Adenosine Kinase Expression in Cortical Dysplasia With Balloon Cells: Analysis of Developmental Lineage of Cell Types

Guoming Luan, MD, PhD, Qing Gao, MD, Feng Zhai, MD, PhD, Jian Zhou, MD, PhD, Changqing Liu, MD, PhD, Yin Chen, and Tianfu Li, MD, PhD

Abstract

Focal cortical dysplasia type IIb (FCDIIb) is a developmental malformation of the cerebral cortex that is associated with pharmacoresistant epilepsy. Overexpression of adenosine kinase (ADK) has been regarded as a pathologic hallmark of epilepsy. We hypothesized that the epileptogenic mechanisms underlying FCDIIb are related to abnormal ADK expression. We used immunohistochemistry to examine the expression of ADK and of heterogeneous cell population markers of astrocytes (glial fibrillary acidic protein), immature glia (vimentin), immature neurons (neuronal class III beta-tubulin, TUJ1), multipotential progenitor cells (nestin), mature neurons (microtubule-associated protein 2), and antiapoptotic gene products (Bcl-2) in surgically resected human epileptic cortical specimens from FCDIIb patients (n = 20). Expression patterns were compared with those in normal autopsy (n = 6) and surgical control (n = 6) brain samples. Balloon cells in FCDII lesions were immunoreactive for ADK (77%) and balloon cells expressing the different cell markers expressing different degrees of ADK. Adenosine kinase expression assessed by Western blot and enzymatic activity were also greater in FCD versus control samples. These results suggest that upregulation of ADK is a common pathologic component of FCDIIb. Adenosine kinase might, therefore, be a target in the treatment of epilepsy associated with FCD.

Key Words: Adenosine kinase, Balloon cell, Development of brain, Focal cortical dysplasia.

INTRODUCTION

Focal cortical dysplasias (FCDs) are developmental malformations of the cerebral cortex that are highly associated with pharmacoresistant epilepsy (1–3). One of the commonest forms of FCD in children is FCD type IIb (FCDIIb) (2, 4), which is characterized by a unique population of abnormal cells known as balloon cells (BCs) (2). Balloon cells have pleomorphic nuclei and large amounts of homogeneous eosinophilic cytoplasm (2). Balloon cells in patients with FCDIIb originate from glioneuronal progenitor cells, strongly suggesting that defects of neuronal and glial differentiation are important in the histogenesis of FCDIIb (5). The origin, pathogenesis, and role of the BC in FCD and epileptogenesis remain to be elucidated. It was previously hypothesized that such structural abnormalities are the result of aberrant patterns of proliferation, differentiation, migration, maturation, and/or apoptosis of neuronal precursors and neurons during cortical development (6–8). The current classification and several other studies indicate that FCDII is a malformation caused by abnormal proliferation or apoptosis (5). Balloon cells are heterogeneous populations that express cell surface markers for pluripotential stem cells and proteins for multipotent progenitors, or immature neurons/glia, which could be caused by persistent postnatal neurogenesis or an early embryonic insult that results in the arrest of proliferation at their early stages (9, 10). An important advance in understanding cell proliferation has been the elucidation of specific molecular pathways that control proliferation, in particular, the mammalian target of rapamycin (mTOR) pathways (11). Activation of mTOR signaling pathways has been observed in focal malformations of cortical development, including FCD and cortical tubers in the tuberous sclerosis complex (12–14).

Adenosine is an endogenous purine nucleoside that modulates a wide range of physiologic functions (15). Most notable among its many roles is its importance in inhibiting seizures (16–22). Adenosine is formed by dephosphorylation of adenosine monophosphate by ecto- and endo-5′-nucleotidase and through hydrolysis of S-adenylylhomocysteine. It is removed by adenosine deaminase and adenosine kinase (ADK) (23). Because of its low Km for adenosine, ADK is the primary route of adenosine metabolism in the brain. Therefore, minor changes in ADK activity translate rapidly into major changes in adenosine (23). Recently, we and others have provided evidence for dysfunctional astrocytic adenosine homeostasis as one of the early pathophysiologic mechanisms of epilepsy (24–27). Adenosine kinase, the main adenosine-removing enzyme, has been highlighted as a diagnostic marker to predict epileptogenesis as well as a potential target for antiepileptogenesis or disease modification (19, 25–28). Our previous results demonstrated that astroglialosis via disruption of adenosine homeostasis per se, and in the absence of any other overt pathology associated with the...
emergence of spontaneous recurrent subclinical seizures, may be a first step in epileptogenesis (26, 27).

