Inflammation and epilepsy: the contribution of astrocytes

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4.3 Overexpression of ADK in Human Astrocytic Tumors and Peritumoral Tissue Is Related to Tumor-Associated Epilepsy

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\textbf{ABSTRACT}

\textbf{Purpose:} Adenosine kinase (ADK), a largely astrocyte-based metabolic enzyme, regulates adenosine homeostasis in the brain. Overexpression of ADK decreases extracellular adenosine and consequently leads to seizures. We hypothesized that dysfunction in the metabolism of tumor astrocytes is related to changes in ADK expression and that those changes might be associated with the development of epilepsy in patients with tumors.

\textbf{Methods:} We compared ADK expression and cellular distribution in surgically removed tumor tissue (n = 45) and peritumoral cortex (n = 20) of patients with glial and glioneuronal tumors to normal control tissue obtained at autopsy (n = 11). In addition, we compared ADK expression in tumor patients with and without epilepsy. To investigate ADK expression, we used immunohistochemistry and Western blot analysis. ADK activity measurement was performed in surgical specimens of astrocytomas World Health Organization (WHO) grade III (n = 3), peritumoral cortex (n = 3), and nonepileptic cortex (n = 3).

\textbf{Key findings:} Immunohistochemistry predominantly showed cytoplasmic labeling in tumors and peritumoral tissue containing infiltrating tumor cells. ADK immunoreactivity was significantly stronger in tumor and peritumoral tissue compared to normal white matter and normal cortex, especially in astrocytoma WHO grade III, as confirmed by Western blot analysis and ADK activity measurements. Importantly, we found a significantly higher expression of ADK in the peritumoral infiltrated tissue of patients with epilepsy than in patients without epilepsy.

\textbf{Significance:} These results suggest a dysregulation of ADK in astrocytic brain tumors. Moreover, the upregulation of ADK observed in peritumoral infiltrated tissue of glioma patients with epilepsy supports the role of this enzyme in tumor-associated epilepsy.
INTRODUCTION

Patients with primary brain tumors commonly have epileptic seizures. The incidence varies between 30-100% depending on tumor pathology (van Breemen, et al. 2007). Although any brain tumor (including also metastases) can causes seizures, patients with glial tumors (particularly slow-growing, low-grade tumors) are more likely to develop epilepsy (van Breemen, Wilms & Vecht 2007). It remains unclear how various types of brain tumors induce epileptogenesis and several hypothesis have been proposed (Beaumont & Whittle 2000, Rajneesh & Binder 2009, Shamji, et al. 2009b). Besides that the mechanism of epileptogenesis is unknown, the majority of patients are refractory to anti-epileptic drugs (AEDs), on which the therapy of epilepsy largely relies. Identifying factors in the pathway that leads to epilepsy may help find preventive therapies.

A dysfunction of adenosine-mediated neuromodulation has recently been suggested to play a role in the development of epilepsy (i.e. epileptogenesis) (Boison & Stewart 2009). Under physiological conditions, adenosine exerts control over a large range of brain functions, acting as endogenous neuromodulator with mainly inhibitory effects on neuronal activity (Boison 2005, Boison 2007, Boison 2008a). Astrocyte-expressed adenosine kinase (ADK), represents the key metabolic enzyme for the regulation of extracellular adenosine levels in the brain by phosphorylating adenosine to 5’-AMP intracellularly, using ATP as a phosphate donor (Boison 2006, Etherington, et al. 2009). A dynamic regulation of ADK expression has been reported under different pathological conditions. Acute injuries (e.g. status epilepticus, ischemia) can rapidly down-regulate ADK, providing a neuroprotective environment, (Boison 2006, Boison 2008c, Pignataro, et al. 2008). In contrast, upregulation of astrocytic ADK has been observed in different experimental models of chronic epilepsy, as well as in human temporal lobe epilepsy (TLE; (Boison 2008c, de Groot, et al. in press, Fedele, et al. 2005b, Gouder, et al. 2004a, Masino, et al. in press). Overexpression of ADK leads to a decrease of extracellular adenosine levels and consequently to seizures and inhibition of ADK has been proposed as therapeutic strategy in epilepsy (Fedele, Gouder, x00Fcttinger, Gabernet, Scheurer, x00Fct, licke, Crestani & Boison 2005b, Theofilas, et al. 2011).

