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Overexpression of ADK in human astrocytic tumors and peritumoral tissue is related to tumor-associated epilepsy

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Abstract

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 tumor patients.

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). Additionally, we compared ADK expression in tumor patients with and without epilepsy. In order to investigate ADK expression, we used immunohistochemistry and western blot analysis. ADK activity measurement was performed in surgical specimens of astrocytomas WHO grade III (n=3), peritumoral cortex (n=3) and non epileptic cortex (n=3).

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.

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.

Keywords

adenosine kinase; human; brain tumors; astrocytoma; peritumoral cortex; epilepsy

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Disclosure
None of the authors has any conflict of interest to disclose.

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 metastasis) can causes seizures, patients with glial tumors (particularly slow-growing, low-grade tumors) are more likely to develop epilepsy (van Breemen et al., 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., 2009). 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, 2008b, 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; (Gouder et al., 2004, Fedele et al., 2005, Boison, 2008b, Aronica et al., 2011, 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 et al., 2005, 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, 2008). Any dysfunction in the metabolism of astrocytes will affect the metabolism of adenosine (Boison, 2010). 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 patients with astrocytic tumors (10 WHO grade I pilocytic astrocytoma, 5 WHO grade II astrocytoma; 8 WHO grade III astrocytoma; 12 glioblastoma multiforme, GBM, 1) 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 neurological diseases. All autopsies were performed within 12 hours after death. Cortical samples (cortex/white matter adjacent to the lesion with reactive changes, such as astrogliosis, but not tumor cells) of 5 patients with non-glial brain tumors (2 meningiomas, 1 metastasis of carcinoma and 1 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). 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).

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, DAKO, Glostrup, Denmark; 1:4000; monoclonal mouse; DAKO; 1:50), 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; rabbit anti-synaptophysin; DAKO; 1:200), CD34 (mouse clone QBEnd10; Immunotech, Marseille, Cedex, France, 1:600) Ki67 (mouse clone MIB-1; DAKO; 1:200), (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark, 1:400), MAP2 (mouse clone HM2; Sigma 1:100) and p53 (Clone DO-7 + BP53-12; Neomarkers; 1:2000), 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, re-hydrated, and incubated for 20 min in 0.3% H_2O_2 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, UK). 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 haematoxylin, 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 2h at RT with Alexa Fluor® 568-conjugated anti-rabbit IgG and Alexa Fluor® 488 anti-mouse IgG (1:100, Molecular Probes, 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 histopathological parameters and specific immunoreactivity (IR) for the different markers. Semi-quantitative evaluation of IR was performed as previously reported (Vandeputte et al., 2002, Ravizza et al., 2006); for more details see supplement.

Since, distinction of tumor astrocytes from reactive astrocytes by only morphological aspects is difficult, immunocitochemistry 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 as well as CD34 positive cells. The peritumoral cortex of astrocytoma and GBM cases used in the study contained Ki67, 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-orthevanadate, 5 mM EDTA (pH 8.0), 5mM NaF and protease inhibitor cocktail (Boehringer Mannheim, Germany). Protein content was determined using the bicinchoninic acid method (Smith, et al. 1985); for more details see supplement.

ADK enzyme activity

The ADK enzyme activity was evaluated in 3 tumor and peritumoral specimens removed from epileptic patients with grade III astrocytoma (gender: m; age: 43, 51, 54 yrs; location: 1 frontal, 2 temporal) and 3 histological normal specimens from non epileptic patients with meningioma (gender: m; age: 53, 39, 58 yrs; location: 2 frontal, 1 temporal). Since macroscopically we cannot, with absolute certainty, differentiate the tumor from peritumoral cortex (normal or infiltrated) histological analysis was performed in sections adjacent to the analyzed sections; hematoxylin and eosin (HE) stain and GFAP, NeuN and Ki67 and p53 immunocitochemistry 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 has 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 previously described (Gouder et al., 2004); for more details see supplement.

