Leukemia Research 39 (2015) 757–762

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Glutaminase activity determines cytotoxicity of L-asparaginases on most leukemia cell lines

Jean Hugues Parmentier[a], Maristella Maggi[b], Erika Tarasco[b], Claudia Scotti[b], Vassilios I. Avramis[c,d], Steven D. Mittelman[a,d,e,*]

[a] Center for Endocrinology, Diabetes & Metabolism, Children's Hospital Los Angeles, Los Angeles, CA, United States
[b] Department of Molecular Medicine, Unit of Immunology and General Pathology, University of Pavia, Italy
[c] Center for Childhood Cancer and Blood Diseases, Children's Hospital Los Angeles, Los Angeles, CA, United States
[d] Department of Pediatrics, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States
[e] Department of Physiology & Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States

ARTICLE INFO

Article history:
Received 27 October 2014
Received in revised form 10 April 2015
Accepted 13 April 2015
Available online 22 April 2015

Keywords:
Asparaginase
Glutaminase
ALL
Leukemia

ABSTRACT

L-Asparaginase (ASNase) is a front-line chemotherapy for acute lymphoblastic leukemia (ALL), which acts by deaminating asparagine and glutamine. To evaluate the importance of glutaminase activity, we exploited a recently developed mutant of *Helicobacter pylori* ASNase (dm HpA), with amino acid substitutions M121C/T169M. The mutant form has the same asparaginase activity as wild-type but lacks glutaminase activity. Wild-type and dm HpA were compared with the clinically used ASNases from *Escherichia coli* (L-ASP) and *Erwinia chrysanthemi* (ERWase). Asparaginase activity was similar for all isoforms, while glutaminase activity followed the rank order: ERWase > L-ASP > wild-type HpA > dm HpA. Cytotoxic efficacy of ASNases was tested on 11 human leukemia cell lines and two patient-derived ALL samples. Two cell lines which we had previously shown to be asparagine-dependent were equally sensitive to the asparaginase isoforms. The other nine lines and the two patient-derived samples were more sensitive to isoforms with higher glutaminase activities. ERWase was overall the most effective ASNase on all cell lines tested whereas dm HpA, having the lowest glutaminase activity, was the least effective. These data demonstrate that asparaginase activity alone may not be sufficient for ASNase cytotoxicity, and that glutaminase activity may be required for full anti-leukemic efficacy.

1. Introduction

L-Asparaginases (ASNases) are naturally occurring bacterial enzymes currently used as a universal component of treatment of childhood acute lymphoblastic leukemia (ALL), especially for remission induction [1–3]. The drug acts through the hydrolysis of asparagine and glutamine, two vital amino acids for leukemia cell growth, to aspartic acid and glutamic acid, respectively, releasing ammonia in the process [4]. Leukemic lymphoblasts are highly sensitive to asparagine depletion due to low levels of asparagine synthetase (ASNS) and thus generally depend on extracellular supply of asparagine. Up-regulation of ASNS and glutamine synthetase, as well as glutamine transporters, are associated with resistance in vitro [5,6]. Level of ASNS mRNA, protein and activity, however, vary widely in pediatric ALL samples [7–12] and are not necessarily associated with in vitro resistance to L-asparaginase, thus indicating additional mechanisms of resistance to ASNases besides modulation of ASNS [13]. Lymphoblasts and lymphocytes, especially B lymphoblastoid cell lines [14,15], are highly dependent on glutamine when compared to other cell types [16]. Glutamine is an essential co-substrate of ASNS, as it donates the amino group required for asparagine synthesis [17], indicating a critical dependency on glutamine supply for asparagine neo-synthesis. Strategies targeting glutamine [18,19] in addition to asparagine may therefore improve treatment outcomes in newly diagnosed, and in relapsed, ALL patients.

Novel ASNases besides *Escherichia coli* ASNase (L-ASP) with different activities have been developed in an attempt to decrease side effects and increase efficacy [20]. *Erwinia chrysanthemi* ASNase or Erwinase (ERWase) has similar asparaginase but more than 10-fold higher glutaminase activity in comparison to *E. coli* ASNase, with favorable K_m and faster catalysis (higher k_{cat}) [21].

