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Inhibition of Glutamine Synthetase Triggers Apoptosis in Asparaginase-Resistant Cells

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Key Words

Sarcoma • Glutamine • Glutamine synthetase • Asparaginase • L-methionine-sulfoximine • Asparagine synthetase

Abstract

The resistance to L-asparaginase (ASNase) has been associated to the overexpression of asparagine synthetase (AS), although the role played by other metabolic adaptations has not been yet defined. Both in ASNase-sensitive Jensen rat sarcoma cells and in ARJ cells, their ASNase-resistant counterparts endowed with a five-fold increased AS activity, ASNase treatment rapidly depletes intracellular asparagine. Under these conditions, cell glutamine is also severely reduced and the activity of glutamine synthetase (GS) is very low. After 24h of treatment, while sensitive cells have undergone massive apoptosis, ARJ cells exhibit a marked increase in GS activity, associated with overexpression of GS protein but not of GS mRNA, and a partial restoration of glutamine and asparagine. However, when ARJ cells are treated with both ASNase and L-methionine-sulfoximine (MSO), an inhibitor of GS, no restoration of cell amino acids occurs and the cell population undergoes a typical apoptosis. No toxicity is observed upon MSO treatment in the absence of ASNase. The effects of MSO are not referable to depletion of cell glutathione or inhibition of AS. These findings indicate that, in the presence of ASNase, the inhibition of GS triggers apoptosis. GS may thus constitute a target for the suppression of ASNase-resistant phenotypes.

Introduction

The antitumor enzyme L-asparaginase (L-asparagine amido-hydrolase, E.C. 3.5.1.1, ASNase) has been employed for many years in the treatment of acute lymphoblastic leukemia (ALL) (see [1] and [2] for review). The cytotoxic effects of ASNase were attributed to asparagine starvation [3, 4] and, consistently, low activity of asparagine synthetase (AS), the enzyme that obtains asparagine from aspartate and glutamine, has been considered the most important determinant of sensitivity to ASNase [2, 5]. In agreement with this hypothesis, methods have been developed to measure AS expression conveniently, so as to foresee clinical sensitivity to ASNase [6], and several ASNase-resistant cell lines have been characterized that exhibit an increased expression and/or activity of AS [5, 7-9].

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However, ASNase sensitivity is not exclusively linked to low AS expression [10] and the expression of other genes has been found more correlated with the establishment of a resistant phenotype than AS itself [11]. Moreover, in cells from ALL patients, increased expression of AS is not associated with ASNase resistance [12] and ASNase-sensitive cells are unable to progress through the cell cycle under the nutritional stress caused by the antitumor drug, despite the ability to upregulate AS [13]. These data suggest that additional factors, other than high expression of AS, contribute to survival and proliferation of ASNase-resistant cells.

As the ASNase enzymes employed for leukemia therapy are also endowed with glutaminase activity [1], a marked depletion of glutamine is observed after in vitro [14, 15] or in vivo [16, 17] treatment with ASNase. Thus, glutamine depletion may be involved in the cytotoxic effects of ASNase [18, 19]. Moreover, glutamine availability may be of particular importance in ASNase-resistant cells that overexpress AS, since the amino acid is the obliged substrate of the synthetase. Interestingly, although ASNase-resistant MOLT-4 human leukemic T lymphocytes overexpress AS, they are also endowed with multiple adaptive mechanisms, all aimed to increase glutamine availability [20, 21]. However, the effective metabolic role of these adaptations was not investigated, thus preventing the possibility to attribute a specific relevance for cell survival to each of them.

The activity of glutamine synthetase (EC 6.3.1.2, GS), the only mammalian enzyme that synthesizes glutamine from glutamate and ammonium [22, 23], is up-regulated by glutamine depletion [23]. To investigate the mechanism and the metabolic importance of this adaptive mechanism, we have monitored GS expression and activity in an ASNase-resistant subline of Jensen rat fibrosarcoma cells. We have demonstrated that, in the presence of ASNase, GS is up-regulated and that, under the same conditions, the inhibition of GS activity triggers apoptosis and overcome ASNase resistance.

Materials and Methods

Cell culture and establishment of ASNase-resistant cells

Jensen rat fibrosarcoma cells, a line that spontaneously present asparagine-independent variants [24], were obtained from Istituto Zooprofilattico Sperimentale della Lombardia. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 0.3 mM asparagine and 10% fetal bovine serum (FBS). For the development of an ASNase-resistant line, Jensen cells underwent a treatment with increasing doses of ASNase. Briefly, Jensen cells were seeded into 175 cm^2 flasks and cultured in McCoy's complete medium 5a supplemented with 10% FBS in the presence of 0.005 IU/ml of E. chrisanthemy ASNase (Erwinase®, Ipsen Ltd., UK). Every third day culture medium was changed to remove loose cells and, once reached the confluence, cells were passed and cultured with a 10x-concentration of ASNase. After about three months of treatment with increasing concentrations of the antitumor enzyme, a cell population was obtained that was resistant to 5 IU/ml ASNase (Asparaginase-Resistant Jensen (ARJ) cells).

