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DIFFERENTIAL EXPRESSION PATTERNS OF CHLORIDE TRANSPORTERS, NA^+-K^+-2CL^--COTRANSPORTER AND K^+-CL^--COTRANSPORTER, IN EPILEPSY-ASSOCIATED MALFORMATIONS OF CORTICAL DEVELOPMENT

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Abstract—Malformations of cortical development are recognized causes of chronic medically intractable epilepsy. An increasing number of observations suggests an important role for cation-chloride co-transporters (CCTs) in controlling neuronal function. Deregulation of their expression may contribute to the mechanisms of hyperexcitability that lead to seizures. In the present study the expression and cell-specific distribution of Na^+-K^+-2Cl^--cotransporter (NKCC1) and K^+-Cl^--cotransporter (KCC2) were studied immunocytochemically in different developmental lesions, including focal cortical dysplasia (FCD) type IIB (n=9), hemimegalencephaly (HMEG, n=6) and ganglioglioma (GG, n=9) from patients with medically intractable epilepsy and in age-matched controls. In normal control adult cortex, NKCC1 displayed low neuronal and glial expression levels. In contrast KCC2 showed strong and diffuse neuropil staining. Notable glial immunoreactivity (IR) was not found for KCC2. NKCC1 was highly expressed in the majority of FCD, HMEG and GG specimens. NKCC1 IR was observed in neurons of different size, including large dysplastic neurons, in balloon cells (in FCD and HMEG cases) and in glial cells with astrocytic morphology. The immunoreactivity pattern of KCC2 in FCD, HMEG and GG specimens was characterized by less neuropil staining and more intrasomatic IR compared with control. KCC2 IR was observed in neurons of different size, including large dysplastic neurons, but not in balloon cells or in glial cells with astrocytic morphology. Double-labeling experiments confirmed the differential cellular distribution of the two CCTs and their expression in $GABA_A$ receptor (α1 subunit)-positive dysplastic neurons. The cellular distribution of CCTs, with high expression of NKCC1 in dysplastic neurons and altered subcellular distribution of KCC2 resembles that of immature cortex and suggests a possible contribution of CCTs to the high epileptogenicity of malformations of cortical development.

Key words: focal cortical dysplasia, hemimegalencephaly, ganglioglioma, immunocytochemistry, cation chloride co-transporter, epilepsy.

Malformations of cortical development (MCDs) are recognized causes of medically intractable epilepsy (Farrell and Vinters, 1997; Blumcke and Wiestler, 2002; Crino et al., 2002; Thom, 2004). A recent classification scheme of MCDs includes focal cortical dysplasia (FCD; with balloon cells, type IIB), glioneuronal tumors (such as ganglioglioma, GG) and hemimegalencephaly (HMEG) among the disorders of proliferation (with abnormal cell types) (Barkovich et al., 2005). Accordingly, recent studies suggest that these MCDs share common pathogenetic mechanisms (Crino, 2005; Majores et al., 2005). Several studies, based on electrocorticographical, immuno-cytochemical and electrophysiological observations support the intrinsic epileptogenicity of these MCDs (Mattia et al., 1995; Palmini et al., 1995; Blumcke and Wiestler, 2002; Avoli et al., 2003; Najm et al., 2004; Cepeda et al., 2005a). In the attempt to detect the still unclear underlying cellular mechanism(s) of epileptic activity in MCDs, attention has been focused on the alterations of the balance between excitation and inhibition and particularly on the local pathways of excitatory amino acid synaptic transmission (for reviews see (Crino et al., 2002; Avoli et al., 2005)).

Recent evidence in human epileptogenic tissue indicates that human dysplastic tissue may retain immature properties, displaying mechanisms of seizure generation similar to that observed during development in the immature brain (for reviews see (Avoli et al., 2005; Cepeda et al., 2006)). Accordingly, electrophysiological studies performed in brain slices from FCD tissue show immature GABA receptor-mediated responses. GABA receptor-mediated synchronization appears to be involved in the mechanism leading to *in vitro* ictal activity in human FCD and this hypothesis is also supported by pharmacological manipulations of GABA type A receptors (D'Antuono et al.,

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Abbreviations: Ab, antibody; CCT, cation-Cl cotransporter; FCD, focal cortical dysplasia; GFAP, glial fibrillary acidic protein; GG, ganglioglioma; HMEG, hemimegalencephaly; IR, immunoreactivity; KCC2, K^+-Cl^--cotransporter; MCD, malformation of cortical development; NeuN, neuronal nuclear protein; NKCC1, Na^+-K^+-2Cl^--cotransporter; TTBS, 20 mM Tris, 150 mM NaCl, 0.1% Tween, pH 7.5; TLE, temporal lobe epilepsy.

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2004; Avoli et al., 2005; Cepeda et al., 2006). The paradoxical excitatory action of GABA observed in the immature brain (Ben-Ari, 2002; Owens and Kriegstein, 2002) depends on the relatively high intracellular chloride ion content which is critically regulated by the cation-Cl cotransporters (CCTs; Payne et al., 2003; Yamada et al., 2004). Interestingly, in both rodent and human brain the CCTs (Na^+-K^+-$2Cl^-$-cotransporter, NKCC1 and K^+-Cl^--cotransporter, KCC2) are developmentally regulated (Clayton et al., 1998; Rivera et al., 1999; Dzhala et al., 2005). In particular, the strong expression of NKCC1 early during development is considered to sustain the excitatory action of GABA and facilitate seizures in the immature brain (Dzhala et al., 2005). Deregulation of CCT levels with upregulation of NKCC1 has been recently reported in brain specimens from temporal lobe epilepsy (TLE) patients (Palma et al., 2006).