* Corresponding author at: Children's Hospital Los Angeles, 4650 Sunset Boulevard, MS #61, Los Angeles, CA 90027, United States. Tel.: +1 323 361 7653; fax: +1 323 361 1350.
E-mail address: smittelman@chla.usc.edu (S.D. Mittelman).

http://dx.doi.org/10.1016/j.leukres.2015.04.008
0145-2126/

Alternatively, other investigators seek to reduce the glutaminase activity of ASNase to reduce toxicity [22–24]. Glutaminase activity of L-ASP was recently shown to be necessary for its anticancer activity against ASNS positive cell types but not ASNS-negative cell types [24]. Recently, we have shown on a panel of 9 human leukemia cell lines that ALL cells have greater dependence on glutamine than asparagine [25]. It is therefore critical to establish whether glutaminase activity is necessary for ALL chemotherapy. In this study, we test whether glutaminase activity is important for cytotoxicity of L-asparaginases on human leukemia cells, using a recently characterized mutant *Helicobacter pylori* ASNase lacking glutaminase activity.

2. Materials and methods

2.1. Cell lines

Human leukemia cell lines were obtained from ATCC (Manassas, VA) and the German Collection of Cell Lines (DSMZ, Braunschweig, Germany; Table 1). We also used patient-derived human leukemia samples, BLQ1 and TxL2 (Ph-positive ALL), which had been passaged through NOD. Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjll}/SzJ mice and cultured as described previously [25,26].

2.2. Double mutant H. pylori construct

The expression and biochemical characterization of recombinant *H. pylori* L-asparaginase (wt HpA) has been previously described [27]. The enzyme showed a strong preference for asparagine (K_m: 0.290 mM) over glutamine (K_m: 46.4 mM) [21,27]. A novel glutamine-inactive form of *H. pylori* ASNase (M121C/T169M mutant, dm HpA) was recently generated by site-directed mutagenesis [28]. The M121C mutant was designed to have the same asparaginase activity as wild type (wt), but lack glutaminase activity. However, only with the addition of the serendipitous mutation T169M was wild-type asparaginase activity maintained in the glutaminase-free mutant. The biochemical characterization of the single and double mutant and a preliminary analysis of the cytotoxicity of the latter on HL-60 cells at 24 h has been described elsewhere [28].

2.3. Measurement of asparaginase and glutaminase activities

L-Asparaginase derived from *E. coli* (L-ASP) was obtained from Amatheon (Miami, FL). Erwinase investigational drug was kindly provided for experimental evaluations by Dr. Paul Plourde (Jazz Pharmaceuticals, Langhorne, Pennsylvania). Quantitative detection of ammonia was performed with Nessler's reagent as previously described [25,29] and adapted to 96-well plates for measurement of asparaginase and glutaminase activities with multiple dilutions. This method allows for direct comparison of asparaginase and glutaminase activities between different ASNase isoforms. The production of ammonia by L-asparaginase over time was expressed relative to the slope of known ammonia standards. The resulting value represented the activity of the enzyme in international units (IUs), in which one IU equaled the amount of enzyme that catalyzed the formation of 1 μmol of ammonia per min. Briefly, 10 mM asparagine or 40 mM glutamine, pH 8.6, were incubated at 37 °C for 15 min with different dilutions of ASNases (0.001–20 IU) in a final volume of 100 μl/well using 96-well plates. The reaction was stopped on ice by adding 5 μl of 1.5 M TCA and microplates were centrifuged at 3700 × *g*. 10 μl of each ammonium sulfate standard or reaction mix were mixed in duplicate with 190 μL of Nessler's Working Reagent, and absorbance was measured at 436 nm. While each ASNase isoform has different optimal pH for their respective asparaginase and glutaminase activities which may differ from the physiological or culture medium pH, we did not observe any significant differences in asparaginase and glutaminase activities measured at pH 8.6 and 6.5. In addition, the ranking order of the isoforms was identical (data not shown).

Data were fitted with a non-linear regression 5-parameter model.

2.4. Determination of cell viability

Cells were plated onto 96-well plates at a density of 100,000–120,000 cells/well in RPMI medium supplemented with 10% FBS, Na Pyruvate, GlutaMax and gentamicin. L-ASNases were tested in a final concentration range from 0.03 to 3 IU/ml for 72 h. Experiments with primary human leukemia cells were conducted over OP9 feeder layers, with leukemia cells collected at 72 h by vigorous trituration [25]. Viable cells were determined by trypan blue exclusion in triplicate wells, counted by a blinded observer. Each dose-response experiment was repeated for $n = 4–5$ for each cell line.