Before the experiments, ARJ cells had been grown for at least 5 passages in the same medium employed for parental Jensen cells in the absence of ASNase. For ASNase treatment, the antitumor enzyme was employed at 5 IU/ml.

Assessment of cell proliferation and viability

Cell proliferation was evaluated by determining the cell number with a Coulter Counter ZM after culture trypsinization and correction for non-viable cells performed with the Trypan blue method.

Cell viability was monitored by a resazurin assay that monitors the metabolic activity of living cells. To this purpose, cells were grown on 96-well multiwell plates (Falcon) and, after the experimental treatment, 7-hydroxy-3H-phenoxazin-3-one-10-oxide (resazurin, Sigma) was added to the incubation medium at a final concentration of 44 μM. The plate was incubated for 2h at 37°C and 5% CO_2 and, after this period, fluorescence was read at 530 nm with a Wallac Victor^2 1420 Multilabel counter. Cell viability was expressed as arbitrary units (A.U.) of fluorescence.

Intracellular amino acid pool

Cells monolayers, grown on 6-well multiwell trays, were washed twice with ice-cold Phosphate Buffered Saline (PBS) and extracted in a 5%-solution of acetic acid in ethanol. The intracellular content of the single amino acid species was determined by HPLC analysis with a Biochrom 20 Amino Acid Analyzer (Amersham Pharmacia Biotech) employing a High Resolution Column Bio 20 Peek Lithium and the Physiological Fluid Chemical Kit (Amersham Pharmacia Biotech) for elution. The column effluent was mixed with ninhydrin reagent, passed through the high temperature reaction coil, and read by the photometer unit. Cell contents of the single amino acid species were expressed as nmol/mg of protein.

Activities of glutamine synthetase (GS) and asparagine synthetase (AS)

Cell cultures, grown in 10-cm diameter dishes, were washed twice in ice-cold PBS, scraped off into the same solution, and collected by a low speed centrifugation. GS and AS assays were performed according to methods by Pishak and Phillips [25] and by Gantt et al. [26], respectively. Briefly, for GS assay the pellet was lysed in 500 μl of a solution containing 50 mM imidazole-HCl, pH 6.8, 0.5 mM EDTA, 1 mM DTT, and 1 mM PMSF. Lysis buffer for AS assay consisted in 500 μl of a solution containing 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.85 mM EGTA, 1 mM DTT, 1 mM PMSF. Both lysis buffers were completed with a cocktail of protease inhibitors (Complete TM,

Mini, Boehringer Mannheim), transferred into a 1.5 ml microtube, disrupted by sonication in ice (Sonicator Ultrasonic Processor XL, Misonix), and clarified by centrifugation at 12000 x g, for 30 min, at 4°C. 500 µg of the supernatant, after protein quantification with the Bio-Rad Protein Assay, with bovine serum albumin (BSA) as a standard, were used to measure enzyme activities by determining the conversion of L-[^{14}C]-glutamic acid to L-[^{14}C]-glutamine (for GS) or of L-[^{14}C]-aspartic acid to [^{14}C]-asparagine (for AS).

GS activity was measured in an imidazole-HCl buffer (50 mM, pH 6.8) supplemented with 10 mM L-[^{14}C]-glutamic acid (2 µCi/ml), 4 mM NH_4Cl, 10 mM ATP, 15 mM $MgCl_2$, and 1 mM 2-mercaptoethanol, to a final volume of 1 ml. AS activity was determined in a Tris-HCl buffer (140 mM, pH 7.5) containing 30 mM L-glutamine, 3 mM L-[^{14}C]-aspartic acid (3 µCi/ml), 10 mM ATP, and 8 mM $MgCl_2$, to a final volume of 1 ml.

The assays were carried on at 37°C for 120 min. The reactions were stopped by heating at 80°C for 3 min. To separate glutamate from glutamine and aspartate from asparagine, each assay solution, cooled at room temperature, was put over an anion exchange column (AG-1X8, 200-400 mesh, Bio-Rad) that was washed 4 times with glutamine 2 mM or asparagine 2 mM for the two enzyme activities, respectively. Radioactive glutamine or asparagine, eluted from the columns, were counted with Wallac 1450 Microbeta Trilux scintillation spectrometer (Perkin Elmer). Synthetase activities were expressed as pmol/mg prot/min.

Glutathione measurements

Total glutathione levels were measured by the method of Griffith [27] with minor modifications. Briefly, after the experimental treatment, cell cultures, grown on 96-well multiwell trays, were washed with PBS, lysed in 50 µl of 0.3% perchloric acid for 30 min at 4 °C, and neutralized with 4 vol of 0.125 M NaH_2PO_4, 6.3 mM NaEDTA, pH 7.5. 50 µl of solution were mixed with 150 µl of 0.3 mM NADPH, 20 µl of 6 mM DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), and 20 µl of glutathione reductase (0.5 U/ml final, Roche Diagnostics SpA, Monza, Italy). The reaction was performed in a Wallac Victor^2 1420 Multilabel counter equipped with a dispenser unit and the product read at 405 nm after 4 min with the same instrument.

