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Effects of exogenous agmatine in human leukemia HMC-1 and HL-60 cells on proliferation, polyamine metabolism and cell cycle

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ABSTRACT

Impairment of agmatine homeostasis is involved in the regulation of cell proliferation in malignant solid tumors. The present study aimed at analyzing the relevance of agmatine homeostasis in pathophysiology of human leukemia. Proliferation of the human leukemia cells HMC-1 and HL-60 was determined in the absence or presence of agmatine. Apoptosis and cell cycle distribution was investigated by determination of caspase-3 activity and/or flow cytometry after staining with propidium iodide. Expression analysis was performed by qPCR and by a microarray genechip. Exogenous agmatine inhibited proliferation of both HMC-1 and HL-60 cells. The antiproliferative effect was due to interference of agmatine with the cell cycle with no evident signs of apoptosis. Comparative analysis of expression of mRNA in untreated HMC-1 cells and in non-leukemic human mast cells revealed a much lower expression of agmatinase and diamine oxidase in HMC-1 cells indicating a significantly reduced agmatine catabolism in the leukemic cells. At the mRNA level, inhibition of proliferation of both cell lines by agmatine was associated with cell type-specific alterations of the expression of enzymes of the polyamine pathway. The present results point to a significant role of agmatine homeostasis in the (patho)physiology of cell proliferation of leukemic cells, at least in HMC-1 and HL-60 cells, that may serve as a potential target for an adjuvant therapy in the treatment of human leukemia.

1. Introduction

Agmatine is the product of L-arginine decarboxylation by arginine decarboxylase (ADC). We recently provided evidence that agmatine homeostasis is crucially involved in the regulation of tumor cell proliferation and growth by acting as a functional antagonist of polyamines [1,2]. Decrease of intracellular agmatine content by RNA interference targeting ADC in human intestinal tumor cells resulted in a significant increase of proliferation [3]. In vivo, in human colon cancer tissue agmatine content was about one-half of that in the normal tissue adjacent to the malignant tumor [4]. The expression of ADC, diamine oxidase and ornithine decarboxylase in human colon neoplastic tissue was, at the mRNA level, about 75% and 50% lower and 150% higher, respectively, than in the adjacent normal tissue [2].

In vitro, administration of exogenous agmatine led to a reduction of ornithine decarboxylase activity [5] and to an inhibition of polyamine uptake [5–7]. Consequently, application of exogenous agmatine inhibited proliferation of cell lines derived from human and rat solid intestinal (Caco2, Cx1, Colo320, HT29, Colo205E, SW480 [1]), hepatic (HepG2, RH7777 [1,8]), breast (MCF-7 [9]) and prostate tumors [10] at concentrations which are achievable in vivo. In leukemia cells relevance of agmatine homeostasis for proliferation has not been defined so far. Therefore, the aims of the present study were (1) to investigate whether exogenous agmatine inhibits proliferation of the human mast cell leukemia cells HMC-1 and the human myeloid leukemia cells HL-60 as models for human hard-to-treat leukemia and (2) to challenge potential mechanisms of action underlying the potential antiproliferative effect.

2. Methods

2.1. Cell culture

The human mast cell leukemia cells HMC-1 were a subclone of the HMC-1 cell line raised by Butterfield et al. [11] possessing at least two functionally activating mutations in tyrosine kinase kit (V560G, D816V [12]). HMC-1 cells were cultured in Optimem I Reduced Serum Medium supplemented with fetal calf serum (FCS) 10%

Abbreviations: DAO, diamine oxidase; ODC, ornithine decarboxylase; ODC-AZ1, ornithine decarboxylase antizyme-1; ADC, arginine decarboxylase; AGMAT, agmatinase.

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(Invitrogen, Karlsruhe, Germany). The cells of the acute promyelocytic leukemia cell line HL-60 [13] were grown and maintained in RPMI-1640 medium, pH 7.4 supplemented with FCS (10%), penicillin (100 Units/ml) and streptomycin (100 μg/ml). The cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C (Hera Cell, Heraeus, Germany).

