvascular endothelial cells (MVECs).

^eTwo-cycle target labeling gene array.

^fWyler classification was not determined in all cases.

MTS, mesial temporal sclerosis.

TABLE 2: Averaged relative gene expression values of AHE, MVEC, and control of the ATP-binding cassette (ABCB1, ABCB2, ABCB3, and ABCC4).

Genes	Symbol	Control ^a	AHE ^a	MVEC ^a
ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1/P-gp	1 ± 0.03	0.91 ± 0.24	37.22 ± 0.97 (<i>P</i> = .0003) ^b
Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	ABCB2/TAP1	1 ± 0.19	1.13 ± 0.33	21.08 ± 2.82 (<i>P</i> = .0017)
Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	ABCB3/TAP2	1 ± 0.11	1.14 ± 0.27	13.39 ± 6.08 (<i>P</i> = .0012)
ATP-binding cassette, sub-family C (CFTR/MRP), member 4	ABCC4/MRP4	1 ± 0.07	2.13 ± 0.68 (<i>P</i> = .001)	5.39 ± 2.44 (<i>P</i> = .005)

^aFold changes were calculated on the bases of normalized values calculated by Genespring 5.0. The mean values of the three controls were set to the value of 1. The data are presented as a mean ± SD.

^bThe statistical differences in comparison to control are indicated (*P*-value, Student's *t*-Test).

U133Plus2.0 arrays, which contains 47,000 transcripts, for 16 hours at 45°C. Arrays were then washed using an Affymetrix Fluidics Station 450 EukGE-WS2v5_450. Affymetrix GeneChip Scanner 3000 (Affymetrix Inc.) was used to measure the fluorescent intensity emitted by the labeled target.

2.7. Microarray Data Analysis. Raw data processing was performed using the Affymetrix Genespring software (Silicon Genetics, California, USA). After hybridization and scanning, probe cell intensities were calculated and summarized

for the respective probe sets by means of the MAS5 algorithm [16]. To compare the expression values of the genes from chip to chip, global scaling was performed, which resulted in the normalization of the trimmed mean of each chip to target intensity (TGT value) of 500 as detailed in the statistical algorithms description document of Affymetrix. Quality control measures were considered before performing the statistical analysis. These included adequate scaling factors (between 1 and 3 for all samples) and appropriate numbers of present calls calculated by application of an algorithm. The efficiency of the labelling reaction and the hybridization

TABLE 3: Comparison of the averaged values of individual epileptic patients and controls between microarray and RT-PCR analysis for genes, which are suspected to be involved in medically intractable epilepsy.

Genes	Symbol	Gene array ¹		RT-PCR ² [Ct values] ³	
		Control	AHE	Control	AHE
ATP-binding cassette, sub-family B (MDR1/TAP), member 1	ABCB1/P-gp	136.3 ± 26.2	164.3 ± 60.5 (<i>P</i> = .469)*	1.2 ± 0.1 [30.1 ± 0.6]	2.3 ± 0.5 (<i>P</i> = .030) [28.3 ± 0.6]
Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	ABCB2/TAP1	58.7 ± 12.3	97.8 ± 15.2 (<i>P</i> = .251)	0.9 ± 0.1 [31.9 ± 0.5]	2.1 ± 0.8 (<i>P</i> = .020) [29.7 ± 0.8]
Transporter 2, ATP-binding cassette, subfamily B (MDR/TAP)	ABCB3/TAP2	42.3 ± 9.6	67.9 ± 12.5 (<i>P</i> = .163)	1.1 ± 0.2 [32.8 ± 0.9]	2.1 ± 0.8 (<i>P</i> = .023) [30.1 ± 1.0]
ATP-binding cassette, sub-family C (CFTR/MRP), member 4	ABCC4/MRP4	104.7 ± 7.2	235.0 ± 69.4 (<i>P</i> = .012)	1.2 ± 0.2 [31.1 ± 0.5]	6.4 ± 2.1 (<i>P</i> = .030) [28.7 ± 0.7]

¹The microarray data are presented as raw values calculated by Microarray Suite 5. The data are given as means ± SD of three controls and eight epileptic patients for each gene, respectively.