Statistical analysis

Statistical analyses were performed with SPSS for Windows (SPSS 15.0 for Windows, SPSS Inc., Chicago, IL, USA) using two-tailed Student's t-test; to assess differences between more than two groups, ANOVA and a non-parametric 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 histopathological characteristics of the patients included are shown in table 1. Of the 45 tumor patients, 26 patients (10 GG, 15 gliomas and 1 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 anti-epileptic drugs (valproic acid, levetiracetam, phenytoin, carbamazepine or oxcarbazepine). Of the patients with glioma, 8 (53%) patients had seizures despite maximal tolerated anti-epileptic drugs. Patients with ganglioglioma all had seizures despite maximal tolerated doses of anti-epileptic 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 grey 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—35 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 co-expressing ADK was quantified in 3 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 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, astrocytoma grade II, III and GG compared to control cortex (table 2). Only in astrocytoma grade III the nuclear IR score was higher compare to 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 observed in control cortex (Fig. 2A, B). Densitometric analysis confirmed the higher expression of ADK in astrocytoma grade III compared to control cortex ($p< 0.05$), astrocytoma grade II showed a trend in the same direction ($p=0.0508$); ADK expression in homogenates from GBM was not significantly different from controls; 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 particular 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) pre-existing neurons (inset b in Fig. 1K). The percentage of p53 positive glial cells co-expressing ADK was quantified in the peritumoral cortex of 3 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 3 histological normal specimens from non epileptic patients.

Enzyme activity of ADK was determined by performing an enzyme-coupled bioluminescent assay. Tumor and periumoral samples from epileptic patients with atrocytoma grade III displayed a significant enhancement of ADK activity compared to the ADK activity detected in non-epileptic cortical control samples from patients with a non-astrocytic tumor (Fig. 2D). Since fresh tumor and peritumoral tissue samples from non-epileptic 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. Since 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 assess whether ADK expression is more directly dependent on presence or absence of seizures or tumor type. Since 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. Additionally, 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 tumour 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, non-infiltrated, cortex. The variable expression levels observed within glial tumors may reflect differences in intra-tumoral vascular perfusion and hypoxia gradients and indeed hypoxia has been shown to down-regulate 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 over-expression 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, Li et al., 2008b). Theofilas et al. (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 being 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. Colocalisation with tumor markers (such as p53) support the expression in tumor astrocytes, however since ADK up-regulation 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 (Shamji, et

al. 2009, van Breemen et al., 2007). 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 tumour and (if possible) the peritumoral epileptic focus.

No significant correlation was found between ADK IR and duration of epilepsy in our cohort, however since 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 which 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 and colleagues (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 atrocytomas and oligogendrogliomas, and a histological characterization of the control tissue was not provided. The findings in the present study are not necessary in conflict with those observations. 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 only reflect 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 non-epileptic patients with astrocytoma were not available. Moreover, the expression patterns and regulation of adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) in both tumor and peritumoral areas deserves further investigation. Besides 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 trough 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, Gessi et al., 2011). Interestingly, increased ADK expression based on quantitative real-time 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. Since Inhibiting 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 anticonvulsive effects of adenosine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Abbracchio MP, Rainaldi G, Giammarioli AM, Ceruti S, Brambilla R, Cattabeni F, Barbieri D, Franceschi C, Jacobson KA, Malorni W. The A3 adenosine receptor mediates cell spreading, reorganization of actin cytoskeleton, and distribution of Bcl-XL: studies in human astroglioma cells. Biochem Biophys Res Commun. 1997; 241:297–304. [PubMed: 9425266]

Aronica E, Zurolo E, Iyer A, de Groot M, Anink J, Carbonell C, van Vliet EA, Baayen JC, Boison D, Gorter JA. Upregulation of adenosine kinase in astrocytes in experimental and human temporal lobe epilepsy. Epilepsia. 2011

Bardot V, Dutrillaux AM, Delattre JY, Vega F, Poisson M, Dutrillaux B, Luccioni C. Purine and Pyrimidine Metabolism in Human Gliomas - Relation to Chromosomal-Aberrations. British Journal of Cancer. 1994; 70:212–218. [PubMed: 8054268]

Beaumont A, Whittle IR. The pathogenesis of tumour associated epilepsy. Acta Neurochir. 2000; 142:1–15.

Boison D. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. Neuroscientist. 2005; 11:25–36. [PubMed: 15632276]

Boison D. Adenosine kinase, epilepsy and stroke: mechanisms and therapies. Trends Pharmacol Sci. 2006; 27:652–658. [PubMed: 17056128]

Boison D. Adenosine as a modulator of brain activity. Drug News Perspect. 2007; 20:607–611. [PubMed: 18301794]

Boison D. Adenosine as a neuromodulator in neurological diseases. Curr Opin Pharmacol. 2008a; 8:2–7. [PubMed: 17942368]

Boison D. The adenosine kinase hypothesis of epileptogenesis. Prog Neurobiol. 2008b; 84:249–262. [PubMed: 18249058]

Boison D, Stewart KA. Therapeutic epilepsy research: from pharmacological rationale to focal adenosine augmentation. Biochem Pharmacol. 2009; 78:1428–1437. [PubMed: 19682439]