Tumor cells in astrocytoma share some common features with reactive astrocytes, suggesting the existence of common astrocytic programs after brain injury or disease and during brain tumorigenesis (Silver & Steindler 2009). However, little is known about the role of adenosine and ADK in brain tumors (Boison 2008b). Any dysfunction in the metabolism of astrocytes will affect the metabolism of adenosine (Boison 2010b). In brain tumors cytogenic abnormalities tend to involve chromosomes carrying genes encoding enzymes of adenine metabolism such as ADK (Bardot, et al. 1994). A possible modification of the adenine metabolism by dysfunctional tumor astrocytes could alter the levels of adenosine in tumor
and/or in the peritumoral tissue. Accordingly, the concentrations of adenosine have been shown to differ between the tumor and peritumoral region (Melani, et al. 2003)

In the present study, we report the expression and cellular distribution of ADK in human astrocytic tumors. ADK protein expression has not been previously studied in primary human glial tumors. In order to relate changes in ADK expression to type and progression of the tumor, both high-, as well as low-grade glial tumors have been included in our study. To explore whether ADK plays a role in tumor-associated- epilepsy, we compared the expression of ADK in tumor tissue and peritumoral cortex of patients with glial and glioneuronal tumors with the expression in control cortex and we evaluated the difference between the expression of ADK in tumor patients with epilepsy and without epilepsy.

MATERIALS AND METHODS

Subjects
We examined immunocytochemically 45 surgical specimens of brain tumor from patients with astrocytic tumors (10 WHO grade I pilocytic astrocytoma, 5 WHO grade II astrocytoma; 8 WHO grade III astrocytoma; 12 glioblastoma multiforme, GBM) and 10 patients with glioneuronal tumors (ganglioglioma, GG); Table 1. In 20 patients, a significant amount of peritumoral tissue/cortex (macroscopically normal-appearing cortex/white matter adjacent to the mass lesion, but microscopically containing infiltrating tumor cells in astrocytoma and GBM cases) was resected as well (Table 1). Normal-appearing control cortex/white matter was obtained at autopsy from 11 adult control patients without a history of seizures or other neurologic diseases. All autopsies were performed within 12 h after death. Cortical samples (cortex/white matter adjacent to the lesion with reactive changes, such as astrogliosis, but not tumor cells) of five patients with nonglial brain tumors (three meningiomas, one metastasis of carcinoma, and one lymphoma) and without refractory epilepsy were also analyzed (control cortex/surgical, Table 1). A chart review was conducted of all patients. Epilepsy was defined as the experience of one or more seizures, and data regarding seizure frequency and seizure type were obtained from patient histories. We collected additional data including age, gender, tumor location, and epilepsy duration. Patients with GG subgroup fulfilled criteria of “long-term epilepsy” [long-term epilepsy-associated tumors; LEATs; according to the definition provided by Luyken et al. (2003)] including patients with epilepsy >2 years (mean duration 11.3 years). Patient data and specimens were obtained from the databases of the departments of Neuropathology of the Academic Medical Center (University of Amsterdam; UVA) in Amsterdam and the University Medical Center in Utrecht (UMCU). 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, and the diagnosis was confirmed according to the revised WHO classification of tumors of the central nervous system (Louis et al., 2007).

**Table 1. Clinical and histopathological features**

<table>
<thead>
<tr>
<th></th>
<th>A II</th>
<th>A III</th>
<th>GBM</th>
<th>GG</th>
<th>Control cortex/autopsy</th>
<th>Control Cortex/surgical</th>
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<td>7/4</td>
<td>3/2</td>
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<td>43 (33-56)</td>
<td>56 (37-72)</td>
<td>27 (9-39)</td>
<td>48 (30-72)</td>
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<td>4</td>
<td>6</td>
<td>10</td>
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<td>-</td>
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<td>Duration epilepsy (months)</td>
<td>6 (4-11)</td>
<td>3.5 (1-9)</td>
<td>21.5 (1-120)</td>
<td>135 (24-192)</td>
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<td>-</td>
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<tr>
<td>Seizure free</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>0</td>
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</table>

All data in number of patients (percentages) or as indicated; 1mean (range); A II: Astrocytoma WHO grade II, A III: Astrocytoma WHO grade III, GBM: Glioblastoma multiforme, GG: Ganglioglioma.