²The results of RT-PCR analysis are expressed as relative gene expression and normalized with 18S rRNA expression as reference gene.

Data are presented as mean ± SD of three controls. *The statistical differences between AHE and controls are indicated (*P*-value, Student's *t*-Test).

³Threshold cycles [CT-values] are given as mean ± SD

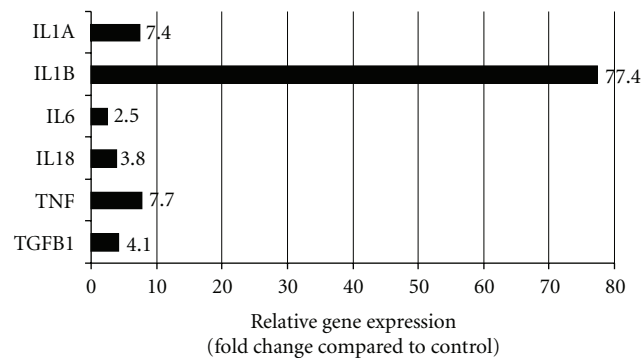


FIGURE 2: Relative gene expression of IL-1 α , IL-1 β , IL-6, IL-18, TNF- α , and TGF- β 1 of control hippocampal tissues (*n* = 3) and AHEs (*n* = 8). The results of gene array analysis were calculated by GeneSpring software. Control mean values were set to one and the fold change of the AHE means calculated.

performance was controlled with the following parameters: present calls and optimal 3'/5' hybridization ratios (around 1) for the housekeeping genes (GAPDH and ACO7), for the poly A spike in controls and the prokaryotic control (BIOB, BIOC, CREX, BIODN). Analysis was performed by setting controls sample data set to zero and filtering and utilizing only genes with transcripts flagged "present" or "marginal". Gene transcripts with a difference of 2-fold or greater "induction" or "reduction" when compared to controls were subsequently annotated and grouped according to their molecular and cellular function.

2.8. Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from hippocampal biopsies and MVEC using QIAzol reagent (Qiagen) according to the manufacturer's protocol. Quantification of ABC transporters was performed by a TaqMan Low Density Array, which is based on an Applied Biosystems 7900HT Micro Fluid Card. The method allows simultaneous analysis of 47 human ABC transporters and the reference

gene 18S rRNA in two replicates of four different samples per run [17].

2.9. Immunohistochemistry and Immunofluorescence. The following antibodies were used for IHC MRP1 (MRP1), MRP2 (M2I-4), MRP3 (M3II-9), MRP4 (M4I-80), MRP5 (M5I-1), all from Alexis, Lausen, Switzerland and IL-1 β (ab2105) and IL-6 (ab6672), both from Abcam, UK. Hippocampal samples were fixed in formalin 4% and embedded in paraffin. Sections were cut at thickness of 2 μ m, mounted on gelatinized glass-slides, deparaffinized, rehydrated, and rinsed (Tris puffer, pH = 7.4). The slides were pretreated for antigen retrieval in 10 mM Na-citrate, pH 6, in microwave for 10 min. Subsequently, sections were treated with 0.3% H₂O₂ in methanol for 10 min to abolish endogenous peroxidase activity and with 2% normal goat serum in Tris for 20 min to block nonspecific IgG binding sites. The primary antibody diluted in Tris (1:50) and applied overnight at 4°C. After rinsing in Tris, the sections were incubated with biotin labelled antimouse IgG (1:100) or antirat IgG (1:100) for 1 hour at RT. After washing in Tris, the sections were incubated with biotin-avidin complex, containing peroxidase (Dako), and washed and stained with haematoxylin for nuclear staining, finally dehydrated and cleared in xylene and cover glass mounted with eukitt (Erne-Chemie, Switzerland).