Boison D. Adenosine dysfunction and adenosine kinase in epileptogenesis. Open Neurosci J. 2010; 4:93–101. [PubMed: 20730044]

Cui XA, Singh B, Park J, Gupta RS. Subcellular localization of adenosine kinase in mammalian cells: The long isoform of AdK is localized in the nucleus. Biochem Biophys Res Commun. 2009; 388:46–50. [PubMed: 19635462]

Dehnhardt M, Palm C, Vieten A, Bauer A, Pietrzyk U. Quantifying the A1AR distribution in peritumoural zones around experimental F98 and C6 rat brain tumours. J Neurooncol. 2007; 85:49–63. [PubMed: 17497078]

Etherington LA, Patterson GE, Meechan L, Boison D, Irving AJ, Dale N, Frenguelli BG. Astrocytic adenosine kinase regulates basal synaptic adenosine levels and seizure activity but not activity-dependent adenosine release in the hippocampus. Neuropharmacology. 2009; 56:429–437. [PubMed: 18957298]

Fedele DE, Gouder N, Guttinger M, Gabernet L, Scheurer L, Rulicke T, Crestani F, Boison D. Astrogliosis in epilepsy leads to overexpression of adenosine kinase, resulting in seizure aggravation. Brain. 2005; 128:2383–2395. [PubMed: 15930047]

Gessi S, Sacchetto V, Fogli E, Merighi S, Varani K, Baraldi PG, Tabrizi MA, Leung E, Maclennan S, Borea PA. Modulation of metalloproteinase-9 in U87MG glioblastoma cells by A3 adenosine receptors. Biochem Pharmacol. 2010; 79:1483–1495. [PubMed: 20096265]

Gessi S, Merighi S, Sacchetto V, Simioni C, Borea PA. Adenosine receptors and cancer. Biochim Biophys Acta. 2011; 1808:1400–1412. [PubMed: 20888788]

Giglioni S, Leoncini R, Aceto E, Chessa A, Civitelli S, Bernini A, Tanzini G, Carraro F, Pucci A, Vannoni D. Adenosine kinase gene expression in human colorectal cancer. Nucleosides Nucleotides Nucleic Acids. 2008; 27:750–754. [PubMed: 18600536]

Gouder N, Scheurer L, Fritschy JM, Boison D. Overexpression of adenosine kinase in epileptic hippocampus contributes to epileptogenesis. J Neurosci. 2004; 24:692–701. [PubMed: 14736855]

Li T, Lan JQ, Boison D. Uncoupling of astrogliosis from epileptogenesis in adenosine kinase (ADK) transgenic mice. Neuron GLIA Biology. 2008a; 4:91–99. [PubMed: 19674507]

Li T, Ren G, Lusardi T, Wilz A, Lan JQ, Iwasato T, Itohara S, Simon RP, Boison D. Adenosine kinase is a target for the prediction and prevention of epileptogenesis in mice. J Clin Invest. 2008b; 118:571–582. [PubMed: 18172552]

Louis, DN.; Ohgaki, H.; Wiestler, OD.; Cavanee, WK. WHO Classification of Tumours of the Central Nervous System. IARC; Lyon: 2007.

Luyken C, Blumcke I, Fimmers R, Urbach H, Elger CE, Wiestler OD, Schramm J. The spectrum of long-term epilepsy-associated tumors: long-term seizure and tumor outcome and neurosurgical aspects. Epilepsia. 2003; 44:822–30. [PubMed: 12790896]

Masino SA, Li T, Theofilas P, Sandau US, Ruskin DN, Fredholm BB, Geiger JD, Aronica E, Boison D. A ketogenic diet suppresses seizures in mice through adenosine A1 receptors. J Clin Invest. 2011; 121:2679–2683. [PubMed: 21701065]

Melani A, De Micheli E, Pinna G, Alfieri A, Della Corte L, Pedata F. Adenosine extracellular levels in human brain gliomas: an intraoperative microdialysis study. Neurosci Lett. 2003; 346:93–96. [PubMed: 12850556]

Pignataro G, Simon RP, Boison D. Transgenic overexpression of adenosine kinase aggravates cell death in ischemia. J Cereb Blood Flow Metab. 2007; 27:1–5. [PubMed: 16685255]

Pignataro G, Maysami S, Studer FE, Wilz A, Simon RP, Boison D. Downregulation of hippocampal adenosine kinase after focal ischemia as potential endogenous neuroprotective mechanism. J Cereb Blood Flow Metab. 2008; 28:17–23. [PubMed: 17457365]

