S-Adenosylhomocysteine Hydrolase Overexpression in HEK-293 Cells: Effect on Intracellular Adenosine Levels, Cell Viability, and DNA Methylation

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Key Words

S-Adenosylhomocysteine hydrolase • Overexpression
• Adenosine • Apoptosis • DNA methylation

Abstract

Background/Aims: S-Adenosylhomocysteine hydrolase (AdoHcyase) catalyzes the reversible hydrolysis of S-adenosylhomocysteine (AdoHcy), which is a potent product inhibitor of S-adenosylmethionine (AdoMet)-dependent methyltransferases. While previous studies have shown that AdoHcyase inhibition or deficiency lead to a decreased AdoMet/AdoHcy ratio resulting in impaired transmethylation, the effect of enhanced AdoHcyase activity on AdoMet/AdoHcy metabolism and methylation reactions has not been studied in detail. **Methods:** To investigate the effect of enhanced AdoHcyase activity, we generated HEK-293 cell lines stably overexpressing AdoHcyase. **Results:** Initial studies revealed that 2-10-fold AdoHcyase overexpression resulted in decreased intracellular AdoHcy and elevated adenosine levels, whereas 16-fold AdoHcyase overexpression increased adenosine and AdoHcy levels, lowered energy charge, and altered cell morphology. Furthermore, we found a correlation

between AdoHcyase activity and cell viability. Caspase-activity assays and DNA fragmentation analysis revealed that the cell death in AdoHcyase overexpressing cells was due to apoptosis. Global DNA methylation was not altered in the different AdoHcyase overexpressing cell lines. **Conclusion:** Taken together, these data show that 2-5-fold enhanced AdoHcyase activity is well tolerated by the cell, while greatly enhanced AdoHcyase activity results in adenosine-induced apoptosis. The fact that enhanced AdoHcyase activity does not increase transmethylation activity suggests that AdoHcyase activity under physiological conditions is not rate limiting for efficient transmethylation.

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Introduction

S-Adenosylhomocysteine hydrolase (AdoHcyase, EC 3.3.1.1) is the only enzyme known in vertebrates that catalyses the hydrolysis of S-adenosylhomocysteine (AdoHcy), which is formed as a product of S-adenosylmethionine (AdoMet)-dependent transmethylation reactions [1]. Because most methyltransferases bind AdoHcy with higher affinity than AdoMet, they are

subject to potent product inhibition by AdoHcy [2] and the ratio AdoMet/AdoHcy is a metabolic indicator for cellular methylation status. Thus, fast removal of AdoHcy is prerequisite for efficient transmethylation reactions [3-5] and the efficiency of transmethylation might profit from a close proximity between the methyltransferase and AdoHcyase. Immunohistology revealed that AdoHcyase is highly concentrated in the nuclei of transcriptionally active cells to maintain efficient mRNA cap methylation [6, 7].

Under normal physiological conditions, AdoHcy is hydrolyzed to adenosine and homocysteine. Since the reaction is reversible and the equilibrium of the reaction favors AdoHcy synthesis, efficient removal of both, adenosine and homocysteine, is prerequisite to maintain the flux in the direction of hydrolysis [8]. Adenosine is efficiently metabolized by adenosine kinase to AMP or by adenosine deaminase (ADA) to inosine. Elevated adenosine levels lead to the induction of apoptosis [9-11] and have been observed in areas where cell damage has occurred, e.g. as a result of ischemia or stroke, and in the interstitial fluid of solid tumors [12, 13]. Homocysteine can be re-methylated to methionine by methionine synthase and by betaine-homocysteine methyltransferase or is transsulfurated to cystathionine by cystathionine β -synthase [14]. Elevated homocysteine levels result in lymphocyte DNA hypomethylation [5] and are an independent risk factor for cardio-vascular disease and Alzheimer's disease [15]. Metabolic changes, that interfere with the efficient removal of adenosine and homocysteine levels lead to an increase in AdoHcy and potent inhibition of methyltransferases [3, 16]. Thereby elevated AdoHcy levels modify gene expression and might increase the methylation of L-arginine to asymmetric dimethylarginine, which appear to play a role in atherogenesis. Thus, elevated AdoHcy levels may be a more important risk factor for cardiovascular disease than homocysteine [17].

The fact that chromosomal deletion that includes the gene encoding AdoHcyase causes embryo lethality in mice shows the biological importance of this enzyme for mammalian survival [18]. Human AdoHcyase deficiency leads to severe biochemical abnormalities (plasma AdoHcy, AdoMet, and methionine levels are elevated) resulting in psychomotor delay, severe myopathy, and demyelination [19]. In contrast, induction of AdoHcyase expression was observed to precede Epstein-Barr virus lytic gene activation in lymphoma cells [20]. The impact of high or elevated AdoHcyase expression and activity on cellular function has not been investigated in detail

until now. Therefore, the aim of the present study was to evaluate whether elevated AdoHcyase activity might also lead to changes in AdoHcy metabolism and thus, influence energy metabolism, cell viability, growth rate, and DNA methylation. As a model system, we used HEK-293 cells stably overexpressing AdoHcyase to different extents, because HEK-293 cells exhibit high intracellular AdoHcy levels and a low AdoMet/AdoHcy ratio compared to other established cell lines [21].

Our results demonstrate that AdoHcyase overexpression results in elevated adenosine levels and decreased cell viability. Furthermore, AdoHcyase overexpressing cells showed different features typical for apoptosis (cell detachment, caspase-like activity, DNA fragmentation), suggesting that cell death is due to apoptosis. The observation that administration of adenosine induced apoptotic features in sham-transfected HEK cells suggests that elevated intracellular adenosine levels play a crucial role in inducing apoptosis.