Two isoforms of ADK have been identified in mammals: a long nuclear isoform and a short cytoplasmic isoform (29). Nuclear ADK-immunoreactive material was observed in a subpopulation of resting astroglial cells, whereas cytoplasmic expression was weak or below detectable levels (19, 22, 24, 30). The cytoplasmic isoform of ADK regulates tissue levels of adenosine, and the concentration of extracellular adenosine may inhibit seizures by adenosine receptor-independent mechanisms (31, 32). By contrast, the nuclear isoform of ADK might play key roles in the regulation of cell proliferation through a combination of epigenetic and additional adenosine receptor–independent mechanisms, such as interaction with the mTOR pathway (33).

Based on the unique role of ADK in modulating an entire network, not only by activation of multiple adenosine receptor–dependent pathways but also through adenosine receptor–independent interaction with the mTOR pathway (and hence regulation of cell proliferation and epigenetic mechanisms), we hypothesized that the epileptogenic mechanisms underlying FCDIIB may be related to changes in ADK expression and that those changes might be associated with the development of epilepsy in patients with this condition.

MATERIALS AND METHODS

Patients and Diagnosis

The local ethics committee (Beijing Sanbo Hospital, Capital Medical University, Beijing, China) approved all studies, and clinical investigations were conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants or their parents or legal guardians. Brain tissue samples of 20 childhood-onset FCDIIB patients diagnosed according to typical clinical, magnetic resonance imaging, and neuropsychologic findings as proposed by Palmini et al (2) were enrolled in this study. Histologic examination revealed abnormal cortical lamination, blurring of the gray-white junction, and the presence of dysmorphic neurons and BCs, consistent with the pathologic diagnosis of FCDIIB (2). None of the patients with FCDIIB in the study had a mixture of cortical dysplasias. Presurgical evaluation in Beijing Sanbo Hospital included magnetic resonance imaging (T1-weighted and T2-weighted axial, coronal sequences and fluid-attenuated inversion recovery images with 5-mm-thick axial, sagittal, and coronal sections), fluorodeoxyglucose positron emission tomography, scalp video-electroencephalography, intracranial electroencephalography monitoring, seizure semiology analysis, as well as neuropsychologic tests. There were 12 male and 8 female patients with a mean age at seizure onset of 3.6 ± 2.7 years (range, 0.25–10 years) and a mean age at surgery of 16.1 ± 9.4 years (range, 2.42–29 years). All 20 FCDIIB patients had refractory epilepsy and underwent surgical resection for intractable seizures (Table). The seizure types of patients included simple partial seizures, complex partial seizures, and secondary generalized tonic-clonic seizures. The duration from the seizure to operation was 11.9 ± 8.4 years (range, 1.67–25 years) (Table). None of the patients had a relevant perinatal history before the onset of the disease. Clinical details of the patients are summarized in the Table. From all FCDIIB patients, brain samples were obtained during neurosurgical operations. Brain tissue obtained

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CPS, complex partial seizure; GTCS, generalized tonic-clonic seizure; SPS, simple partial seizure; F, female; M, male.
from all patients was paraffin embedded. For 6 of the patients, parts of the brain samples were cryoprotected, in addition, for Western blot analysis.

For control tissue samples, we included normal-appearing control cortex/white matter obtained at autopsy from 6 children without a history of seizures or other neurologic diseases; they had an average age of 10.25 ± 5.58 years (range, 3.5–17 years). Because the age ranges are not substantially overlapping with the FCDII patients, we added 6 adults aged 28.66 ± 7.03 years (range, 20–37 years) to address whether age influenced the ADK expression. All autopsies were performed within 12 hours after death. To address the possibility that time from death to tissue processing may have affected ADK expression, we also added surgical control specimens. Six neocortical specimens were obtained from normal-appearing cortex/white matter well outside the lesion tissue from resection specimens for intractable epilepsy in patients with FCD; the average age of these patients was 13.66 ± 6.65 years (range, 4–24 years). Of note, we specifically avoided perilesion tissue within the border zone between the lesion and normal-appearing cortex (Fig. 1B, blue circle) because the extent of neuropathologic abnormalities in this area may vary among subjects.