Rajneesh KF, Binder DK. Tumor-associated epilepsy. Neurosurgical Focus. 2009; 27:E4. [PubMed: 19645560]

Ravizza T, Boer K, Redeker S, Spliet WG, van Rijen PC, Troost D, Vezzani A, Aronica E. The IL-1beta system in epilepsy-associated malformations of cortical development. Neurobiol Dis. 2006; 24:128–143. [PubMed: 16860990]

Ren G, Li T, Lan JQ, Wilz A, Simon RP, Boison D. Lentiviral RNAi-induced downregulation of adenosine kinase in human mesenchymal stem cell grafts: a novel perspective for seizure control. Exp Neurol. 2007; 208:26–37. [PubMed: 17716659]

Sai K, Yang D, Yamamoto H, Fujikawa H, Yamamoto S, Nagata T, Saito M, Yamamura T, Nishizaki T. A(1) adenosine receptor signal and AMPK involving caspase-9/-3 activation are responsible for adenosine-induced RCR-1 astrocytoma cell death. Neurotoxicology. 2006; 27:458–467. [PubMed: 16469385]

Shamji MF, Fric-Shamji EC, Benoit BG. Brain tumors and epilepsy: pathophysiology of peritumoral changes. Neurosurg Rev. 2009; 32:275–284. discussion 284–276. [PubMed: 19205766]

Silver DJ, Steindler DA. Common astrocytic programs during brain development, injury and cancer. Trends Neurosci. 2009; 32:303–311. [PubMed: 19398132]

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985; 150:76–85. [PubMed: 3843705]

Studer FE, Fedele DE, Marowsky A, Schwerdel C, Wernli K, Vogt K, Fritschy JM, Boison D. Shift of adenosine kinase expression from neurons to astrocytes during postnatal development suggests dual functionality of the enzyme. Neuroscience. 2006; 142:125–137. [PubMed: 16859834]

Synowitz M, Glass R, Farber K, Markovic D, Kronenberg G, Herrmann K, Schnermann J, Nolte C, van Rooijen N, Kiwit J, Kettenmann H. A1 adenosine receptors in microglia control glioblastoma-host interaction. Cancer Res. 2006; 66:8550–8557. [PubMed: 16951167]

Theofilas P, Brar S, Stewart KA, Shen HY, Sandau US, Poulsen D, Boison D. Adenosine kinase as a target for therapeutic antisense strategies in epilepsy. Epilepsia. 2011; 52:589–601. [PubMed: 21275977]

van Breemen MS, Wilms EB, Vecht CJ. Epilepsy in patients with brain tumours: epidemiology, mechanisms, and management. Lancet Neurol. 2007; 6:421–430. [PubMed: 17434097]

Vandeputte DA, Troost D, Leenstra S, Ijlst-Keizers H, Ramkema M, Bosch DA, Baas F, Das NK, Aronica E. Expression and distribution of id helix-loop-helix proteins in human astrocytic tumors. Glia. 2002; 38:329–338. [PubMed: 12007145]

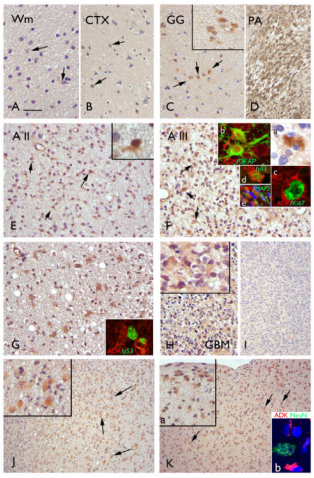

Fig. 1.

Expression of ADK immunoreactivity (IR) in glial and glioneuronal tumors. Representative photomicrographs of ADK IR in histologically normal white matter (WM, A) and cortical grey 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 in **J** and inset a in **K**); inset b in **K** shows ADK expression in tumor astrocytes (red) surrounding a pre-existing neuron (green). Scale bar in A: A, 40 μm; B–H: 80 μm; I–K: 160μm.