Materials and Methods

Materials

HEK-293 cells (human embryonic kidney 293 cells) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, Germany). HFF cells (human foreskin fibroblasts) were kindly provided by Prof. Dr. B. Schittek (Department of Dermatology, University of Tuebingen, Germany). The media and reagents used for cell culture and transfection were provided by Invitrogen (Karlsruhe, Germany). AdoMet, AdoHcy, adenosine, N⁶-methyladenosine, nucleoside phosphorylase, ammonium phosphate, and caspase substrates were obtained from Sigma (Taufkirchen, Germany). ADA and xanthine oxidase were purchased from Roche (Mannheim, Germany), AMP, ADP, and ATP from Boehringer Mannheim (Germany), and heptanesulfonic acid sodium salt from Fluka (Buchs SG, Switzerland). HPLC grade methanol and acetonitrile were from Merck (Darmstadt, Germany). The recombinant plasmid pPROKcd20 [22], which contains an *EcoRI* DNA fragment encoding the recombinant human placental AdoHcyase inserted into the bacterial expression plasmid pPROK-1 (Clontech, Palo Alto, CA) was kindly provided to our laboratory by Dr. Michael Hershfield (Duke University, Durham, NC).

Construction of AdoHcyase overproducing plasmid (pcDNAcd20)

To generate HEK-293 clones with high-level stable expression of human placental AdoHcyase the *EcoRI* fragment encoding AdoHcyase from pPROKcd20 (cd20) was subcloned into the mammalian expression vector pcDNA 3.1 (+). Presence and proper orientation of AdoHcyase in the plasmid was initially screened by restriction endonuclease mapping and later

confirmed by DNA sequencing. Prior to transfection, pcDNAc20 was linearized using the restriction enzyme *ScaI*, since linearizing the plasmid will decrease the likelihood of the vector integrating into the genome in a way that disrupts AdoHcyase. HEK-293 cells sham-transfected with the expression vector pcDNA 3.1 (pcDNA cells) served as control cells.

Cell culture and transfection

HEK-293 cells were cultured in minimum essential medium (MEM) containing glutamine supplemented with 10% heat-inactivated newborn calf serum (NCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. HFF cells were cultured in DMEM containing 10% heat-inactivated fetal calf serum and gentamycin (20 µg/ml). Cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C and subcultured at a split ratio of 1:3-1:4 every 3-4 days. Two days before transfection, 3 million cells were plated in culture dishes (diameter 9.4 cm) in 10 ml MEM supplemented with NCS, so that the cells were 90-95% confluent at the time of transfection. Transfection was performed according to the manufacturer's instructions. Complexes were prepared by adding 19 µg plasmid DNA with 48 µl Lipofectamine 2000 to give a DNA (µg) to Lipofectamine (µl) ratio of 1:2.5. Isolation of stable cell lines was achieved by passaging the cells at a 1:10 dilution into fresh growth medium 24 h after transfection and addition of selective medium (+ Geneticin 700 µg/ml) 48 h after transfection. Cells were fed with selective medium until Geneticin-resistant foci were identified. Colonies were picked and expanded in 24-well plates, to identify AdoHcyase overexpressing clones.

Prior to all other experiments cells were rinsed with PBS and then cultured with serumfree MEM for 24 h. This allows us to compare our results to previous studies [3, 10, 21].

Metabolite measurement

AdoMet, AdoHcy, adenosine, AMP, ADP, and ATP levels in cultured HEK-293 cells were measured in perchloric acid cell extracts. Cells were suspended by rinsing with culture medium and centrifuged for 10 min at 200xg. The pelleted cells were washed with PBS (200xg, 2 min) and subsequently lysed by addition of 1.2 ml pre-cooled 0.6 M perchloric acid. The protein precipitate was removed by centrifugation (10 min, 20,000xg). Metabolite concentration in the supernatant was determined via HPLC as described previously [21]. Cell number was determined with a hemocytometer, because the metabolite concentration was expressed as nanomoles per 1·10⁷ cells.

Determination of cell viability

Cell number and viability of detached and adherent cells was monitored using a cell analyzer system (CASY® Model TT, Schärfe System, Reutlingen, Germany). In order to define cursor positions specific for HEK-293 cells, calibration was performed using dead and vital cells. Cursor positions were set to 10.25 to 50.00 µm (evaluation cursor) and 6.00 to 50.00 µm (normalization cursor). Culture medium (containing detached cells) was collected and aliquots of 400 µl were diluted in 10 ml CASY®-ton. All samples were run in duplicate.

RNA isolation and reverse transcription

RNA was isolated with Tri-Reagent/Trizol. Total cellular RNA was reverse transcribed by incubating a 20 µl reaction mixture composed of 300 ng of RNA as template, 1x NH₄-reaction buffer for PANScript Polymerase (PAN, Aidenbach, Germany), 1 mM dNTPs (Eppendorf, Hamburg, Germany), 0.25 µg random hexamers (Promega, Mannheim, Germany), 15 U RNase inhibitor (Eppendorf, Hamburg, Germany), and 12.5 U AMV reverse transcriptase (PeqLab, Erlangen, Germany) at 20°C for 10 min and 42°C for 1 h. The reaction was stopped by incubation at 95°C for 5 min.

LightCycler-Analysis

For LightCycler reaction a master mix of the following reaction components was prepared to the indicated end-concentration: 12.6 µl H₂O, 2.4 µl MgCl₂ (4 mM), 0.5 µl forward primer (0.5 µM), 0.5 µl reverse primer (0.5 µM), and 2.0 µl Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). 2 µl cDNA were added to 18 µl LightCycler master mix as PCR template. The following primers were used: *AdoHcyase forward*: GGT ATC GGT TGA AGA ATG G, *reverse*: GGT ACT TGT CTG GAT GGG TC, *18S rRNA forward*: CGG CTA CCA CAT CCA AGG AA, *reverse*: GCT GGA ATT ACC GCG GCT. The LightCycler experimental run protocol was performed as described previously [3]. Relative AdoHcyase mRNA expression was calculated according to a mathematical model established by Pfaffl [23] using 18S rRNA as non-regulated reference gene.