**Tissue preparation for immunocytochemistry**

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 5 µm, mounted on organosilane-coated slides (SIGMA, St. Louis, MO) and used for immunohistochemical staining as described below.

**Antibodies**

Antibodies specific for glial fibrillary acidic protein (GFAP; polyclonal rabbit, 1:4,000; DAKO, Glostrup, Denmark; monoclonal mouse, 1:50; DAKO), vimentin (mouse clone V9, 1:1,000; DAKO), neuronal nuclear protein (NeuN; mouse clone MAB377, 1:2,000; Chemicon, Temecula, CA, U.S.A.), synaptophysin (mouse clone Sy38, 1:200; DAKO; rabbit anti-synaptophysin, 1:200; DAKO), CD34 (mouse clone QBEnd10, 1:600; Immunotech, Marseille, Cedex, France), Ki67 (mouse clone MIB-1, 1:200; DAKO), (HLA)-DP, DQ, DR (mouse clone CR3/43, 1:400; DAKO), MAP2 (mouse clone HM2, 1:100; Sigma), and p53 (Clone DO-7 + BP53-12, 1:2,000; Neomarkers, Fremont, CA, U.S.A.) were used in the routine immunohistochemical analysis of glial and glioneuronal tumors. For the detection of ADK, we used a polyclonal rabbit antibody [1:500; (Gouder et al., 2004; Studer et al., 2006; Ren et al., 2007; Aronica et al., 2011)].
**Immunohistochemistry**

For single-label immunohistochemistry, paraffin-embedded sections were deparaffinized, rehydrated, and incubated for 20 min in 0.3% H2O2 diluted in methanol to quench the endogenous peroxidase activity. Antigen retrieval was performed by incubation for 10 min at 121°C in citrate buffer (0.01 m, pH 6.0); sections were washed with phosphate-buffered saline (PBS) and incubated for 30 min in 10% normal goat serum (Harlan Sera-Lab, Loughborough, Leicestershire, United Kingdom). Sections were incubated with the primary antibodies overnight at 4°C. Hereafter, sections were washed in PBS and we used the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3’-diaminobenzidine (DAB; Sigma) as chromogen. Sections were counterstained with hematoxylin, dehydrated, and coverslipped. Sections incubated without the primary antibody were essentially blank.

For double-labeling studies, after incubation of ADK combined with GFAP (or NeuN, Ki67, p53, MAP2) overnight at 4°C, sections were incubated for 2 h at RT with Alexa Fluor® 568-conjugated anti-rabbit IgG and Alexa Fluor 488 anti-mouse IgG (1:100; Molecular Probes, Leiden, The Netherlands). Sections were then analyzed by means of a laser scanning confocal microscope (Leica TCS Sp2; Wetzlar, Germany) equipped with an argon-ion laser.

**Evaluation of immunostaining**

All labeled tissue sections were evaluated by two independent observers, with respect to the presence or absence of various histopathologic parameters and specific immunoreactivity (IR) for the different markers. Semiquantitative evaluation of IR was performed as reported previously (Vandeputte et al., 2002; Ravizza et al., 2006); for more details see Data S1. Because distinction of tumor astrocytes from reactive astrocytes according to only morphology is difficult, immunocytochemistry for NeuN, Ki67, p53, and CD34 (in GG) was performed for the evaluation and characterization of the peritumoral tissue. The peritumoral cortex of GG did not contain Ki67, P53, or CD34 positive cells. The peritumoral cortex of astrocytoma and GBM cases used in the study contained Ki67 and P53 positive cells, indicating tumor infiltration.

**Western blot**

For immunoblot analysis, freshly frozen human histologically normal cortex (n = 6, autopsy material; n = 1, surgical temporal cortex), astrocytomas grade II (n = 5), astrocytomas grade III (n = 5), and GBM (n = 12) samples were homogenized in lysis buffer containing 10 mm Tris (pH 8.0), 150 mm NaCl, 10% glycerol, 1% NP-40, 0.4 mg/ml Na-orthovanadate, 5 mm EDTA (pH 8.0), 5 mm NaF, and protease inhibitor cocktail (Boehringer, Mannheim, Germany).
tein content was determined using the bicinchoninic acid method (Smith et al., 1985); for more details see Data S1.