2.2. Proliferation assay

HMC-1 and HL-60 cells were incubated in the absence (control cells) or presence of agmatine for 72 h. At the end of the experiment cell proliferation was determined by using the Quick Cell Proliferation Assay Kit (BioVision, Mountain View, CA; USA) according to the manufacturer's instructions.

2.3. Protein determination

Protein content of the cells was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Bonn, Germany) according to the manufacturer's instructions.

2.4. Caspase-3-assay

HMC-1 and HL-60 cells were incubated in the absence (control cells) or presence of 1 mM agmatine for 72 h. At the end of the experiment caspase-3 activity was assessed by using the Caspase-3/CPP32 colorimetric assay kit according to the manufacturer's protocol (BioVision, Mountain View, CA, USA).

2.5. Cell cycle phase distribution

HMC-1 and HL-60 cells were incubated in the absence (control cells) or presence of 1 mM agmatine for 72 h, fixed in cold 70% alcohol in PBS, washed, digested with DNase-free RNase and stained with propidium iodide in dark according to the manufacturer's protocol (Cell Cycle Analysis Kit, GenScript Corporation, Piscataway, NJ, USA). Cells were analyzed immediately by flow cytometry on FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA).

2.6. Isolation of hematopoietic mast cell-committed progenitors from peripheral blood

Ten healthy volunteers recruited at the blood bank of the University Hospital of Bonn were anonymous blood donors for the isolation of hematopoietic mast cell-committed progenitors for comparison with HMC-1 cells. All blood donors were examined according to the German guidelines for blood donation. Peripheral blood obtained by venipuncture was drawn into syringes containing EDTA as anti-coagulant. Hematopoietic mast cell-committed progenitors were isolated as described previously [12]. Briefly, mast cell-committed progenitors were separated by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden) followed by immunomagnetic positive selection with mAb against human CD117 (tyrosine kinase Kit) according to the manufacturer's instructions (Magnetic cell sorting, MACS; Miltenyi Biotech, Bergisch Gladbach, Germany). The isolated cell population was homogenous as evaluated by microscopy.

2.7. Quantitative polymerase chain reaction

Quantitative RT-PCR was performed as described previously [2]. In brief, RNA from human hematopoietic mast cell-committed progenitor cells, HMC-1 and HL-60 cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with DNase treatment according to the manufacturer's instructions. Total RNA of each sample was reverse transcribed according to the manufacturer's instructions (Superscript II, Invitrogen, Karlsruhe, Germany; random hexamer primers, MWG, Ebersberg, Germany). For qPCR 35 μl of amplification mixture (QuantitectSybrGreen Kit, Qiagen) was used, containing 20–120 ng of reverse transcribed RNA and 300 nM of the respective primers (see Supplementary Table 1) according to the manufacturer's instructions. Reactions (triplicates, 10 μl) were run on a '7900 HT Fast Real-Time PCR System' (Applied Biosystems, Foster City, CA, USA). The cycling conditions were: 15 min polymerase activation at 95 °C and 45 cycles at 95 °C for 30 s, at 58 °C for 30 s and at 72 °C for 30 s. Each assay included a standard curve and no-template controls. The relative mRNA expression (R) was calculated from the ratio "agmatine-treated" over "control", $R = E^{\text{Ct control−treated (target)}} / E^{\text{Ct control−treated (housekeeper)}}$ with the efficiency $E = 10^{-1/\text{slope}}$ measured with a standard curve in all experiments. Results for the housekeeping gene ß-actin were determined by the same method (for primers, see Supplementary Table 1). The identity of the PCR products was initially confirmed by agarose gel electrophoresis followed by dideoxy chain termination sequencing and then after each real-time reaction by melt point analysis.

2.8. Expression analysis

To identify gene expression changes induced by treatment of both cell lines with 1 mM agmatine for 72 h, we compared gene expression profiles of HMC-1 and HL-60 cells with and without agmatine treatment using Illumina Human HT-12 v3 Expression BeadChip (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.