Preparation of total protein

HEK cells were harvested by rinsing with medium, HFF cells were harvested by trypsinization and then collected by centrifugation at 200xg for 10 min. The pelleted cells were washed with PBS and homogenized in potassium phosphate buffer (50 mM, pH 7.0) by sonication. The homogenate was centrifuged at 20,000xg for 15 min and supernatant was processed for immunoblot analysis and photometrical determination of enzyme activity as described below.

Protein quantification

The protein concentration was determined according to the method of Bradford using bovine serum albumin (BSA) as the standard.

Immunoblotting

Protein expression of AdoHcyase in transfected HEK-293 cells was detected by immunoblotting as described previously [21]. Briefly, total cell lysates were separated by 12% SDS-polyacrylamid gel electrophoresis (PAGE). After electrophoresis the proteins were electroblotted onto a nitrocellulose membrane. The membrane was blocked with 5% defatted milk powder in PBS overnight, then washed 3 times with Tween/PBS. The first antibody (rabbit anti-bovine AdoHcyase; [24]) was carried out for 3.5 h in PBS+0.5% BSA at a dilution of 1:500. Subsequently, the membrane was washed and the second antibody (goat-anti-rabbit coupled to horseradish peroxidase, Dako) reaction was performed for

1.5 h in PBS+0.5% BSA at a dilution of 1:1,500. The membrane was washed and the staining was performed using 4-chloro-1-naphthol, H₂O₂ and ortho-toluidine. When color appeared on the membrane, the reaction was stopped by washing with water. We confirmed equal loading of protein in parallel experiments using a cyclophilin A antibody (upstate #07-313) at a dilution 1:2,000.

Enzymatic activity of AdoHcyase

The AdoHcyase activity in crude cell extracts was measured photometrically in a total volume of 500 µl in 50 mM potassium phosphate buffer (pH 7.0) at room temperature. The reaction mixture contained total cell lysate (whole protein 150-400 µg), ADA (1 U), nucleoside phosphorylase (0.09 U), and xanthine oxidase (0.08 U). Therefore, adenosine generated by AdoHcy hydrolysis is processed to uric acid, which can be measured at 292 nm. The reaction was started by the addition of 50 µM AdoHcy.

Enzymatic activity of ADA

The ADA activity of total cell lysates was determined photometrically in a total volume of 500 µl in 50 mM potassium phosphate buffer (pH 7.0) at room temperature. The reaction mixture contained cell lysate (whole protein 100-200 µg), 0.09 U nucleoside phosphorylase, and 0.08 U xanthine oxidase. Inosine generated by ADA is processed to uric acid, which can be measured at 292 nm. The reaction was started by addition of 50 µM adenosine.

Fluorometric assay of caspase-like activity

Culture medium (containing detached cells) was collected and adherent cells were suspended in medium. Adherent and detached cells were collected by centrifugation at 200xg for 10 min and suspended in 1 ml of PBS buffer. An aliquot of 500 µl was then withdrawn for determination of DNA laddering. The remaining 500 µl were centrifuged again (500xg, 2 min) and the cell pellet was suspended in a buffer containing 20 mM HEPES (pH 7.4), 84 mM KCl, 10 mM MgCl₂, 0.2 mM EDTA, 0.2 mM EGTA, 0.5% Tergitol (Type NP-40), 1 mM DTT, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 5 µg/ml aprotinin [25]. Cells were lysed by freezing (-20°C) and thawing and the lysate was clarified by centrifugation for 15 min at 4°C and 20,000xg. Caspase activity was determined by incubation of 50 µM of the fluorogenic substrate Ac-DEHD-AMC (caspase 2), Ac-DEVD-AMC (caspase 3), Ac-VEHD-AMC (caspase 6), Ac-LETD-AFC (caspase 8), or Ac-LEHD-AFC (caspase 9) as described previously [10]. To verify the involvement of caspases we used the universal caspase inhibitor z-VAD-fmk at a final concentration of 20 µM.

Assessment of DNA laddering

The cells were prepared as described under "Fluorometric assay of caspase-like activity". Subsequently, cells were washed with PBS and the cell pellet was lysed in 300 µl lysis buffer (0.5% Triton X-100, 5 mM Tris, 20 mM EDTA, pH 7.5) on ice for 20 min. After centrifugation (20,000xg, 15 min, 4°C), the supernatant containing the cytoplasmic DNA was extracted with phenol/chloroform. DNA was precipitated by addition of

0.1 M of sodium acetate (3 M, pH 5.5) and 2.5 volumes of absolute ethanol. The precipitated DNA was pelleted by centrifugation (20,000xg, 30 min, 4°C) and washed with 70% ethanol. The dried pellet was suspended in 10 µl 10 mM Tris/1 mM EDTA (pH 8) and incubated with RNaseA (30 min, 37°C) and proteinase K (60 min, 37°C). The isolated DNA fragments were separated in a 1.2% agarose gel.