**ADK enzyme activity**
The ADK enzyme activity was evaluated in three tumor and peritumoral specimens removed from epileptic patients with grade III astrocytoma (gender: male; age: 43, 51, 54 years; location: one frontal, two temporal) and three histologic normal specimens from nonepileptic patients with meningioma (gender: male; age: 53, 39, 58 years; location: two frontal, one temporal). Because we cannot, with absolute certainty, differentiate the tumor from peritumoral cortex (normal or infiltrated) macroscopically, histologic analysis was performed in sections adjacent to the analyzed sections; hematoxylin and eosin (H&E) stain and GFAP, NeuN, Ki67, and p53 immunocytochemistry were used for the evaluation and characterization of the tissue. The tumor specimens included in the analysis contain only tumor astrocytes, and the diagnosis of grade III astrocytoma had been confirmed on additional formalin-fixed paraffin-embedded material. The peritumoral specimens of astrocytoma consist of macroscopically normal-appearing cortex/white matter adjacent to the lesion, but microscopically infiltrated, but not entirely replaced by tumor cells. The peritumoral specimens of meningioma consist of microscopically normal-appearing cortex/white matter adjacent to the lesion. The evaluation of the enzymatic activity for ADK was performed as described previously (Gouder et al., 2004); for more details see Data S1.

**Statistical analysis**
Statistical analyses were performed with SPSS for Windows (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL, U.S.A.) using a two-tailed Student’s t-test; to assess differences between more than two groups, analysis of variance (ANOVA) and a nonparametric Kruskal-Wallis test, followed by the Dunn’s post hoc test, were performed. Correlations between immunostaining and different clinical variables were assessed using the Spearman’s rank correlation test. The value of p < 0.05 was defined as statistically significant.

**RESULTS**

**Patients**
The clinical and histopathologic characteristics of the patients included are shown in Table 1. Of the 45 tumor patients, 26 patients (10 GG, 15 gliomas, and one pilocytic astrocytoma) had epilepsy and of the 20 patients (4 gangliogliomas and 16 gliomas) of whom peritumoral tissue was analyzed, 15 had epilepsy. The majority of the patients with epilepsy had secondary generalized seizures. All patients with epilepsy used antiepileptic drugs (valproic acid,
levetiracetam, phenytoin, carbamazepine, or oxcarbazepine). Of the patients with glioma, 8 patients (53%) had seizures despite maximal tolerated antiepileptic drugs. Patients with ganglioglioma all had seizures despite maximal tolerated doses of antiepileptic drugs.

ADK immunoreactivity
Control tissue
In control white matter, ADK IR was present in sparse glial cells with only a weak staining (Fig. 1A). Similar to white matter, control cortical gray matter displayed a weak astroglial staining (Fig. 1B). The IR score was similar in control cortex from autopsy and surgical samples (Table 2).

Tumor tissue
Thirty-five tumor specimens were studied and the mean IR score for each tumor type is summarized in Table 2. GG showed weak to moderate ADK IR within the tumor area; ADK was detected in the astroglial component of the tumor with a predominant cytoplasmic localization (Fig. 1C). Cytoplasmic and, to a lesser extent, nuclear expression was observed in pilocytic astrocytoma (grade I; Fig. 1D), astrocytomas grade II and grade III (Fig. 1E,F; Table 2), and GBM (Fig. 1H; Table 2). Double labeling confirmed ADK expression in GFAP-positive tumor cells (Fig. 1F, inset b) as well as in tumor cells that express the proliferation marker Ki-67 (Fig. 1F, inset c) and show nuclear accumulation of p53 protein (Fig. 1F, inset d) and MAP2 positivity (Fig. 1F, inset e). The percentage of p53 positive glial cells coexpressing ADK was quantified in three patients with astrocytoma grade III, showing a high percentage of p53 tumor cells with ADK positivity (95 ± 4). Cells of the microglial/macrophages lineage (HLA-DR positive cells) did not display ADK IR (not shown). Astrocytoma grade II showed ADK positive cells (Fig. 1E); however, the amount of positive cells was variable among the specimens, as reflected by the IR score (Table 2). More consistent ADK expression was observed in the astrocytoma grade III (Fig. 1F; Table 2). GBM showed a variable ADK IR; although both nuclear