In additional experiments, cell death was measured by flow cytometry. Cells plated at 500,000 cells/well in 24-well plates were incubated for 48 h with ASNases. Cells were then washed twice in ice-cold PBS. DAPI was added and the samples were run on LSR II Analyzer (Becton Dickinson).

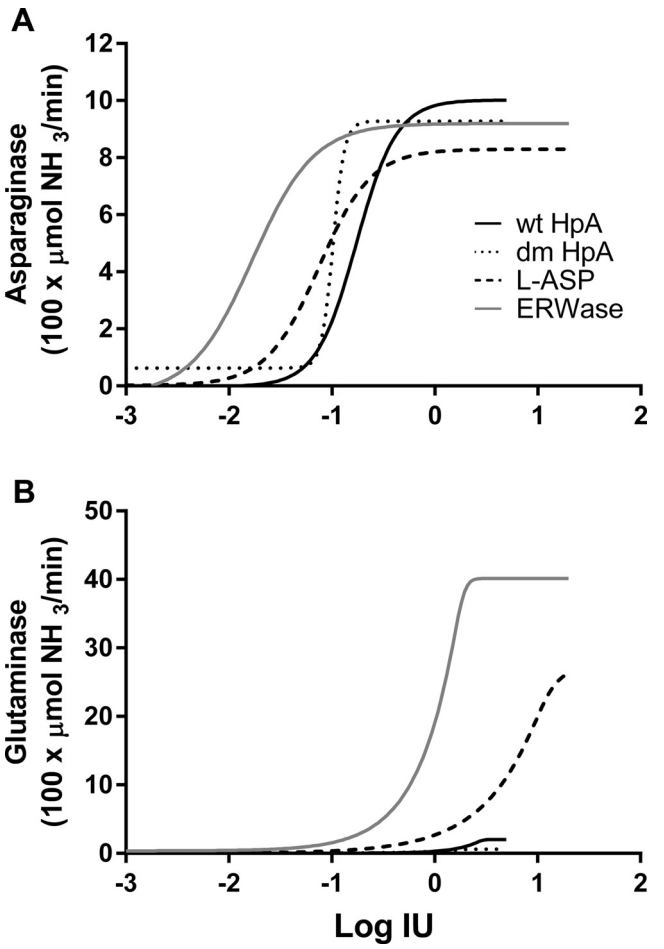

Fig. 1. Dose response fit of asparaginase activity (A) and glutaminase activity (B) of four ASNases ($n = 2$). The *X*-axis represents the Log IU for each isoforms calculated from 10 to 12 different concentrations ranging from 0.001 to 20 IU. Graphs shown are a non-linear regression 5-parameter model.

2.5. Statistical analysis

Two-way ANOVA with Bonferroni post-hoc test was used to analyze the percentage of remaining viable leukemia cells or percent inhibition. Inferences for concentration analysis were determined with Wilcoxon signed-rank test. A *p*-value of less than 0.05 was considered significant. IC_{50} and percentage inhibition at 3 IU/ml were calculated from normalized data using non-linear regression with GraphPad Prism. Correlation was determined with Pearson's test.

3. Results

3.1. ASNases have similar maximal asparaginase activity with different glutaminase activity

To test the dual activity of each ASNase isoform simultaneously, asparaginase and glutaminase activities were determined as described in Material and Methods. Under our assay conditions, asparaginase activity reached a plateau for all isoforms between 0.1 and 1 IU (Fig. 1A), and was not significantly different between ASNase isoforms. Glutaminase activity also plateaued, but at higher enzyme concentrations (Fig. 1B). Glutaminase activity of ERWase was higher than for L-ASP, whereas for wt HpA it was small compared to other isoforms, and for dm HpA it was close to background activity even at 5 IU. This is summarized in Table 2 where asparaginase and glutaminase activities are expressed as a percentage of ERWase (arbitrarily fixed at 100%) at a dose of 3 IU. Glutaminase activity showed the following ranking order: ERWase > L-ASP > wt HpA > dm HpA.