Determination of global DNA methylation

Methylation patterns in global DNA were determined using cytosine extension assay as described previously [3, 26]. Briefly, genomic DNA (2 µg) was digested with 20 units *Hpa*II (Fermentas) for 15 h. A second DNA aliquot was incubated without enzyme and served as background control. For cytosine extension assay 0.5 µg DNA were incubated with 1 x NH₄-reaction buffer for PANScripT Polymerase, 1 mM MgCl₂, 0.5 units PANScripT DNA Polymerase (PAN), and 0.1 µl [³H]dCTP (40-60 Ci/mmol, NEN Life Science Products) in a total volume of 25 µl for 1 h at 56°C. Duplicate 10 µl aliquots from each reaction were filtered through Whatman DE-81 ion exchange filters and filters were washed 3 times with 3 ml 0.5 M sodium phosphate buffer (pH 7.0). Radioactivity incorporated in DNA and adsorbed on the filters was determined by liquid scintillation counting.

Calculation and statistics

The energy charge of the adenylate system was calculated according to Atkinson and Walton [27], defined as

$$\frac{[\text{ATP}] + 0.5 \cdot [\text{ADP}]}{[\text{AMP}] + [\text{ADP}] + [\text{ATP}]}$$

Data were analyzed by Student's t-test if variability is the same in each group, because t-test assumes that standard deviations of two datasets are equal. If variances of two datasets are significantly different, we used Welch's alternate t-test, because this test does not assume equal variances (InStat). A difference between groups was considered to be significant when p-value was <0.05.

Results

Identification of AdoHcyase overexpressing clones

To generate AdoHcyase overexpressing cells, we stably transfected HEK-293 cells with the mammalian expression vector pcDNAcd20 (cd20 cells) [22]. AdoHcyase activity assay was used to identify those clones overexpressing AdoHcyase. No difference in AdoHcyase activity was seen between non-transfected HEK-293 cells and HEK-293 cells sham-transfected with the expression vector pcDNA 3.1 (pcDNA cells). Although HEK-293 cells are immortalized cells, AdoHcyase activity in this cell line is comparable to

Table 1. AdoHcyase activity in HEK-293 cells, sham-transfected HEK-293 cells (pcDNA), and cells transfected with an expression vector encoding human AdoHcyase (cd20). Data represent averages $\pm$ SD for n experiments. Each sample was measured in duplicate. * $p < 0.05$ versus pcDNA 1 control cells.

Cell line	n	mU/mg protein	Cell line	n	mU/mg protein
HEK-293	11	0.89 ± 0.26	cd20 1	3	$8.86 \pm 1.37^*$
pcDNA 1	3	0.81 ± 0.03	cd20 2	1	10.49
pcDNA 2	1	0.92	cd20 3	3	$5.69 \pm 1.33^*$
pcDNA 3	1	1.02	cd20 4	3	$4.03 \pm 1.13^*$
pcDNA 4	1	0.98	cd20 5	3	$1.82 \pm 0.18^*$
pcDNA 5	1	1.16	cd20 6	3	$13.31 \pm 0.69^*$
pcDNA 6	1	1.00	cd20 7	4	$8.74 \pm 2.78^*$
pcDNA 7	1	1.02	cd20 8	1	8.64
pcDNA 8	1	0.73	cd20 9	1	6.10
pcDNA 9	1	0.65	cd20 10	1	3.09
pcDNA 10	1	0.72	cd20 11	1	7.77
			cd20 12	1	10.38

Fig. 1. AdoHcyase mRNA expression (A) and protein expression (B) in HEK-293 (lane 1), pcDNA control cells (lane 2) and five different cd20 clones (lane 3: cd20 1, 4: cd20 3, 5: cd20 4, 6: cd20 5, 7: cd20 6), overexpressing AdoHcy to different extents. Changes in relative mRNA expression were determined by real-time RT-PCR. Data are mean $\pm$ SD for 4 experiments. * $p < 0.05$ versus pcDNA control cells. For the western blot analysis, total cellular protein was separated by SDS-PAGE and electroblotted onto nitrocellulose membrane. AdoHcyase was detected immunologically by a specific antibody. Equal protein loading was confirmed using an antibody to cyclophilin A.

enzymatic activity in various cancer cell cells (MCF-7, HeLa, SK-HEP-1) [21] and also to those in less modified cells like HFF (0.56 ± 0.07 mU/mg protein, $n=5$). Transfection of HEK-293 cells with the mammalian expression vector pcDNAcd20 (cd20 cells), encoding the recombinant human placental AdoHcyase, clearly showed that AdoHcyase activity was enhanced in the isolated clones to different extents (Table 1). To determine whether increased AdoHcyase activity might result in changes in AdoMet/AdoHcy metabolism, cell viability, and DNA methylation in an enzyme activity-dependent manner, we selected five clones, cd20 1, cd20 3, cd20 4, cd20 5, and cd20 6, overexpressing AdoHcyase to different extents. Highest intracellular AdoHcyase activity of 13.31 mU/mg protein was found in cd20 6 cells. In this cell line AdoHcyase activity was increased 16-fold compared to pcDNA control cells (0.81 mU/mg protein). The cell lines cd20 1- cd20 5 exhibited an enzyme activity between 1.82 (cd20 5) and 8.86 mU/mg protein (cd20 1). LightCycler and western blot analysis revealed that the mRNA and protein expression patterns were to a large extent coherent with the activity measurements (Fig. 1).

Cell morphology of different AdoHcyase overexpressing cell lines

We next examined the consequences of elevated AdoHcyase activity on cell morphology of pcDNA and the different cd20 cell lines using phase-contrast microscopy. pcDNA cells and cd20 1- cd20 5 cells showed a vital appearance and fibroblastoid morphology

Fig. 2. Effect of AdoHcyase overexpression on cell morphology. All cell lines were cultured in MEM supplemented with NCS and Geneticin. Normal growth was observed in pcDNA control cells and AdoHcyase overexpressing cell lines cd20 1- cd20 5 using phase-contrast microscopy. In contrast, cd20 6 cells did not form coherent monolayers and showed a very slow growth rate.

typical for HEK-293 cells, growing as coherent monolayers with 70 to 90% confluence (Fig. 2). In contrast, cd20 6 cells grew in a totally different manner forming monolayers with only 50-60% confluence. At higher cell density, cd20 6 cells completely detached from the culture dishes. Notably, we observed that cd20 6 cells were only viable in the presence of culture medium supplemented with 20% NCS and exhibited a markedly prolonged doubling time (~ 1 week versus 24-48 h of pcDNA control cells).