<table>
<thead>
<tr>
<th>IR</th>
<th>A II (n=5)</th>
<th>A III (n=8)</th>
<th>GBM (n=12)</th>
<th>GG (n=10)</th>
<th>Control/autopsy (n=11)</th>
<th>Peritumoral cortex (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>2.6 ± 0.6 *</td>
<td>4.0 ± 0.5 **</td>
<td>2.2 ± 0.4 *</td>
<td>2.9 ± 0.3 *</td>
<td>1.1 ± 0.1</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.6 ± 0.2</td>
<td>2.8 ± 0.2 *</td>
<td>0.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
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</table>

A II: Astrocytoma WHO grade II, A III: Astrocytoma WHO III, GBM: Glioblastoma multiforme, GG: Ganglioglioma. Values represent the mean ± SEM of the number of samples indicated in parenthesis. * = P< 0.05 (compared to control cortex); ** = P< 0.05: significant difference when compared to control cortex and GBM.
Fig. 1. Expression of ADK immunoreactivity (IR) in glial and glioneuronal tumors. Representative photomicrographs of ADK IR in histologically normal white matter (WM, A) and cortical gray matter (CTX, B), ganglioglioma (C), pilocytic astrocytoma (grade I; PA; D), astrocytoma grade II (A II; E), astrocytoma grade III (A III; F, G), and glioblastoma multiforme (GBM; H–J). Sections were counterstained with hematoxylin. Control white matter and cortex show only a weak ADK IR in sparse cells. ADK IR is clearly detectable in tumor cells in GG, PA, A II, and A III with both nuclear and cytoplasmic IR (arrows and insets in C, E, F). Inset b in (F): merged confocal image, showing ADK (red) expression in GFAP (green) positive tumor cells; inset c in (F): merged confocal image, showing ADK (red) expression in Ki67 (green) positive tumor cells. Inset d in (F): merged confocal image, showing ADK (red) expression in p53 (green) positive tumor cells. Inset e in (F): merged confocal image, showing ADK (red) expression in MAP2 (green) positive tumor cells. Panel (G) shows strong IR in the peritumoral region with tumor infiltration of A III. Inset in (G): merged confocal image, showing ADK (red) expression in p53 (green) positive tumor cells. Variable ADK IR is observed in GBM tumor cells (H–J). Panel (H) shows both nuclear and cytoplasmic ADK IR in GBM (high magnification in inset); however, low IR is also observed in GBM, particularly in the central area of the tumor (I). Panels (J and K) show ADK-positive cells in the peritumoral region with tumor infiltration of a GBM (arrows; inset J and inset a in K); inset b in (K) shows ADK expression in tumor astrocytes (red) surrounding a preexisting neuron (green). Scale bar in A: 40 μm; B–H: 80 μm; I–K: 160 μm.
and cytoplasmic staining was observed in GBM, the central area of the tumor showed often low IR (Fig. 1H,I). The tumor cytoplasmic IR scores were higher in pilocytic astrocytoma, and astrocytoma grade II, III, and GG compared to control cortex (Table 2). Only in astrocytoma grade III the nuclear IR score was higher compared to that of control tissue. ADK cytoplasmic IR score was not significantly different between the different tumor subtypes.

On Western blot, homogenates from astrocytomas grade II and astrocytomas grade III cases displayed a denser band than that observed in control cortex (Fig. 2A,B). Densitometric analysis confirmed the higher expression of ADK in astrocytoma grade III compared to control cortex (p < 0.05) and astrocytoma grade II showed a trend in the same direction (p = 0.0508); ADK expression in homogenates from GBM was not significantly different from that of controls, and ADK expression was not significantly different between individual tumor groups (Fig. 2C).
Peritumoral tissue
The ADK IR score in peritumoral cortex was significantly stronger compared to control cortex (Table 2; p < 0.05). A particularly strong expression was observed in the peritumoral brain tissue, which is infiltrated by tumor cells (Fig. 1G,J,K). Double labeling confirmed ADK expression in tumor cells with nuclear accumulation of p53 protein (inset in Fig. 1G) and surrounding (NeuN positive/ADK negative) preexisting neurons (inset b in Fig. 1K). The percentage of p53-positive glial cells coexpressing ADK was quantified in the peritumoral cortex of three patients with astrocytoma grade III, showing a high percentage of p53 tumor cells with ADK positivity (91 ± 3).