**Tissue Preparation**

Formalin-fixed paraffin-embedded tissue samples (1 representative paraffin block per case containing the complete lesion or the largest part of the lesion resected at surgery) were sectioned at 4 μm and mounted on precoated glass slides (Star Frost; Waldemar Knittel GmbH, Braunschweig, Germany). Sections of all specimens were processed for hematoxylin and eosin and for immunohistochemical staining for a neuronal marker NeuN, glial marker glial fibrillary acidic protein (GFAP), ADK, vimentin, nestin, neuronal class III α tubulin (TUJ1), microtubule-associated protein 2 (MAP-2), and Bcl-2. Brain tissue from FCDIIB patients (n = 6) and autopsy controls (n = 6) was snap-frozen in liquid nitrogen and stored at −80°C until use for Western blot analysis.
Immunohistochemistry

Antibodies to GFAP (polyclonal rabbit, 1:4000; DAKO, Glostrup, Denmark), vimentin (mouse monoclonal, 1:100; Invitrogen, Carsbad, CA), nestin (mouse monoclonal, 1:50; Santa Cruz Biotechnology, Dallas, TX), (TUJ1, mouse monoclonal, 1:100; Chemicon, Carpinteria, CA), MAP-2 (mouse monoclonal, 1:200; Thermo Fisher Scientific Inc, Waltham, MA), Bcl-2 (mouse monoclonal, 1:100; Leica Biosystems Newcastle Ltd, UK), and ADK (polyclonal rabbit, 1:500; provided by Professor Detlev Boison, RS Dow Neurobiology Laboratories, Legacy Research Institute, Portland, OR) (17, 19, 24, 30) were used in the immunohistochemical analysis. Double label immunocytochemistry was performed as described previously (19, 24, 30). After incubation with primary antibodies, sections were incubated for 2 hours at room temperature with Alexa Fluor 568 and Alexa Fluor 488 (anti-rabbit IgG or anti-mouse IgG, 1:200; Molecular Probes, Eugene, OR). Images were visualized using a Leica microscope under Ex/Em wavelengths of 500/550 nm (green), collected using an Optronics DEI-750 three-chip camera equipped with a BQ 8000 sVGA frame grabber, and analyzed using Bioquant software (Nashville, TN).

Evaluation of Immunohistochemical Staining

Balloon cells were defined using morphometric parameters (maximal cell diameter, somatic area, and process extension) based on hematoxylin and eosin staining for quantitative cell counting analysis (2). The contiguous images spanned a 1-cm² region of interest (ROI) and were generated under light microscopy and phase-contrast optics to maximize identification of cell morphology. The staining intensity of immunoreactivity was graded using the following scale: negative, weak positive, and positive. During manual counting of BCs on the hematoxylin and eosin–stained slides and immunohistochemical analyses, the total numbers of morphologically identified BCs were determined in each ROI for each case and the mean number of BCs in ROIs was determined across all 20 cases.

Western Blot Analysis

Preparation and analysis of Western blots were performed as previously described (17, 24, 30). Freshly frozen histologically normal autopsy cerebral cortex and FCDIIB samples (n = 6 per group) were homogenized in lysis buffer containing 10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 10% glycerol, 1% NP-40, Na-orthovanadate (10.4 mg/mL), 5 mmol/L EDTA (pH 8.0), 5 mmol/L NaF, and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein content was determined using the bicinchoninic acid method. For electrophoresis, equal amounts of proteins (30 µg/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis. Separated proteins were transferred to nitrocellulose paper for 1 hour 30 minutes using a semidry electroblotting system (BioRad, Transblot SD, Hercules, CA). Blots were incubated overnight in TTBS (20 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween, pH 7.5)/5% nonfat dry milk, containing the primary antibody (1:5000). After several washes in TTBS, the membranes were incubated in TTBS/5% nonfat dry milk/1%...
bovine serum albumin, containing the goat anti-rabbit or goat anti-mouse antibodies coupled to horseradish peroxidase (1:2500; DAKO) for 1 hour. After washes in TTBS, immuno-
reactivity was visualized using Lumi-light PLUS Western blotting substrate (Roche Diagnostics) and digitized using a Luminescent Image Analyzer (LAS-3000; Fuji Film, Japan).