Changes in AdoMet, AdoHcy, and adenosine metabolism in AdoHcyase overexpressing cells

In a set of experiments we explored the possibility that elevated AdoHcyase activity might lead to changes in AdoMet, AdoHcy, and adenosine metabolism, because AdoHcyase is the only enzyme known in vertebrates that catalyzes the hydrolysis of AdoHcy to adenosine and homocysteine. Therefore, we determined intracellular metabolite concentration in the different cell lines *via*

HPLC. Consistent with our previous studies in non-transfected HEK-293 cells [21], sham-transfected pcDNA cells showed an AdoMet/AdoHcy ratio of 9.40 and an intracellular adenosine level of 0.051 nmol/10⁷ cells (Table 2). While AdoHcyase overexpression slightly but significantly decreased AdoHcy levels in cd20 1- cd20 5 cells, AdoHcy concentration was 2.8-fold increased in cd20 6 cells although this cell line showed enormous AdoHcyase overexpression. Therefore, AdoMet/AdoHcy ratio was slightly elevated in cd20 1- cd20 5 cell lines, whereas it was decreased in cd20 6 cells. The different cd20 cell lines exhibited elevated adenosine levels with lowest adenosine levels in cd20 5 cells (0.071 nmol/10⁷ cells) and highest levels in cd20 6 cells (0.813 nmol/10⁷ cells).

ADA activity in the different cell lines

Having shown that AdoHcyase overexpression results in elevated adenosine levels, we sought to determine the ADA activity in the different cell lines to

Table 2. Intracellular AdoMet, AdoHcy, and adenosine concentration in different cell lines. The different cell lines were incubated in serumfree medium for 24 h. Thereafter, cells were harvested and extracted with perchloric acid. The metabolites were separated by HPLC as described in Materials and methods. Data are mean $\pm$ SEM for n determinations. * $p < 0.05$ versus pcDNA cells.

Cell line	n	AdoMet nmol/ 10^7 cells	AdoHcy nmol/ 10^7 cells	adenosine nmol/ 10^7 cells	AdoMet/AdoHcy
pcDNA	10	1.73 $\pm$ 0.17	0.184 $\pm$ 0.010	0.051 $\pm$ 0.006	9.40 $\pm$ 0.75
cd20 1	7	1.78 $\pm$ 0.15	0.124 $\pm$ 0.008*	0.191 $\pm$ 0.015*	14.59 $\pm$ 1.29*
cd20 3	6	1.92 $\pm$ 0.14	0.142 $\pm$ 0.017*	0.184 $\pm$ 0.021*	13.98 $\pm$ 0.96*
cd20 4	6	1.54 $\pm$ 0.11	0.116 $\pm$ 0.009*	0.088 $\pm$ 0.009*	13.60 $\pm$ 1.13*
cd20 5	6	1.72 $\pm$ 0.17	0.149 $\pm$ 0.010*	0.071 $\pm$ 0.007	11.81 $\pm$ 1.42
cd20 6	5	2.37 $\pm$ 0.37	0.510 $\pm$ 0.110*	0.813 $\pm$ 0.068*	4.88 $\pm$ 0.28*

Table 3. Endogenous adenine nucleotide levels and energy charge (EC) in the different cell lines. All cell lines were cultured in serumfree medium for 24 h. Thereafter, cells were harvested and extracted with perchloric acid. Intracellular adenine nucleotides were analyzed by HPLC as described in Materials and methods. Data are mean $\pm$ SEM for n determinations. * $p < 0.05$ versus pcDNA cells.

Cell line	n	AMP nmol/ 10^7 cells	ADP nmol/ 10^7 cells	ATP nmol/ 10^7 cells	EC
pcDNA	9	8.03 $\pm$ 0.69	23.57 $\pm$ 1.52	45.28 $\pm$ 4.54	0.74 $\pm$ 0.01
cd20 1	7	6.15 $\pm$ 0.86	19.74 $\pm$ 0.60	49.78 $\pm$ 4.62	0.78 $\pm$ 0.02
cd20 3	6	8.68 $\pm$ 2.08	22.69 $\pm$ 3.10	47.46 $\pm$ 3.14	0.75 $\pm$ 0.03
cd20 4	6	5.30 $\pm$ 0.56*	18.34 $\pm$ 1.19*	40.72 $\pm$ 2.37	0.77 $\pm$ 0.01
cd20 5	6	7.68 $\pm$ 0.67	21.30 $\pm$ 1.21	37.65 $\pm$ 3.41	0.72 $\pm$ 0.02
cd20 6	4	17.15 $\pm$ 0.72*	37.24 $\pm$ 4.38*	65.33 $\pm$ 11.99	0.68 $\pm$ 0.03*

Fig. 3. ADA activity in HEK-293, pcDNA control cells, and cd20 cell lines. Enzyme activity was determined photometrically in total cell lysates as described in Materials and methods. Data represent averages $\pm$ SD for 3-7 experiments. Each sample was measured in duplicate. p-values are given versus pcDNA control cells.

exclude the possibility that transfection also causes alterations in ADA activity. As shown in Fig. 3 ADA activity was ~ 6 mU/mg protein in HEK-293 and sham-transfected pcDNA cells and was unchanged in cd20 1, cd20 4, cd20 5 cell lines and only slightly decreased in cd20 3 cells. In contrast, in cd20 6 cells ADA activity was lowered by $\sim 50\%$.