ADK activity
To study the possible relationship between increased ADK IR observed in astrocytic tumors and in peritumoral infiltrated tissue with enhanced adenosine metabolism, we evaluated the enzymatic activity of ADK in homogenates derived from tumor and peritumoral specimens removed from epileptic patients with grade III astrocytoma and three histologically normal specimens from nonepileptic patients. Enzyme activity of ADK was determined by performing an enzyme-coupled bioluminescent assay. Tumor and peritumoral samples from epileptic patients with astrocytoma grade III displayed a significant enhancement of ADK activity compared to the ADK activity detected in nonepileptic cortical control samples from patients with a nonastrocytic tumor (Fig. 2D). Because fresh tumor and peritumoral tissue samples from nonepileptic patients with astrocytoma were not available, we could not establish the relationship between ADK activity and epilepsy with this method.

ADK expression and epilepsy
We compared the expression and distribution of ADK IR in tumor tissue and peritumoral tissue of glial tumors patients (astrocytoma grade III and GBM) with epilepsy and without epilepsy. Because all patients with ganglioglioma had epilepsy, and in our cohort only one patient with supratentorial pilocytic astrocytoma had epilepsy, we could not analyze these groups. No differences were observed within the tumor area between glioma patients with and without epilepsy analyzed (Table 3). In contrast we found a significant higher ADK expression in peritumoral tissue of glioma patients with epilepsy compared to the patients without epilepsy (Table 3; p < 0.05). The number of astrocytomas grade II and III with and without epilepsy was, however, too small to perform meaningful statistical comparisons in subgroups and to assess whether ADK expression is more directly dependent on the presence or absence of seizures or tumor type. Because fresh peritumoral tissue samples from these patients were not available, we could not establish this finding on Western blot or by
determination of ADK activity. Furthermore, no significant correlations were found between ADK IR and other clinical variables such as age at surgery, age at seizure onset, duration of epilepsy, and AED regimens.

**DISCUSSION**

We assessed the cellular distribution and expression of ADK in epilepsy-associated primary glial brain tumors. We detected changes in ADK protein expression and function in astrocytic tumors and peritumoral cortex compared to control tissue. In addition, ADK expression in the peritumoral cortex of glioma patients with epilepsy was significantly higher than in glioma patients without epilepsy.

To our knowledge this is the first study to describe the cellular distribution and expression of ADK in primary brain tumors. A previous study (Melani et al., 2003) evaluated adenosine concentration in the extracellular fluid of tumor and peritumoral tissue of patients with high-grade gliomas by intraoperative microdialysis. In this study the concentration of adenosine has been shown to be significantly reduced in the tumor tissue when compared to the control tissue, suggesting an altered purine metabolism in the tumor area (Melani et al., 2003). The extracellular adenosine levels may reflect differences in ADK expression; accordingly we observed higher ADK expression in tumors compared to control, noninfiltrated cortex. The variable expression levels observed within glial tumors may reflect differences in intratumoral vascular perfusion and hypoxia gradients, and indeed hypoxia has been shown to downregulate the expression of ADK in astroglial cells (Boison, 2008b; Pignataro et al., 2008). Interestingly, increased ADK expression and activity (compared to control cortex) was detected at the margin of the tumor and in the invasion front.

Immunocytochemical analysis showed ADK expression in tumor astrocytes with both nuclear and cytoplasmic labeling; however, expression was predominant in the cytoplasm. ADK exists in two isoforms: ADK-long and ADK-short isoforms (Cui et al., 2009). It has been demonstrated that ADK-long is mainly localized in the nucleus and has an essential role in methylation reactions, being possibly involved in epigenetic controlling mechanisms. ADK-short, on the other hand, is cytoplasmically localized and regulates the extracellular adenosine concentrations (Boison, 2007; Cui et al., 2009). Therefore, the latter one is believed to be more involved in the regulation of neuronal excitability. Accordingly, several studies demonstrated that overexpression of ADK in mice resulted in a decrease in the adenosinergic tone and subsequently increased seizure activity (Fedele et al., 2005; Pignataro et al., 2007; Li et al., 2008a,b). Theofilas et al. (2011) showed that overexpression of the cytoplasmic ADK-short isoform alone is sufficient to evoke seizures. Furthermore, both experimental and human studies indicate that dysregulation of ADK is a common mechanism, given that it is
operative in several forms of epilepsy (Aronica et al., 2011).