2.9. Amplification, labeling and BeadChip hybridization of RNA samples

RNA isolation was carried out using RNeasy Mini Kit (Qiagen, Hilden, Germany) with DNase treatment according to the manufacturer's instructions. Total RNA concentration was quantified using NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA) spectrophotometer. Illumina TotalPrep-96 RNA Amplification Kit (Ambion, Austin, TX, USA) was used to transcribe 200 ng total RNA according to the manufacturer's recommendation. A total of 750 ng of cRNA was hybridized at 58 °C for 16 h to the Illumina HumanHT-12v3 Expression BeadChip. Bead-Chips were scanned using the Illumina iScan Reader and the iScan Control Software (Illumina, San Diego, CA, USA).

2.10. Data processing

Data has been processed with GenomeStudio Gene Expression Module v1.0. The normalizations executed by Illumina GenomeStudio were all applied to the expression values on the original scale. Parameters used for calculating differential gene expression (reference group: untreated cells) were Cubic Spline normalization with no background subtraction applying the Illumina custom error model with multiple testing corrections using Benjamini and Hochberg False Discovery Rate (for details see GenomeStudio Gene Expression Module V1.0 User Guide; http://www.illumina.com/). No background normalization was executed [14]. Cubic spline normalization was preferred according to Schmid et al. [15] who had compared different normalization methods for Illumina BeadChip HumanHT-12v3. Quality filter criteria for significantly regulated genes were chosen as follows: only genes were included with a detection *P*-value (probability that the signal from a given probe is greater than the average signal from the negative controls) of $P < 0.05$ and a differential score of ≥ 13 or ≤ 13, respectively, corresponding to a *P*-value < 0.05 [differential score = $10 * \text{sgn}(\mu_{\text{ref}} - \mu_{\text{cond}}) * \log_{10}(P)$] based on the difference between the average signal in the reference group (untreated cells) vs. the comparison group (treated cells).

2.11. Pathway analysis

Subsequently, we analyzed whether genes regulated by agmatine treatment were enriched in certain pathways or biological processes. For this purpose, the program Ingenuity Pathways Analysis (IPA, version 8.6, http://www.ingenuity.com), a web-based interface that provides computational algorithms to identify biological functions and canonical pathways in a dataset on the basis of functional annotation and molecular interactions were used. Functional knowledge comes from peer-reviewed publications and is stored in the Ingenuity Knowledge Base. Gene lists of significantly regulated genes including their expression values for either HMC-1 or HL-60 cells were uploaded onto IPA applying the "core analysis" function. In the functional analysis, biological functions and disease processes are grouped to different categories from the Ingenuity Knowledge Base. Fisher's exact test with Benjamini–Hochberg correction for multiple testing was used to calculate statistical significance of enriched biological functions and diseases assigned to the datasets. In the canonical pathways analysis the most significant metabolic and cell signalling pathways across the entire input dataset are displayed. The ratio for a canonical pathway is calculated from the number of molecules in a given pathway from the input list divided by the total number of molecules that make up that pathway. The significance of a given pathway for a dataset is a measurement for the likelihood that the pathway is associated with the dataset by random chance, and it is given as −log (*P*-value).

2.12. Statistical analysis

Unless stated otherwise data are expressed as mean ± SEM. The statistical significance of the difference between control and treated groups was determined by student's *t*-test for unpaired data and *P* values <0.05 were considered significant.

3. Results

3.1. Cell proliferation

Incubation with agmatine for 72 h concentration-dependently inhibited proliferation of both HMC-1 and HL-60 cells being more effective in HMC-1 than in HL-60 cells (Fig. 1). Proliferation of HMC-1 cells was significantly inhibited by agmatine 0.1 and 0.3 mM to 87.9 ± 1.1% and 83.7 ± 1.4% of the corresponding controls, respectively, whereas these concentrations were hardly effective in HL-60 cells (inhibition to 95.5 ± 1.4% and 94.0 ± 2.1%, respectively, compared to corresponding controls; Fig. 1). Agmatine 1 mM sig-