The present histological study analyzed the expression of both NKCC1 and KCC2 in a large series of developmental lesions, including FCD (type IIB), GG and HMEG from patients with medically intractable epilepsy. We report the specific cellular distribution of the two CCTs in both neuronal and glial components of these developmental lesions and discuss the potential role of CCTs in the epileptogenesis of MCDs.

EXPERIMENTAL PROCEDURES

Subjects

The cases included in this study were obtained from the files of the Departments of Neuropathology of the Academic Medical Center (University of Amsterdam) and the University Medical Center in Utrecht. We examined a total of 24 specimens removed from patients undergoing surgery for severe FCD ($n=9$), HMEG ($n=6$) and GG ($n=9$). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. Two neuropathologists reviewed all cases independently. For the GG we used the revised WHO classification of tumors of the nervous system (Kleihues and Cavanee, 2000). For the FCD we followed the classification system proposed by Palmini et al. (2004), for grading the degree of FCD. Normal-appearing control cortex/white matter from temporal region was obtained at autopsy from six adult control patients (male/female: 3/3; mean age 31; range 17–41) without history of seizures or other neurological diseases. All autopsies were performed within 10 h after death. We also included control material from patients with age <10 years (1 month, 2 months, 3 months, 6 months, 2 years and 8 years). We also selected three cases (3 GG) that contained sufficient amount of perilesional zone (normal-appearing cortex/white matter adjacent to the lesion), for comparison with the autopsy specimens. This material represents good disease control tissue, since it is exposed to the same seizure activity, drugs, fixation time, and age and gender are the same.

The clinical features (derived from the patient's medical record) are summarized in Table 1. Seizures were resistant to maximal tolerated doses of antiepileptic drugs. All patients underwent presurgical evaluation (van Veelen et al., 1990). In all patients the lesion was localized by brain MRI; electroencephalographic recordings were performed to detect the epileptogenic area. We classified the postoperative seizure outcome according to Engel (1993). Class I consisted of patients who remained completely seizure-free and class II includes patients who are almost seizure free or have rare or nocturnal seizures only. Follow-up period ranged from 1 to 15 years.

Table 1. Summary of clinical features of MCD patients

Patient/sex/age	Lesion Type	Duration Epilepsy	Seizure type	Location	Engel class
1/M/10 (y)	FCD	10 (y)	CPS	Temporal	I
2/M/14	FCD	4	CPS/SGS	Temporal	I
3/M/11	FCD	9	CPS/SGS	Temporal	I
4/M/21	FCD	21	CPS/SGS	Temporal	I
5/M/24	FCD	17	CPS	Temporal	I
6/F/16	FCD	9	CPS/SGS	Temporal	I
7/F/29	FCD	10	CPS	Temporal	I
8/F/26	FCD	14	CPS/SGS	Temporal	II
9/F/24	FCD	17	CPS	Temporal	II
10/M/27	GG	27	CPS/SGS	Temporal	I
11/M/16	GG	3	CPS	Temporal	I
12/M/26	GG	18	CPS	Temporal	I
13/M/10	GG	9	CPS	Temporal	I
14/F/26	GG	25	CPS/SGS	Temporal	I
15/F/25	GG	24	CPS	Temporal	I
16/F/35	GG	18	CPS/SGS	Temporal	I
17/F/17	GG	16	CPS/SGS	Temporal	II
18/F/24	GG	18	CPS	Temporal	II
19/F/3 (mo)	HMEG	3 (mo)	IF	CH/R	I
20/M/6	HMEG	6	IF	CH/R	I
21/M/7	HMEG	7	IF	CH/R	I
23/M/2	HMEG	2	IF	CH/L	II
2/F/24	HMEG	24	CPS/SGS	CH/L	II
24/M/96	HMEG	96	CPS/SGS	CH/L	II

Age of HMEG patients is presented in months; FCD and GG patients in years. CH, cerebral hemisphere; CPS, complex partial seizure; IF, infantile spasm; R/L, right/left; SGS, secondary generalized seizure.

Tissue preparation

Tissue was fixed in 10% buffered formalin and embedded paraffin. To avoid differences in labeling related to technical variables such as tissue fixation, we used the same fixation protocol for both autopsy and surgical material; small samples of selected cortical regions (temporal cortex) were collected at autopsy and immediately fixed in formalin for 24 h (same fixation time used for the surgical specimens). Paraffin-embedded tissue was sectioned at 6 μm, mounted on organosilane-coated slides (Sigma, St. Louis, MO, USA) and used for histological and immunocytochemical reactions as described below. Frozen tissue from control cortex, FCD and GG (stored at −80 °C) was used for Western blot analysis.

Antibody (Ab) characterization

Glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9, DAKO; 1:1000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemicon, Temecula, CA, USA; 1:2000), synaptophysin (mouse clone Sy38; DAKO; 1: 200), CD34 (mouse clone QBEnd10; Immunotech, Marseille, Cedex, France, 1:600), human leukocyte antigen (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark, 1:400), and HLA-DR (mouse clone Tal1b5, Sigma; 1:100) were used in the routine immunocytochemical analysis of FCD, GG and HMEG specimens to document the presence of a heterogeneous population of cells.

For the detection of the CCTs the following antibodies (Abs) were used: NKCC1 rabbit polyclonal Ab raised against a 22 amino