Immunolabeling of MTLE tissue: 20- μ m sections were obtained using a vibrating microtome (VT 1000S, Leica, Heilderberg, Germany) and processed as free-floating sections for double immunolabeling with mouse anti-P-gp C494 (Signet Laboratories, Dedham, MA, diluted 1:10 to 1:40 in BB) and rabbit anti-GFAP (diluted 1:1 in BB; prediluted antibody from ImmunoStar, Hudson, WI). The incubation steps were as follows: (1) 30 min in BB, (2) 30 minutes in 0.5% Triton X-100 in PBS, (3) 1 hour in a mixture of the primary antibodies at RT, and (4) 1 hour in the fluorophore conjugates goat antimouse and goat anti-rabbit IgGs at RT (diluted 1:200 in BB; Alexa Fluor 488 and 555, respectively; Invitrogen) at RT. The sections

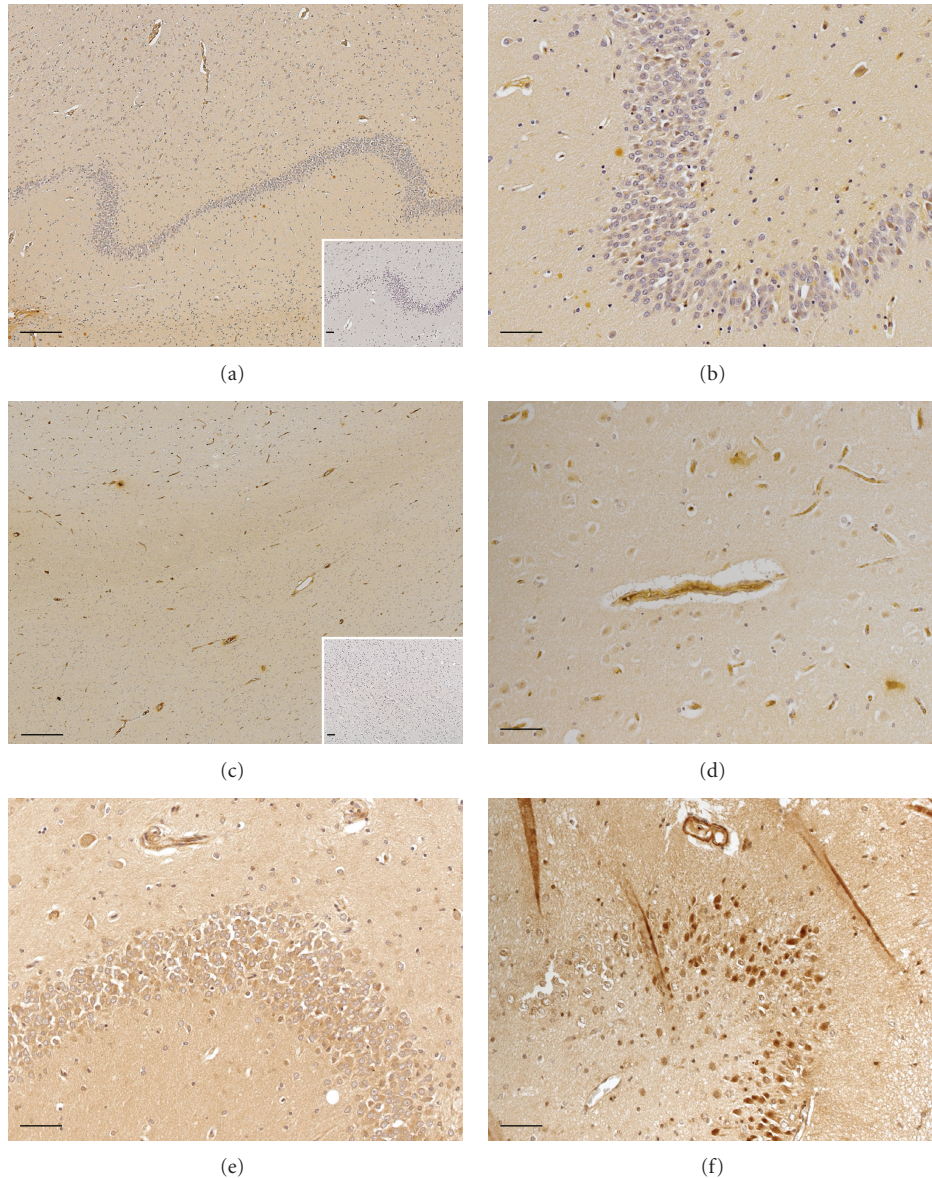


FIGURE 3: Photomicrographs of IL-1 β (a, b, c, d) and IL-6 (e, f) in hippocampus (dentate gyrus) from a control (a, b, e) and epileptic patient (no. 5) (c, d, f). Only a weak IL-1 β staining was found in control patient (a, b, e) in contrast to the epileptic patient (c, d, f). The staining was mainly confined to the neurovascular unit. Figures show transverse sections at different magnifications (a, c, e: $\times 4$; b, d and f: $\times 20$). Scale bar: 100 μm . Isotype antibody controls are shown as inserts in a and c.

were washed 3 times for 10 min in PBS between each step and counterstained with TOPRO-3 (diluted 1:10,000 in PBS; Invitrogen) added to the final wash. The sections were then transferred onto Vectabond treated slides (Vector Laboratories), carefully drained, coverslipped in Vectashield mounting medium (Vector Laboratories), and sealed with nail varnish.

3. Results

The GeneChip expression analysis was performed from control hippocampi ($n = 3$), hippocampal MTLE biopsies ($n = 8$), and isolated MVECs ($n = 2$) thereof. A total

of 27,000 out of 54,000 mRNA transcripts of known or predicted function were found to be present in all samples. The expressed genes were normalized and compared with control. Gene transcripts with >2 -fold induction or reduction were analyzed. The comparison of the gene expression profiles of AHEs, MVECs, and controls is shown in the multidimensional hierarchical cluster analysis (Figure 1). This analysis revealed remarkable differences between the three groups. When AHEs were compared with controls, a total of 1,253 genes were found to be significantly up-regulated and 637 genes down-regulated. Among the many up-regulated genes we found also GFAP (7.6-fold) as a bona fide marker for astrogliosis. The comparison between MVEC