FIGURE 3. Adenosine kinase (ADK), glial fibrillary acidic protein (GFAP) immunostaining in balloon cells (BCs) in the brain tissue of focal cortical dysplasia type IIb (FCDIIb) patients. (A–C) GFAP staining in BCs and reactive astrocytes within the lesion. GFAP-positive BCs (A, arrows) and reactive astrocytes surround BCs (A, arrowheads); GFAP-negative BCs (B, arrows) and reactive astrocytes in the perivascular area (B, arrowheads); weakly GFAP-positive BCs (C, arrows) and a GFAP-positive dysmorphic enlarged neuron (C, arrowheads). (D–F) ADK staining in BCs and reactive astrocytes. Concomitant with reactive astrogliosis, there is cytoplasmic localization of ADK immunoreactivity in cells with typical astroglial morphology (D, arrowheads). Balloon cell immunostaining is variable, that is, positive in (D), arrows; negative in (F), arrow; and weakly positive in (F), arrowheads. There is also cell membrane localization of ADK immunoreactivity in BCs (E, arrow). (G–L) In autopsy control (G–I) and surgical control (J–L) specimens, there is weak immunostaining for ADK in control cortical gray matter (G, H, J, K) and sparse glial cells in control white matter (I, L). Scale bars = (A–F, H, I, K, L) 40 μm; (G, J) 160 μm.
Expression of β-actin (monoclonal mouse, 1:50,000; Sigma, St. Louis, MO) was used as reference. Adenosine kinase levels were normalized to internal standards and reported relative to control.

ADK Enzyme Activity Analysis
Adenosine kinase enzyme activity was evaluated in 6 specimens from FCDIIB patients and 6 control specimens. The evaluation of the enzymatic activity for ADK was performed as previously described (21, 28, 30). Briefly, tissues were homogenized individually at 4°C in 200 μL of 50 mmol/L NaCl, 20 mmol/L Na2HPO4, and 5 mmol/L EDTA, pH 6.5. The homogenates were then centrifuged at 100,000 × g for 10 minutes. The protein contents of the supernatants were quantified using the bicinchoninic acid method. Then, 75 μL of each sample was applied to a Micro Bio-Spin 6 column (catalog no. 732–6221; Bio-Rad) and equilibrated with 500 μL of the adenosine 5'-triphosphate assay mix dilution buffer (FL-AAB; Sigma, Buchs, Switzerland). Each sample was centrifuged at 1,000 × g for 4 minutes and diluted with FL-AAB to reach a final protein concentration of 1.0 mg/mL; 5 μL of each sample was used for ADK activity determinations with an enzyme-linked bioluminescent assay according to the previously described method. Adenosine kinase activity values were normalized to endogenous lactate dehydrogenase activity, used as an internal standard and determined with a commercial assay kit according to the manufacturer’s protocol (Roche Applied Science, Mannheim, Germany).

Statistical Analysis
Statistical analyses were performed with SPSS for Windows (SPSS 11.5, SPSS Inc, Chicago, IL) using 2-tailed Student t-test; p < 0.05 was considered significant.

RESULTS
Histopathologic Features of FCDIIB
Normal-appearing neocortex has a characteristic layering of the human isocortex (Fig. 1C). By contrast, marked cortical dyslamination is seen in FCDIIB lesions (Fig. 1D), with enlarged dysmorphic neurons with a huge nucleus and abnormal intracytoplasmic Nissl aggregates (Fig. 1D, inset, arrow); BCs are the characteristic hallmarks of FCDIIB (Fig. 1D, inset, arrowhead). Magnetic resonance T2-FLAIR image reveals hyperintensity in the lesion area (Fig. 1A, red circle), and the difference between the lesion area (Fig. 1B, red circle) and the normal-appearing neocortex (Fig. 1B, blue circle) usually can be recognized in the surgical specimen.

Typical BCs in FCDIIB With Variably Nuclear Atypia
In FCDIIB specimens, BCs were observed in the lesion areas (Fig. 2A). There were typical BCs with eccentric nuclei and glassy eosinophilic cytoplasm (Fig. 2B, arrows and insets) admixed with abnormal cells showing both dysplastic neuron and BCs with the features of eccentric nucleolated nucleus and the enlarged pale but slightly basophilic cytoplasm (Fig. 2B,...
There were occasional BCs showing binucleation with nuclear budding (Fig. 2C, arrows and insets), BCs with a nuclear bridge connecting 2 nuclei (Fig. 2D, arrows and insets), BCs with a multinucleation and/or micronucleation (Fig. 2E, arrows and insets), and groups of BCs arranged in a line (Fig. 2F, arrows).