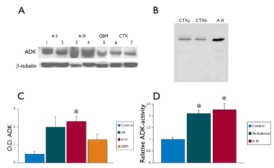

Fig. 2. Western blot analysis and ADK enzyme activity in astrocytic tumors

A: Western blot analysis: representative immunoblot of ADK (~ 40 kDa) in total homogenates of tumor specimens (astrocytoma grade II, A II, lanes 1–2 ; astrocytoma grade III, A III, lanes 3–4; glioblastoma multiforme, GBM, lane 5) and control cortex (CTX; lanes 6–7). **B: representative immunoblot of ADK in total homogenates of control (CTXa, control autopsy specimen; CTXb, control surgical specimen) and A III specimens.**

C: densitometric analysis of ADK; values (optical density units; O.D.) are mean ± SEM of 6 control cortex, 5 AII, 5 AIII and 12 GBM (*p<0.05 compare to control). **ADK expression was not significantly different between individual tumor groups.**

D: ADK enzyme activity measured using an enzyme-coupled bioluminescent assay. The results were normalized to the ADK activity found in control non-elipeptic samples and expressed as mean ± SEM of AIII (n= 3), control cortex (n= 3) and peritumoral cortex (n= 3); (*p<0.05 compare to control).

Table 1

Clinical and histopathological features

	PA (n = 10)	A II (n= 5)	A III (n= 8)	GBM (n= 12)	GG (n= 10)	Control cortex/autopsy (n= 11)	Control Cortex/surgical (n= 5)
Gender (m/f)	6/4	4/1	5/3	11/1	7/3	7/4	3/2
Age Yrs [1]	16 (6–42)	34 (22–43)	43 (33–56)	56 (37–72)	27 (9–39)	48 (30–72)	54 (43–75)
Location						-	
Frontal	1	5[a]	2[a]	2[a]	-	4	2
Parietal	2	-	1	2[a]	-	1	-
Temporal	-	-	-	4[a]	10	5	2
Occipital	-	-	1	1[a]	-	1	-
Thalamus	1	-	-	1[a]	-	-	-
Parieto-occipital	-	-	1	1[a]	-	-	-
Temporo-parietal	-	-	2[a]	-	-	-	1
Parieto-occipital	1	-	-	-	-	-	-
Fronto-temporal	-	-	1[a]	1[a]	-	-	-
Cerebellum	5	-	-	-	-	-	-
Peritumoral tissue/glial tumors	-	**5**[*]	**3**[*]	**6**	**4**	-	-
Epilepsy	1	5	4	6	10	-	-
Duration epilepsy(months) [1]	60	6 (4–11)	3.5 (1–9)	21.5 (1–120)	135 (24–192)		

All data in number of patients (percentages) or as indicated

[1] mean (range); PA: Pilocytic Astrocytoma (WHO grade I); A II: Astrocytoma WHO grade II; A III: Astrocytoma WHO grade III; GBM: Glioblastoma multiforme; GG: Ganglioglioma. All specimens were used for immunocytochemistry

[*] additional specimens of peritumoral tissue from one patient with astrocytoma grade II (gender: m; age: 47 yrs; location: frontal; seizure free) and astrocytoma grade III (gender: m; age: 35 yrs; location: temporo-parietal; seizure free) have been included in the immunocytochemical evaluation of ADK

[a] specimens used for western blot analysis. Additional samples were used in the evaluation of ADK enzyme activity (see methods).

Table 2

ADK immunoreactivity in glial and glioneuronal tumors

	PA (10)	A II (n= 5)	A III (n= 8)	GBM (n=12)	GG (n= 10)	Control cortex/autopsy (n=11)	Control cortex/surgical (n-5)	Peritumoral cortex glial tumors (n=20)
IR Cytoplasma	2 (2–6) *	2 (2–6) *	4 (2–6) **	2 (1–6)	3 (2–4) *	1 (1–2)	1 (1–2)	4 (1–9)*
IR Nucleus	1 (0–1)	1 (0–1)	2 (1–6) *	1 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–2)

PA: Pilocytic Astrocytoma (WHO grade I); A II: Astrocytoma WHO grade II; A III: Astrocytoma WHO III; GBM: Glioblastoma multiforme; GG: Ganglioglioma. Values represent the median immunoreactive score (range).

* P< 0.05 (compared to control cortex, both autopsy and surgical samples).

Table 3

ADK immunoreactivity in patients with and without epilepsy

Immunoreactivity (IR)	Glial tumors (n = 25)		Peritumoral cortex glial tumors (n = 20)	
	With epilepsy (n= 15)	Without epilepsy (n= 10)	With epilepsy (n= 15)	Without epilepsy (n= 5)
IR Cytoplasma	3 (1–6)	2 (1–4)	4 (1–9)*	2 (1–2)
IR Nucleus	1 (0–6)	1 (0–2)	0 (0–2)	0 (0–1)

Values represent the median immunoreactive score (range).

* P <0.05: significant difference in ADK expression in peritumoral cortex of glial tumors in patients with and without epilepsy.