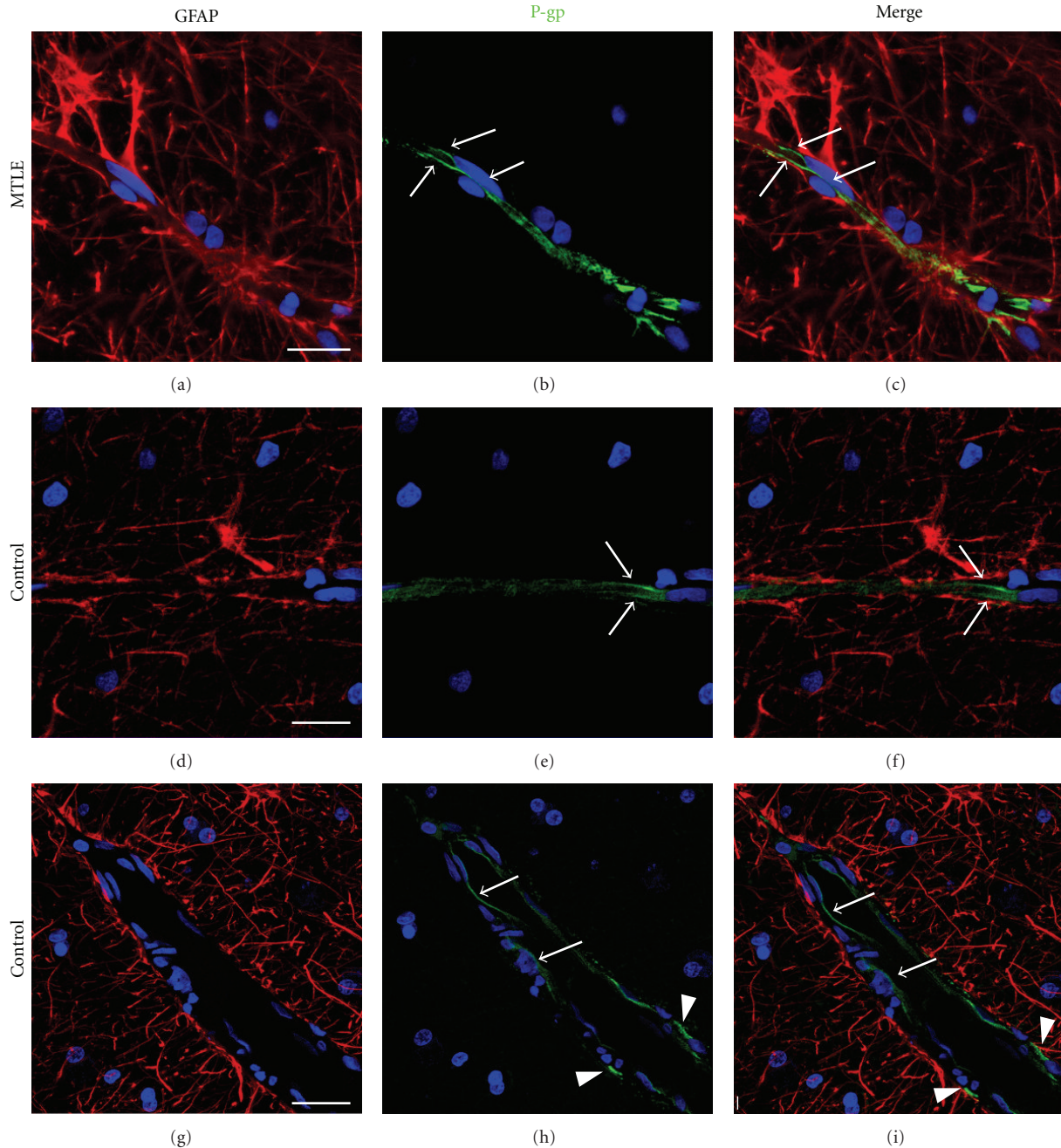
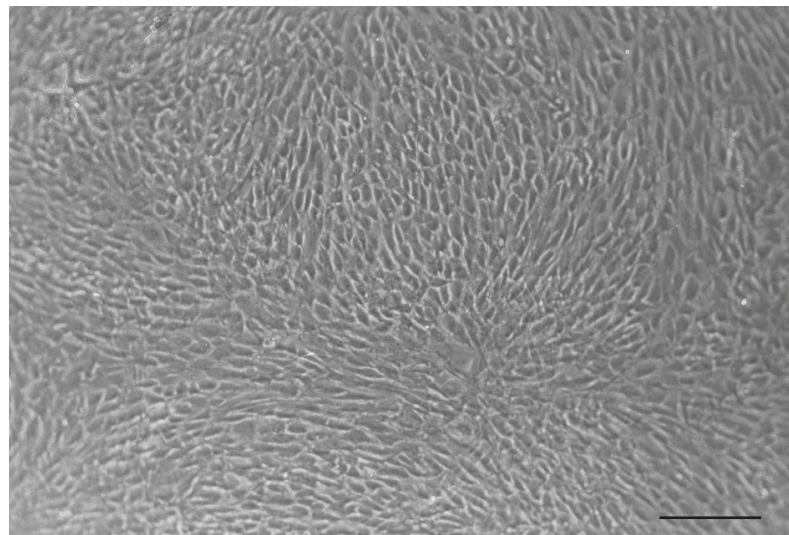


FIGURE 4: Laser scanning confocal microscopy of P-gp and GFAP expression in MTLE (a–c) and control (d–i) tissue sections. (a–c) High expression of P-gp which appears restricted to the luminal side of microvascular endothelial cells (b, c; arrows) and is not detectable on GFAP-positive astrocytes (a, c). A control capillary (d–f) and a small arteriole (g–i) showing P-gp expression preferentially localized on the endothelial luminal front (e, f, h, i; arrows) and absence of P-gp in the GFAP-positive perivascular astrocytes (d, f, g, i). Note in (h, i) the expression of P-gp on smooth muscle cells (arrowheads). Scale bars: a–f, 10 μm ; g–h 20 μm .