Effect of AdoHcyase overexpression on energy metabolism

To further explore possible alterations in adenosine metabolism, we measured intracellular AMP, ADP, and ATP concentration. HPLC analysis showed comparable AMP, ADP, and ATP levels and thus, comparable energy charges in pcDNA cells and cd20 1- cd20 5 cells (Table 3). In contrast, cd20 6 cells exhibited significantly higher AMP and ADP levels (Table 3), resulting in a decreased energy charge (0.68 versus 0.74 (pcDNA)).

AdoHcyase overexpression induces cell death

Based on the above findings of elevated adenosine concentrations in cell lines with elevated AdoHcyase activity and on previous studies showing induction of apoptosis by adenosine, we hypothesized that increases in intracellular adenosine might result in cell death. For this purpose, we first determined the number of dead cells in the supernatant of the different cell lines. As shown in Fig. 4, number of detached dead cells in the supernatant was unchanged in cd20 4 and 5 cells, ~ 2 -fold higher in cd20 1 and cd20 3 cell lines, and ~ 3.6 -fold higher in cd20 6 cells compared with pcDNA cells. Thus, cell counting revealed an obvious correlation among cell viability, AdoHcyase activity ($r = 0.92$, $p < 0.01$; Fig. 5B), and

Fig. 4. Cell viability in the different cell lines. The total number of dead cells in the supernatant was quantified by CASY® cell counter + analyzer. Therefore, culture medium (containing detached cells) was collected and two aliquots of 400 µl were diluted in 10 ml CASY®ton. Data represent averages ± SD for 3 independent experiments. * $p < 0.01$ versus pcDNA control cells.

intracellular adenosine levels ($r = 0.94$, $p < 0.01$; Fig. 5A). Moreover, these data confirmed the results obtained by microscopic investigation, that enormous AdoHcyase overexpression in cd20 6 cells results in decreased cell viability.

Implication of caspases in AdoHcyase overexpression-induced cell death

After having shown that AdoHcyase overexpression is associated with the decrease of cell viability, we examined whether the cell death is necrosis or apoptosis. In order to analyze different apoptotic parameters we chose cd20 1 and cd20 6 cells because these cell lines (i) exhibit highest AdoHcyase expression and activity and (ii) show significantly reduced viability. Since, caspases have been implicated as the principal mediators of apoptosis, our first attempt was to assess caspase-like activity in AdoHcyase overexpressing cells. As shown in Fig. 6, DEHDase (caspase 2-like), DEVDase (caspase 3-like), LETDase (caspase 8-like), and LEHDase (caspase 9-like) activity were significantly higher in AdoHcyase overexpressing cd20 1 and cd20 6 cells than in pcDNA control cells. In contrast, VEHDase (caspase 6-like) activity was very low in pcDNA, cd20 1, and cd20 6 cells, suggesting that this enzyme does not play a

Fig. 5. Correlation between dead cells in supernatant and adenosine levels (A) and AdoHcyase activity (B).

significant role in this process. Since previous studies have shown that adenosine induces apoptosis in several cell types [9, 10], we hypothesized that induction of caspase-like activity in AdoHcyase overexpressing cells might be the result of increased intracellular adenosine levels. For that purpose, we also determined the effect of extracellular adenosine on caspase-like activity in pcDNA cells. In fact, treatment of pcDNA cells with adenosine provided a degree of DEHDase, DEVDase, LETDase, and LEHDase activation similar to cd20 1 cells (Fig. 6). As shown in Fig. 6, z-VAD-fmk (a cell permeable broad caspase inhibitor), markedly inhibited adenosine-stimulated caspase activation.

Fig. 6. Changes in DEHDase (caspase 2-like, A), DEVDase (caspase 3-like, B), VEHDase (caspase 6-like, C), LETDase (caspase 8-like, D), and LEHDase (caspase 9-like, E) activity by AdoHcyase overexpression in cd20 1 and cd20 6 cells or incubation of pcDNA control cells with adenosine or adenosine plus pan-caspase inhibitor z-VAD-fmk. The enzyme activity was determined by cleavage of the specific substrates and release of the fluorogenic AMC or AFC. Data represent mean $\pm$ SEM of 5-15 independent experiments; * p <0.01 versus pcDNA control cells, § p <0.01 versus pcDNA+adenosine.

AdoHcyase overexpression induces DNA fragmentation

As another feature of apoptosis, DNA fragmentation was analyzed (Fig. 7). DNA gel electrophoresis shows that AdoHcyase overexpression in cd20 1 (lane 5) and cd20 6 cells (lane 7) caused internucleosomal DNA fragmentation, producing a DNA ladder characteristic of

apoptosis. Incubation of pcDNA control cells with adenosine 1 mM or adenosine 1 mM plus pentostatin 1 μ M also induced DNA fragmentation (lanes 2 and 4). This result is consistent with our hypothesis that AdoHcyase overexpression-induced cell death is due to apoptosis which is probably caused by increased intracellular adenosine levels.

Fig. 7. Agarose gel electrophoresis of DNA extracted from sham-transfected HEK-293 cells (pcDNA control cells, lanes 1, 3, and 6), pcDNA cells incubated with 1 mM adenosine (2), pcDNA cells incubated with 1 mM adenosine plus pentostatin 1 μ M (4), cd20 1 cells (5), and cd20 6 cells (7). Low-molecular-weight DNA was extracted and separated on a 1.2% agarose gel containing ethidium bromide.