**Overexpression of the ADK Concomitant With Astrogliosis Within the Lesions of FCDIIB**

As in previous studies (24, 30), in autopsy control (Fig. 3I) and surgical control (Fig. 3L) white matter, ADK immunoreactivity was present in sparse glial cells with only a weak staining. Similarly, autopsy control (Fig. 3G, H) cortical gray matter (Fig. 3J, K) displayed weak astroglial staining.

In FCDIIB specimens, 45.2% ± 10.6% BCs were GFAP positive. The GFAP–strongly positive BCs (Fig. 3A, arrows), GFAP-negative BCs (Fig. 3B arrows), and GFAP–weakly positive BCs (Fig. 3C, arrows) were observed in the lesion area. Marked GFAP-positive reactive astrogliosis was observed around BCs (Fig. 3A, arrowheads) and in perivascular areas (Fig. 3B, arrowheads). Occasional GFAP–positive dysmorphic enlarged neurons were seen (Fig. 3C, arrowheads). A total of 77.2% ± 13.1% of BCs were ADK positive in FCDIIB specimens. Concomitant with reactive astrogliosis, cytoplasmic localization of ADK immunoreactivity was observed in cells with typical astroglial morphology (Fig. 3D, arrowheads).

Western blot analysis was also performed to quantify the total amount of ADK in the total homogenates of autopsy control, surgical control, and surgical cortex from FCDIIB patients (Fig. 5A, B). There was significantly greater ADK expression (p < 0.05) in FCDIIB versus autopsy control and surgical specimens (Fig. 4C). No difference between the surgical control and autopsy control specimens was found (Fig. 4C). In addition, no difference of ADK expression between the children and adult autopsy group was indicated (Fig. 4D; p > 0.05).
ADK Activity Within Lesions is Increased in FCDIIB Patients

To study the possible relationship between increased ADK immunoreactivity observed in FCDIIB tissue with enhanced adenosine metabolism, the enzymatic activity of ADK was evaluated in homogenates derived from autopsy control, surgical control, and surgical cortex from FCDIIB. The FCDIIB samples from epileptic patients displayed significantly greater ADK activity versus the ADK activity detected in surgical control and autopsy control specimens (p < 0.05) activity (Fig. 6).

TUJ1

In FCDIIB specimens, 38.5% ± 8.3% BCs were TUJ1 positive (Fig. 7A). TUJ1-positive BCs (Fig. 7B, arrows) and TUJ1-negative BCs (Fig. 7B, arrowheads) were observed in the lesions. TUJ1 immunoreactivity was mainly localized within the cytoplasm (Fig. 7B, arrows). Double labeling confirmed that there was 36.7% ± 5.6% dually immunopositive for ADK/TUJ1 in ADK-positive BCs (Fig. 8A–D); other ADK-positive BCs did not express TUJ1 (Fig. 8I–L). In addition, some TUJ1-positive BCs did not express ADK (Fig. 8E–H).

Vimentin

In FCDIIB specimens, 39.1% ± 7.6% BCs were vimentin positive (Fig. 9A). Vimentin-positive BCs (Fig. 9B, arrows) and vimentin-negative BCs (Fig. 9B, arrowheads) were observed in the lesions. Vimentin immunoreactivity mainly localized within the cytoplasm (Fig. 9B, arrows). Double labeling confirmed that there was 81.5% ± 15.9% dual immunopositivity for ADK/vimentin in ADK-positive BCs (Fig. 10A–D); other ADK-positive BCs did not express vimentin (Fig. 10E–H, arrows). In addition, some vimentin-positive BCs did not express ADK (Fig. 10I–L).

Nestin

In FCDIIB specimens, 26.2% ± 6.9% BCs were nestin positive (Fig. 11A). Nestin-positive BCs (Fig. 11B, arrows) and nestin-negative BCs (Fig. 11B, arrowheads) were observed in the lesions. Nestin immunoreactivity mainly localized within the cytoplasm (Fig. 11B, arrows). Double labeling confirmed that there was 78.5% ± 13.1% dual immunopositivity for ADK/nestin in ADK-positive BCs (Fig. 12A–D); other ADK-positive BCs did not express nestin (Fig. 12I–L). In addition, some nestin-positive BCs did not express ADK (Fig. 12E–H).