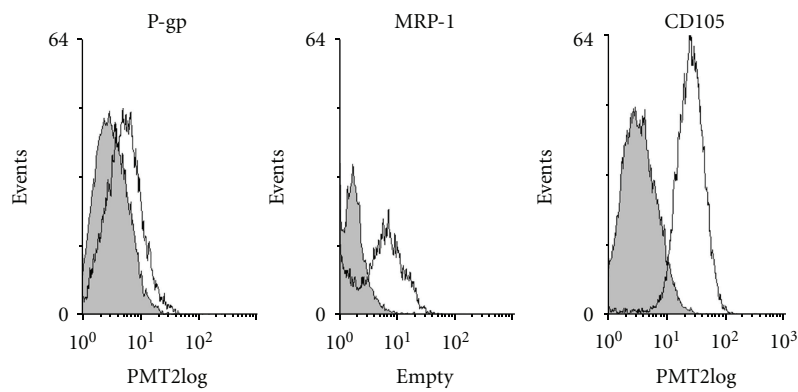
and controls revealed 7,862 up-regulated and 9,504 down-regulated genes. These much higher numbers compared to AHE reflect enrichment of endothelial mRNA upon eliminations of glial genes from the highly purified MVEC population.

Various multidrug transporters of the ATP-binding cassette protein, subfamilies A (ABCB) and B (ABCC), were

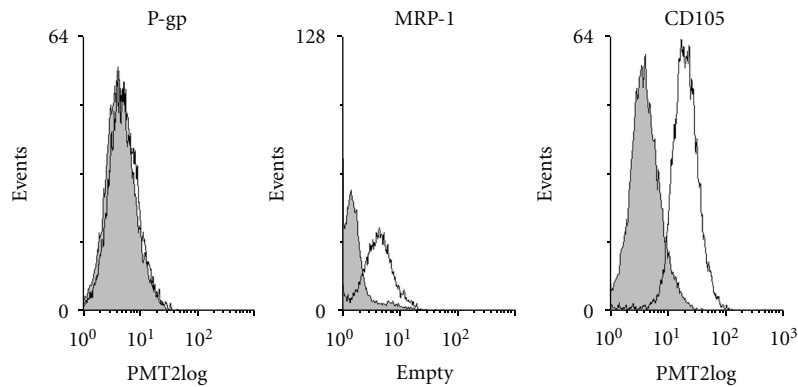
found to be overexpressed in AHEs and MVECs compared to the control group. A significant up-regulation of ABCB1/P-gp, ABCB2, and ABCB3 was found in MVECs of epileptic hippocampi (Table 2). Among the ABCC transporters, only ABCC4 was significantly up-regulated in both AHEs and MVECs (Table 2). These data were validated by quantitative RT-PCR analysis, as summarized in Table 3. The trend



(a)



(b)



(c)

FIGURE 5: P-glycoprotein and MRP-1 expression of brain-derived microvascular endothelial cells (MVECs) of an MTL and control patient. Morphology of primary hippocampal MVECs after 10 days in culture from an epileptic patient (a). Confluent MVECs exhibit a cobblestone morphology with some spindleform-like, elongated cells. Scale bar: 60 μm . P-gp and MRP-1 expression was assessed by flow cytometry of primary hippocampal MVECs isolated either from a patient who underwent selective amygdalohippocampectomy (sAHE) (b) or a control tissue specimen (c). The expression of the endothelial marker endoglin (CD105) was shown to prove the purity of the MVEC cultures (b and c). The open profiles indicate the specific antibodies and the filled profiles the isotype antibodies.

which was found by micro array analysis became statistically significant by RT-PCR for P-gp, MRP-2, and MRP4 (Table 3). As we had to deal with a large number of differentially expressed genes we evaluated those genes which were found to be highly overexpressed in epileptic patients compared to controls. Surprisingly, immunological factors such as cytokines were significantly up-regulated in AHEs ($n = 8$) compared to controls ($n = 3$). This includes proinflammatory interleukins (IL-1 α , IL-1 β , IL-6, and IL-18), and cytokines (TNF- α and TGF β -1) as shown in Figure 2.