The dysregulation of ADK in astrocytic brain tumors together with the upregulation of ADK observed in peritumoral infiltrated tissue of glioma patients with epilepsy supports the role of this enzyme in tumor-associated epilepsy. Importantly, significantly higher expression of ADK was detected in peritumoral tissue of glioma patients with epilepsy than in the peritumoral tissue of patients without epilepsy. Colocalization with tumor markers (such as p53) support the expression in tumor astrocytes; however, since ADK upregulation has been detected in reactive astrocytes (Aronica et al., 2011), we cannot exclude the contribution of a reactive glial cell population to the increase expression/activity observed within the peritumoral cortex.

The peritumoral region has been shown to be relevant for the generation and propagation of seizure activity (van Breemen et al., 2007). The epileptogenicity of the peritumoral zone is supported by both functional and immunocytochemical studies, showing network alterations and revealing cytoarchitectural and neurochemical changes in the cortex resected from patients with intractable epilepsy associated with different types of glial tumors (van Breemen et al., 2007; Shamji et al., 2009). The observed changes in ADK expression may additionally contribute to the epileptogenicity of this region, supporting a surgical approach that should aim to maximize simultaneous resection of both the tumor and (if possible) the peritumoral epileptic focus.

No significant correlation was found between ADK IR and duration of epilepsy in our cohort; however, because our study does not focus on long-term epilepsy-associated tumors (LEATs; Luyken et al., 2003) future investigations on a large cohort of LEATs are necessary to address the relationship between ADK expression and/or activity and duration and/or severity of epilepsy. Additional analysis of large series of tumors that could be stratified on the basis of the presence and absence of chronic epilepsy is also essential to further assess the value of ADK expression/activity as biomarker of epileptogenicity.

A key question is whether the increased ADK protein expression leads to an increase in enzymatic activity. Bardot et al. (1994) evaluated purine metabolic enzyme activities and found no differences in enzyme activity of ADK between low- and high-grade tumors and tissue taken far from the tumor tissue in human patients. However, in this study the low- and high-grade tumors studied included both astrocytomas and oligodendrogliomas; a histologic characterization of the control tissue was not provided. As discussed above, variable levels of ADK expression were observed within the tumor and particularly in GBM. However, higher levels of ADK activity could be detected in astrocytoma grade III and in peritumoral cortex compared to control tissue. Future studies are required to further understand whether the different expression levels observed in GBM reflect only differences in hypoxia gradients.
within the tumor, or may be associated with different glioma cell phenotypes. We acknowledge limitations to the interpretation of these results, since we analyzed the ADK activity in a small cohort of patients and we could not establish the relationship between ADK activity and epilepsy, because fresh tumor and peritumoral tissue samples from nonepileptic patients with astrocytoma were not available. Moreover, the expression patterns and regulation of adenosine receptors (A1, A2A, A2B, and A3) in both tumor and peritumoral areas deserve further investigation. In addition to epileptogenesis, ADK might also play a role in tumor growth and apoptotic cell death in astrocytoma, regulating proliferation of glial and endothelial cells, as well as the antitumor immune response through activation of receptors expressed in both astroglial and microglial cells (Abbracchio et al., 1997; Synowitz et al., 2006; Dehnhardt et al., 2007; Gessi et al., 2010, 2011). Interestingly, increased ADK expression based on quantitative real-time polymerase chain reaction (PCR) data was also found in human cancer samples outside the brain, such as in colorectal cancer (Giglioni et al., 2008). It was further demonstrated that extracellular adenosine reduced the viability of cultured astrocytoma cells (Sai et al., 2006), suggesting that overexpression of ADK might be a strategy of tumor cells to improve survival capabilities.

In conclusion, this study provides information on the cellular distribution and expression of ADK in primary brain tumors, suggesting a dysregulation of ADK in astrocytic brain tumors, as well as a potential involvement in the epileptogenicity of these tumors. Further understanding of the role of adenosine dysfunction and ADK in tumor-associated epilepsies requires the development of suitable animal models displaying both the clinical manifestations and neurochemical changes similar to those observed in human cerebral tumors. Because inhibition of ADK has proven to be an effective therapy for epilepsy in different animal models (Boison, 2010), the use of appropriate experimental models of tumor-associated epileptogenesis is essential to evaluate the possible use of adenosine augmentation therapies in patients with brain tumors and epilepsy.

Consequently, adenosine-augmenting therapeutic strategies might combine antiproliferative effects with the well-known anticonvulsant effects of adenosine. **Acknowledgements**

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We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.
REFERENCES