Microtubule-Associated Protein 2

In FCDIIB specimens, 12.2% ± 5.6% BCs were MAP-2 positive (Fig. 13A). Microtubule-associated protein 2-positive BCs (Fig. 13B, arrows) and MAP-2-negative BCs (Fig. 13B, arrowheads) were observed in the lesions. Microtubule-associated protein 2 immunoreactivity was mainly localized within the cytoplasm (Fig. 13B, arrows). Double labeling

FIGURE 7. Neuronal class III β tubulin (TUJ1) immunostaining in balloon cells (BCs) within the brain tissue of focal cortical dysplasia type IIB (FCDIIB) patients. (A) Low-magnification view of a representative region of cortex shows TUJ1 immunostaining in BCs. (B) Magnified view of the lesion shows TUJ1-positive BCs (arrows) and TUJ1-negative BCs (arrowheads). Scale bars = (A) 80 μm; (B) 40 μm.
confirmed that there were no dually immunopositive ADK/MAP-2 BCs among ADK-positive BCs (Fig. 14A).

**Bcl-2**

In FCDIIB specimens, 52.3% ± 15.2% BCs were Bcl-2 positive (Fig. 15A). Bcl-2–positive BCs (Fig. 15B, arrows) and Bcl-2–negative BCs (Fig. 15B, arrowheads) were observed in the lesions. Bcl-2 immunoreactivity in BCs was mainly localized within the cytoplasm (Fig. 15B, arrows). Double labeling confirmed that there was 66.6% ± 11.7% dual immunopositivity for ADK/Bcl-2 in ADK-positive BCs (Fig. 16A–D); other ADK-positive BCs did not express Bcl-2 (Fig. 16E–H). In addition, all the Bcl-2–positive BCs expressed ADK (Fig. 16A–D).

**DISCUSSION**

Adenosine kinase has been extensively studied in experimental epilepsy models and in patients with intractable epilepsy such as temporal epilepsy, Rasmussen encephalitis, and tumor-related epilepsy (17–19, 24, 27, 28, 30). Adenosine kinase is, therefore, a potential target for antiepileptogenesis or disease modification by activation of multiple adenosine receptor–dependent pathways (19, 25–28). On the other hand, ADK plays a role in the regulation of cell proliferation and epigenetic...
mechanisms through adenosine receptor-independent interactions with the mTOR pathway (33).

Balloon cells in patients with FCDIIB are thought to originate from glioneuronal progenitor cells, strongly suggesting that defects of neuronal and glial specifications are important in the histogenesis of FCDIIB (10, 34). In the present study, abnormal neurologic cells admixed with typical BCs showed both dysplastic neurons and BCs with eccentric nucleolated nuclei and enlarged pale but slightly basophilic cytoplasm; these features suggest the possibility that they represent a transitional form of neuroglial cell. There were occasional BCs showing binucleation with nuclear budding (a marker of gene amplification), a nuclear bridge connecting 2 nuclei (a marker of chromosome rearrangement), and multinucleation and/or micronucleation (a marker of chromosome breakage and/or loss) (35). These patterns likely reflect different genetic mishaps that may be involved in BC formation.

Adenosine is a powerful inhibitory substance released during seizures and implicated in seizure arrest, postictal refractoriness, and suppression of epileptogenesis (27). We and others have demonstrated adenosine dysfunction in astrogliosis, including a decrease in the density of adenosine 1 receptors (36), upregulation of the major adenosine-removing enzyme ADK (17, 19, 22, 24), and reduced adenosine tone in the epileptic focus, which can promote further seizures (20). In particular, it has been shown that ADK is a critical molecular link between astrogliosis and neuronal dysfunction of epilepsy (19, 27).