The immunohistochemistry staining of human hippocampal section for IL-1 β revealed a strong expression, mainly localized at the cerebrovascular unit from epileptic hippocampus (Figures 3(c) and 3(d)), and staining for IL-6 showed positive expressing cells to be evenly distributed in vessels, neurons, and glial cells of epileptic hippocampus (Figure 3 (f)).

At the protein level, high P-gp expression was found in an MTLE patient which was restricted to the luminal side of MVECs and not detectable on GFAP-positive astrocytes (Figures 4(a) and 4(c)). In a control patient, a capillary (Figures 4(d)–4(f)) and a small arteriole (Figure 4(g)) were showing P-gp expression at lower level and mainly on the luminal front (Figures 4(e), 4(f), 4(h), and 4(i)).

The morphology of cultured MVECs derived from epilepsy patients had a cobblestone morphology with some spindleform-like, elongated cells (Figure 5(a)) which was also seen in control MVEC cultures (data not shown) and their purity was demonstrated by expression of the endothelial marker CD105 (Figures 5(b) and 5(c)).

Interestingly cultured MVECs isolated from MTLE patients revealed by flow cytometry an enhanced P-gp expression in comparison to controls (Figures 5(b) and 5(c)). In contrast, no difference was found in their level of MRP1 expression. These data are consistent with the findings on the mRNA level.

4. Discussion

In our study resected hippocampi and hippocampal MVECs, forming the BBB from epileptic patients, were evaluated for their global gene expression profile. Our analysis was based on the “multitransporter up-regulation hypothesis”. We also singled out those genes that were found highly up-regulated.

In examining the gene expression profile of the multidrug transporters, we included all transporters that have been described in the literature: 13 ABCB and 12 ABCB transporters. Some of them have been associated with medically intractable epilepsy: ABCB1/P-gp. [18–22]; MRP1 and MRP2[18–20]; MRP3 and MRP5 [19]. Most of these findings are based on IHC. However, Dombrowski et al. performed a gene expression analysis of MVEC isolated from human epileptogenic hippocampi and discovered an up-regulation of P-gp, MRP2, –3 and –5, which is partly consistent with our findings. In addition, we observed an up-regulation of ABCB2, ABCB3, ABCB4, and MRP4. Until now these transporters have not been identified as being overexpressed in epileptogenic hippocampi, suggesting that

of the list of gene products contributing to the “transporter hypothesis” may need to be enlarged.

With regard to the localization of these transporters, our results show an up-regulation, mainly in endothelial cells. Several studies have addressed the question of where these transporters are exactly localized; however, the answers are still highly controversial (luminal versus basolateral or even both).

At the protein level, IHC of hippocampal sections revealed that P-gp and MRP1-5 were largely confined to the endothelial cells of the hippocampus. The overexpression of these genes was validated by quantitative real-time RT-PCR, and the results were compared to that of the gene array. No significant differences were found by gene array when the averaged values of P-gp of individual epileptic patients and controls were compared. However, P-gp values obtained by RT-PCR revealed a statistically significant difference between them. The same situation was also observed by comparing the MRP4 values. This finding suggests that the RT-PCR method is more sensitive; further support for this is given by the threshold cycles (CT-values). When comparing MRP1, MRP2, and MRP3, no significant differences were obtained between the two methods. In contrast, a statistical significance for MRP5 was found by gene array analysis, but not by the RT-PCR analysis.