Influence of an altered AdoHcyase activity on global DNA methylation

The level of DNA methylation is determined as the extent of [3 H]dCTP incorporation into DNA after treatment with the methyl-sensitive restriction enzyme, *HpaII*, that cuts DNA leaving a guanine overhang at unmethylated recognition sites [26]. Thus, the amount of radioactivity incorporated into the DNA reflects the number of unmethylated cytosine residues. As shown in Fig. 8, long-term AdoHcyase overexpression did not alter global DNA methylation in cd20 1- cd20 5 cells. Although, cd20 6 cells showed slightly decreased [3 H]dCTP incorporation compared to pcDNA cells, these changes were not quite significant ($p = 0.088$).

Discussion

AdoHcyase plays a pivotal role in regulating cellular levels of AdoHcy. The cellular consequences of high or elevated AdoHcyase activity have not been investigated in detail previously. Stably transfected HEK-293 cells with the mammalian expression vector pcDNAcd20 (cd20 cells) showed increased AdoHcyase expression and activity in cd20 cells compared to non-transfected HEK-293 cells and sham-transfected HEK-293 cells (pcDNA cells). LightCycler and western blot analysis revealed that AdoHcyase activity positively correlates with AdoHcyase expression level in the different isolated clones. Since, during the creation of stable cell lines, the vector has to integrate into the genome, the extent of AdoHcyase overexpression and thus, activity strongly depends on the

Fig. 8. Comparison of the global DNA methylation in pcDNA control cells and cd20 cell lines. DNA methylation was determined using cytosine extension assay as described in Materials and methods.

number of integrated vector fragments (encoding AdoHcyase). This observation is consistent with previous results showing that AdoHcyase activity positively correlates with AdoHcyase mRNA levels [3, 21, 28]. Moreover, the isolation of several clones with different AdoHcyase activities is a valuable tool to study if AdoHcy metabolism is altered in an enzyme-activity dependent

manner. We selected five clones, which showed an increase in AdoHcyase activity between 2-fold (cd20 5) and 16-fold (cd20 6) to study AdoHcy metabolism, cell morphology, cell viability, and DNA methylation.

AdoHcyase overexpression readily explains the decreased AdoHcy concentrations in cd20 1-5 clones. Since the AdoMet levels are unchanged, the ratio AdoMet/AdoHcy is increased in cd20 1-5 cell lines, suggesting a higher rate of transmethylation. For instance, increased AdoHcyase activity elevates N-acetyl-S-farnesyl-L-cysteine methylation in whole cell lysates [29]. Increased AdoHcy levels in cd20 6 cells might be the result of enormously elevated adenosine levels in this cell line because adenosine is a product inhibitor of AdoHcyase [30].

The elevation of adenosine levels could be due to increased AdoHcy hydrolysis. Since ADA activity and AMP levels were unchanged in cd20 1- cd20 5 cells it is unlikely that increased adenosine levels are the result of impaired ADA or adenosine kinase activity. Since the measurement of adenosine in perchloric acid cell extract does not differentiate between protein-bound and free adenosine [31], another explanation for the increased adenosine levels is that adenosine is released from the adenosine-binding enzyme AdoHcyase during preparation for HPLC analysis because AdoHcyase binds adenosine with a maximal capacity of 2 mol adenosine per mol enzyme [32]. Conceptually, increases in AdoHcyase protein levels due to AdoHcyase overexpression result in an increased adenosine-binding capacity and thus, in increased adenosine levels within the cell. We are aware of the fact that adenosine metabolism is complex and besides AdoHcyase, AMP dephosphorylation by cytosolic 5'-nucleotidase and transmembraneous adenosine transport may provide adenosine. Furthermore, the adenosine produced is further metabolized by adenosine kinase and deaminase. Thus, decreased ADA activity might further enhance intracellular adenosine level in cd20 6 cells. Since cd20 6 cells exhibit an impaired growth rate, we speculate that in this cell line AdoHcyase overexpression results in impaired protein synthesis rate which might cause decreased ADA activity. This hypothesis is supported by the observation that AdoHcyase protein expression is stimulated to a lower extent than AdoHcyase mRNA expression in cd20 6 cell line (Fig. 1A). Interestingly, cyclophilin A protein expression also seems to be slightly reduced in this cell line (Fig. 1B). Although, we cannot completely exclude that decreased ADA activity in cd20 6 cells is due to pcDNA integration at an unfavorable position, the

observation that transient AdoHcyase overexpression also increased intracellular adenosine levels to 0.196 nmol/10⁷ cells, suggests that artifacts concerning the stably transfected cd20 cell lines are rather unlikely. Interestingly, cd20 6 cell line also exhibits elevated AMP and ADP levels. Since the ATP level is slightly but not significantly higher than pcDNA control, elevated AMP and ADP levels are rather due to rephosphorylation of adenosine than the result of ATP degradation. However, we cannot exclude the possibility that increased AMP levels result in elevated adenosine levels by AMP dephosphorylation. Elevated AMP and ADP levels lead to a significantly impaired energy charge of 0.68 in this cell line, whereas all other cell lines exhibited an energy charge between 0.7 and 0.8 which is within the physiological range obtained by *in vivo* experiments in different human and rat tissues and cell cultures [13, 21, 33]. In general, cells with energy charges greater than 0.7 to 0.8 divide and synthesize protein at near-maximal rates [34], suggesting that the lower energy charge in cd20 6 cells might lead to decreased growth rate. The greatly reduced growth rate of this cell line might also be a result of changes in AdoHcy and adenosine metabolism, because elevated AdoHcy levels decrease transmethylation activity [3-5] and high adenosine levels are known to induce cell death in several cell types [9-11]. Changes in cell morphology might be attributable to increased adenosine and AdoHcy levels, because an increase in the concentration of both metabolites as a result of ADA or AdoHcyase deficiency has previously been associated with histological changes in muscle, liver, and small lymphoid organs [19, 35].