Extracellular levels of adenosine are regulated largely by an astrocyte-based adenosine cycle, and astrocytic ADK is the major adenosine-removing enzyme (37). Adenosine kinase is the primary route of adenosine metabolism in the brain. Therefore, minor changes in ADK activity translate rapidly into major changes in adenosine (33). Upregulation of ADK leads to reducing the “tone” of ambient adenosine, leading to insufficient activation of adenosine receptors (25). Overexpression of ADK per se might be sufficient to trigger electrographic seizures. This concept is supported by the following: i) Transgenic mice overexpressing ADK display increased sensitivity to brain injury and seizures; conversely, after pharmacologic induction of an otherwise epileptogenesis-precipitating acute brain injury, transgenic mice with reduced forebrain ADK are resistant to subsequent epileptogenesis. ii) Wild-type mice overexpressing the virus-induced ADK in astrocytes of the CA3 area of the hippocampus formation displayed electrographic seizures (38). iii) Intrahippocampal implants of stem cells engineered by biallelic genetic disruption of the ADK gene (Adk−/−) prevent epileptogenesis in the limbic mouse model induced by injection of kainate into the amygdala (19). iv) A ketogenic diet suppresses seizures in transgenic mice overexpressing ADK and ADK−/− mice through inhibition of ADK expression (thus increasing ambient levels of adenosine) and thereby increasing activation of adenosine 1 receptors (17). v) Adenosine kinase is overexpressed in tissues from patients with temporal lobe epilepsy, Rasmussen encephalitis, and glial tumors, as well as peritumoral regions infiltrated by glia (24, 28, 30). Basal adenosine is reduced in epileptic compared with control human hippocampus, consistent with ADK contributing to epileptogenesis (20, 24, 30). Thus, ADK is a potential target for diagnosis and therapeutic intervention.

To our knowledge, this is the first study to describe the cellular distribution and expression of ADK in the brains of FCD patients. Immunohistochemical analysis showed that ADK expression in BCs and reactive astrocytes was dominantly found in the cytoplasm. The cellular distribution and expression of ADK in FCD are similar with the findings of ADK expression in gliomas and Rasmussen encephalitis (28, 30).

Adenosine kinase exists in two isoforms: ADK long and ADK short isoforms (29). Nuclear ADK expression was observed in a subpopulation of resting astroglial cells, whereas cytoplasmic expression was weak or below detectable levels (19, 22, 24). Adenosine kinase long is mainly localized in the nucleus and has an essential role in methylation reactions, being possibly involved in the regulation of cell proliferation and epigenetic mechanisms (29, 33, 39), whereas ADK short is localized in the cytoplasm and regulates the extracellular
adenosine concentrations. Accordingly, reduced adenosine tone has been detected in mice constitutively overexpressing a transgene for the cytoplasmic isoform of ADK (19, 22).

The functions of ADK isoforms in the developing human brain are still unclear. Developmental studies performed in mice indicate that there is a switch from neuronal expression during the perinatal period to near exclusive astrocytic expression in the adult brain (31). These observations point to a dual functionality of this enzyme and suggest a key role for ADK that may affect important cellular functions of neural progenitor cells, such as proliferation, survival, and neural plasticity. Interestingly, strong expression of ADK has been detected in human fetal brains (gestational week 13; temporal cortex); high levels observed by Western blot analysis in total cortical homogenates may reflect the expression in the deep compartments of the cortical wall (i.e. ventricular/subventricular zone) at early stages of corticogenesis (39). We detected high levels of expression of the cytoplasmic isoform of ADK in BCs (77% positive) and reactive astrocytes within the lesion of FCD IIB, which could contribute to epileptogenesis by a reduction of adenosine tone. On the other hand, the presence of ADK protein in BCs may be because, in patients with FCD IIB, they are derived from radial glial cells in the ventricular zone and retain an embryonic phenotype (40). The expression of ADK in the BCs may suggest that these cells fail to mature fully and therefore continue to express embryonic genes and proteins. These immature cells may lack some of the

FIGURE 10. Double labeling for adenosine kinase (ADK) and vimentin in lesion areas in patients with focal cortical dysplasia type IIB (FCD IIB). (A–L) Colocalization of ADK and vimentin confirms ADK expression in some vimentin-positive balloon cells (BCs) (A–D, arrows; E–H, arrowheads) and also in some vimentin-negative BCs (E–H, arrows) within the lesion area. In addition, some vimentin-positive BCs do not express ADK (I–L). (A, E, I) vimentin (green); (B, F, J) ADK (red); (C, G, K) DAPI (blue). Scale bar = 40 µm.
ADK expression throughout development correlate with the maturation of hippocampal cells in mice (31). In the present study, ADK and nestin were coexpressed in a subpopulation of BCs. Our data suggest that these ADK-positive BCs are in the transitional stage of the maturation process.