Unexpectedly, we observed a strong up-regulation of various cytokines (IL-1 α , IL-1 β , IL-6, IL-18, TNF- α , and TGF- β 1) in the hippocampus of epilepsy patients. The fact that MTLE-HS has not been considered to be an immunological disorder makes this finding even more interesting. Only few papers addressed a possible link between immunology and epilepsy, using different approaches. Two studies have been published on the gene expression profile of hippocampus in MTLE [23, 24]. In one of the studies, the mRNA expression was compared in the hippocampi of three patients with sclerosis and three without, using a small array representing 588 genes. This study revealed 9 overexpressed genes and 12 underexpressed genes. Among these genes are those associated with gliosis (GFAP), cell death/survival, and neuronal plasticity, but none associated with inflammation [23]. In the second study, five sclerotic hippocampi from MTLE patients were analysed, using the Affymetrix GeneChip U133A [24]. Unfortunately, this study does not report on the full expression pattern in these specimens, but only the expression pattern that was also present in the hippocampi from rats having pilocarpine-induced seizures. It is important to mention that the lack of a suitable control (“healthy” hippocampus) makes it difficult to interpret these results.

With respect to the link between MTLE and immunology, one of the most remarkable findings is the role of IL-1 β in the development of febrile seizures in animal models as well as the assumption that a polymorphism of IL-1 β at position –511 may favour a high production of IL-1 β in patients with MTLE-HS, possibly a major trigger for the development of HS [12]. In another study, the role of cytotoxic T cells and auto-antibodies against neurons in the brain in Rasmussen encephalitis was examined suggesting an immunotherapy as a treatment approach [25].

In a recent article, possible pathways underlying neuronal death in HS have been postulated, including the participation of cytokines and chemokines, leading to a disturbance of the glutamatergic system, and subsequently to the persistence of seizures by chronic neuronal overexcitation [10, 26–28]. Among the different factors potentially involved in the molecular pathway underlying glutamate release from astrocytes, chemokines, chemokine receptors, IL-1 β , and prostaglandin E2 have been found to be highly up-regulated in both epileptogenic hippocampus and MVEC. In experimental models and in clinical cases of epilepsy, proinflammatory and anti-inflammatory cytokines have been described in brain and plasma after seizure induction. Experimental findings demonstrated that proinflammatory cytokines IL-1 β , IL-6, and TNF- α in microglia and astrocytes are increased and followed by a cascade of downstream inflammatory events which may recruit cells of the adaptive immune system [29, 30]. In addition to inflammatory disease, recent evidence shows the activation of innate immune system and associated inflammatory reactions in epilepsy may modulate some of the molecular and structural changes occurring during and after seizure activity [30]. Moreover, experimental strategies aiming to block CNS or systemic inflammatory pathways reduce status epilepticus duration and seizure frequency [31].

Nevertheless, little is known about the possible underlying mechanisms which might be responsible for the fact that inflammatory factors are strongly overexpressed [32]. How inflammatory reactions intrinsic to the brain compared with those mediated by peripheral immune cells can be contributed to the epileptogenesis is poorly understood [33]. BBB failure might be a link between these two mechanisms.

In summary, whether these findings contribute to the persistence of seizures despite treatment with AEDs, or whether this event must be regarded as an epiphenomenon, is currently an open issue. It remains elusive, however, if such changes are causes or consequences of the disease. Since the underlying pathophysiology of MTLE-HS remains an issue of debate, more research is needed with the common goal of finding new targets and therapeutic strategies. The findings that inflammatory mediators contribute to the onset and recurrence of seizures in experimental models, as well as the presence of inflammatory molecules in human epileptogenic tissue, do highlight the possibility of targeting specific inflammation-related pathways to control seizures that are otherwise resistant to the available AEDs. Drugs that block IL-1 β actions have entered clinical trials as potential therapeutics for autoimmune and inflammatory pathologies and may also have therapeutic potential in refractory epilepsies associated with proinflammatory processes in the brain [34].

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