We hypothesized that elevated adenosine levels might induce cell death and analyzed different features typical for apoptosis (cell detachment, caspase-like activity, DNA fragmentation) in AdoHcyase overexpressing cell lines. In fact, counting the number of dead cells in the supernatant revealed an obvious correlation between cell number, AdoHcyase activity (Fig. 5B), and intracellular adenosine concentration (Fig. 5A). Furthermore, 2-5-fold elevated AdoHcyase activity (cd20 4 and cd20 5 cell lines) did not decrease cell viability whereas, a 7-16-fold increased AdoHcyase activity in cd20 1, cd20 3, and cd20 6 cells was associated with significantly reduced cell viability. In view of these findings and the fact that transient expression studies have demonstrated that 6-fold increased AdoHcyase activity is associated with slightly elevated number of dead cells in supernatant (data not shown), it seems likely that up to 5-fold increases in AdoHcyase activity are compatible with physiological cell function.

In addition, our results demonstrate that the cell death is due to apoptosis, because induction of caspase-like activity and DNA fragmentation was observed in cd20 1 and cd20 6 cells. The fact that administration of adenosine induced apoptotic features in sham-transfected HEK cells (pcDNA control cells) is consistent with our hypothesis that elevated intracellular adenosine levels play a crucial role in inducing apoptosis. The observation that incubation with adenosine for 24 h only slightly induced DNA fragmentation is not amazing since adenosine is rapidly metabolized by ADA in cell culture [36]. Therefore, DNA laddering can be enhanced by co-incubation with pentostatin, an inhibitor of ADA (Fig. 7).

Determining the specific caspase activity pattern, we can narrow down the spectrum of signal transduction pathways. The specific enhancement of caspase 8-like activity suggests the involvement of the “death receptor pathway” which proceeds from death receptors through caspase 8 to caspase 3 [37]. Although caspase 9-like activity is also induced, we suppose that this caspase is probably not involved here, because enzyme activity even in AdoHcyase overexpressing cells and adenosine-treated pcDNA cells is lower than in other cell types under control conditions [10]. Enhanced caspase 2-like activity might result in the permeabilization of mitochondria, resulting in the release of mitochondrial pro-apoptotic factors [38]. Among the “effector” caspases, we determined caspase 3-like and caspase 6-like activity. While caspase 6-like activity was not increased in cd20 1 cells and was only slightly increased in cd20 6 cells and after stimulation with adenosine, caspase 3-like activity was enhanced in cd20 1, cd20 6 cells, and adenosine-treated pcDNA cells which is consistent with previous studies, demonstrating DEVDase activation after adenosine administration [10]. However, the details of the mechanisms by which AdoHcyase overexpression and adenosine target caspases and induce apoptosis in HEK-293 cells are intriguing and await additional exploration. Previous studies have shown that adenosine initiates apoptosis through different mechanisms: (i) activation of adenosine receptors, (ii) intracellular conversion to AMP and stimulation of AMP-activated kinase, (iii) conversion to AdoHcy and subsequent inhibition of AdoMet-dependent methyltransferases [9-11].

Since AdoHcy is a potent product inhibitor of most, if not all, AdoMet-dependent methyltransferases, elevated AdoHcyase activity might also play a role in the regulation of methyltransferase activity. For instance, elevated AdoHcy levels result in DNA hypomethylation *in vitro*

and *in vivo* [5, 39] suggesting that decreases in intracellular AdoHcy levels due to AdoHcyase overexpression might lead to increased methyltransferase activity and DNA hypermethylation. However, AdoHcyase overexpression did not significantly alter global DNA methylation. Since *de novo* methylation is a slow and inefficient process [40], a delay might be required for a build up of *de novo* methylation events. However, the use of cell lines stably overexpressing AdoHcyase, which were cultivated for several weeks before DNA methylation status was determined, allowed us to exclude this possibility. One explanation could be that the AdoHcy concentration is much lower than the K_i for AdoHcy of DNA methyltransferases even under control conditions (pcDNA cells) such that, further decreased AdoHcy levels do not stimulate DNA methyltransferases. Another explanation could be that decreased AdoHcy levels increase DNA methyltransferase activity to an extent, which is not sufficient to induce *de novo* methylation. This hypothesis is supported by previous studies showing that increasing DNA methyltransferase expression and activity by at least 9-fold can induce *de novo* methylation [40].

In summary, the present study demonstrates that slightly enhanced AdoHcyase activity is well tolerated by the cell, while greatly enhanced AdoHcyase activity elevates intracellular adenosine levels to an extent, which results in adenosine-induced apoptosis. The fact that enhanced AdoHcyase activity does not increase DNA methylation suggests that AdoHcyase activity under non-stimulated conditions is not rate limiting for efficient DNA methylation.

Abbreviations

AdoHcyase (S-Adenosylhomocysteine hydrolase); AdoHcy (S-Adenosylhomocysteine); AdoMet (S-Adenosylmethionine); HEK-293 (Human embryonic kidney 293 cells); HFF (Human foreskin fibroblasts); ADA (Adenosine deaminase); HPLC (High performance liquid chromatography); MEM (Minimum essential medium); NCS (Newborn calf serum); PBS (Phosphate buffered saline); BSA (Bovine serum albumin); PAGE (Polyacrylamid gel electrophoresis); DTT (1,4-dithio-DL-threitol); PMSF (Phenylmethyl sulfonyl fluoride); AMC (7-amino-4-methylcoumarin); AFC (7-amino-4-trifluoromethylcoumarin); z-VAD-fmk (Benzyl-oxy carbonyl-VAD(O-Me) fluoromethylketone).

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