Because nestin is transiently expressed in multipotent neuroepithelial stem cells in the developing CNS, it may act as a neural stem cell marker. Vimentin is expressed in less mature glial cells. In early CNS development, vimentin appears transiently after nestin expression (44). In agreement with previous reports (9, 10), we found that BCs are immunoreactive to vimentin and TUJ1, which are generally considered to be markers for less mature glial and neuronal lineage, respectively. In addition, fewer BCs were labeled for the mature neuronal marker MAP-2. Immunofluorescence studies demonstrated that a fraction of ADK-positive BCs colocalized with vimentin and TUJ1, whereas no colocalization of ADK with MAP-2 was detected. These findings lend further support to the hypothesis that BCs failed to express their cellular commitment during development (45), that is, ADK was expressed in less mature glial cells and neurons with no ADK colocalization with mature neurons.

The detection of antiapoptotic gene products, such as Bcl-2 in BCs and its colocalization with ADK, raises another interesting hypothesis regarding the pathogenesis of BCs. Putative apoptotic cell death occurs within the subependymal zone (46). Bcl-2 protein is associated with neuronal immaturity (47), and recent studies suggest that Bcl-2 may act as a protective signaling molecule against cell death either through an antiapoptotic activity (48) or as a survival-promoting protein (49). The present finding that BCs are located outside the subependymal zone supports the notion that Bcl-2 may facilitate the survival of BCs by suppressing their programmed cell death. Double labeling showed that ADK and Bcl-2 were coexpressed in a subpopulation of BCs; and all the Bcl-2–positive BCs expressed ADK, which indicates that the immature BCs with the antiapoptotic property of Bcl-2 expression also expressed ADK.

FIGURE 11. Nestin immunostaining in balloon cells (BCs) within the brain tissue of focal cortical dysplasia type IIb (FCDIIb) patients. (A) Low-magnification view of a representative region of cortex shows nestin immunostaining in BCs. (B) Magnified view of the lesion shows nestin-positive BCs (arrows) and nestin-negative BCs (arrowheads). Scale bars = (A) 80 μm, (B) 40 μm.
We found no difference in ADK expression between children and adult autopsy control cortex. One possible reason is that the age of the children in the study were more than 3 years. For the developmental and age-related differences, further study is needed on younger children (e.g. 1 month, 6 months, 1 year, 2 years, 3 years, etc.).

In conclusion, we report ADK expression patterns on BCs with heterogeneous and differential profiles of lineage and neurochemical marker proteins in human FCD, providing insight into the developmental and cellular phenotypes of BCs. Increased expression of the major adenosine-removing enzyme ADK in FCDIIB patients likely plays an important role in the epileptogenesis of FCD. Upregulation of ADK is a common pathologic hallmark of FCDIIB, and ADK might be a target in the treatment of epilepsy associated with FCD.

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FIGURE 13. Microtubule-associated protein 2 (MAP-2) immunostaining in balloon cells (BCs) in the brain tissue of focal cortical dysplasia type IIB (FCDIIB) patients. (A) Low-magnification view of a representative region of cortex shows MAP-2 staining in BCs. (B) Magnified view of the lesion showing MAP-2-positive BCs (arrows) and MAP-2-negative BCs (arrowheads). Scale bars = (A) 80 μm; (B) 40 μm.

FIGURE 14. Double labeling for adenosine kinase (ADK) and microtubule-associated protein 2 (MAP-2) in lesions of patients with focal cortical dysplasia type IIB (FCDIIB). (A–H) Colocalization analysis of ADK and MAP-2 demonstrates that ADK and MAP-2 expression in balloon cells (BCs) does not overlap within the lesion area (A–H). (A, E) MAP-2 (green); (B, F) ADK (red); (C, G) DAPI (blue). Scale bar = 40 μm.
FIGURE 15. Bcl-2 immunostaining in balloon cells (BCs) in the brains of focal cortical dysplasia type IIB (FCDIIB) patients. (A) Low-magnification view of a representative region of cortex shows Bcl-2 staining in BCs. (B) Magnified view of the lesion shows Bcl-2–positive BCs (arrows) and Bcl-2–negative BCs (arrowheads). Scale bars = (A) 80 μm; (B) 40 μm.

FIGURE 16. Double labeling for adenosine kinase (ADK) and Bcl-2 in lesion areas in patients with focal cortical dysplasia type IIB (FCDIIB). (A–H) Colocalization analysis of ADK and Bcl-2 demonstrates that there is ADK expression in Bcl-2–positive balloon cells (BCs) (A–D) and also in some Bcl-2–negative BCs (E–H) within the lesion. (A, E) Bcl-2 (green); (B, F) ADK (red); (C, G) DAPI (blue). Scale bar = 40 μm.
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