Reverse Transcription

Total RNA from subconfluent cultures (10-cm^2 wells) was isolated with OMNIZOL® (EuroClone Ltd., U.K.). RNA (1 µg) was added with dNTPs (500µM each) and dN6 random examers (250 ng), heated at 65°C for 5 min, and placed on ice for 3 min. The mixture was then incubated for 5 min at 25°C with 5 mM dithiothreitol, 1X First-strand buffer (Invitrogen, Life-Technologies), 40 units of RNAse inhibitor (Invitrogen, Life-Technologies), 200 units of SuperScript III Reverse Transcriptase (Invitrogen), and water, to a final volume of 20 µl. The reaction was run for 50 min at 50°C and stopped by heating at 70°C for 15 min. The duplex of RNA-DNA was treated with 2.5 units of RNAse H (US Biochemicals, Cleveland, OH) at 37°C for 20 min, and the amount of single-strand cDNA was evaluated with a Victor^2 1420 Multilabel Counter (Wallac, Perkin Elmer) using the fluorescent probe Oligreen (Molecular Probes, Eugene, OR) and phage M13+ as single-strand DNA standard.

Quantitative PCR

For real time PCR, 30 ng of cDNA from each sample were amplified in a total volume of 25 µl with 2X Platinum Sybr Green Quantitative PCR SuperMix-UDG (Invitrogen, Life-Technologies), along with both glyceraldehyde-3-phosphate dehydrodenase (GAPDH) or Glutamine Synthetase (GS) primers mix. The forward and reverse primers employed were 5' GCC TTC TAA TGG CTT CCC TG 3' and 5' TAA CCT CGG CAT TTG TCC CT 3' for GS (NM_017073); 5' GGT GCT GAG TAT GTC GTG GAG 3' and 5' GCG GAG ATG ATG ACC CTT TT 3' for GAPDH (NM_017008). The final concentration of the primers was 0.5 µM. The primers were designed according to the known sequences reported in GenBank with the help of Primer 3 program [28].

Quantitative PCR was performed in a 36-well Rotor-Gene 3000™ (Corbett Research, Software Rotor-Gene 3000, version 5.0.60, Mortlake, Australia). After an initial step at 50°C for 2 min, samples were taken at 95°C for 2 min in order to allow denaturation of secondary structures. The reaction continued for 40 cycles, consisting of a denaturation step at 95°C for 15 sec and a unique annealing/extension step at 60°C for 30 sec. Fluorescence was acquired at the end of the 60°C annealing/extension step. A no-template, no-reverse transcriptase control was included in each experiment. At the end of the amplification cycles a melt curve analysis was added.

The analysis of data was performed according to the Relative Standard Curve Method [29]. Data are expressed as ratio of GS mRNA level normalized to the corresponding GAPDH mRNA level.

Western Blot analysis

Cells, grown in 10-cm diameter dishes, were washed with ice-cold PBS, scraped in the same solution, and collected through low-speed centrifugation. The pellet was resuspended in 0.5 ml of a lysis buffer containing 50 mM imidazole-HCl at pH 6.8, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF. Cell lysate, obtained by brief sonication in a ice-cold bath, was centrifuged at 12000 x g for 30 min at 4°C. Protein concentration of the supernatant was determined with the Bio-Rad protein assay, with BSA as a standard. Sample aliquots of the supernatant, containing 20 µg of proteins, were employed immediately or frozen at -20°C. Protein samples were suspended in SDS-PAGE sample buffer and boiled for 4 min. Proteins were separated on a 10% SDS-PAGE and elettrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Bio Rad). Non-specific binding sites were blocked with an incubation in Tris-buffered saline (TBS; 50 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 1% casein, 0.33% gelatin, 3% BSA for 2 h at 30°C; a monoclonal antibody for GS (BD Transduction Laboratories, Becton & Dickinson, Franklin Lakes, NJ) or a polyclonal antibody for β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the same solution at the concentration recommended by the manufacturer (1:5000) and incubated overnight at 4°C. Antibody binding was detected with a Biotin-Streptavidin-HRP conjugate system. Briefly, after washes in TBS-Tween 0.1%, PVDF

membranes were incubated for 30 min at 37°C in the blocking solution containing anti-mouse biotin antibody (DAKO), diluted 1:1000, and, after 4 washes in TBS-Tween, incubated in the same solution containing streptavidin-HRP conjugate (1:7000, DAKO). Immunoreactive bands were developed using a luminol-based chemiluminescence system (ECL, Amersham).

Evaluation of cell death mode by confocal microscopy

Cell death was evaluated by confocal laser scanning microscopy (CLSM) with calcein-AM and propidium iodide (both from Molecular Probes, Eugene, OR) or with annexin V-FITC (Sigma) according to a previously described technique [30]. Observations were carried out on a Molecular Dynamics Multiprobe 2001 system (Sunnyvale, CA) equipped with an argon laser and based on a Nikon inverted microscope.

For experiments, cells, seeded on four-chamber slides (Nalge Nunc International, Rochester, NY) at a density of 50 x 10^3 cells/cm^2 and grown for 1 d, were incubated in complete growth medium supplemented calcein-AM (2 μM), propidium iodide (2 μg/ml), or annexin V-FITC (0.5 μg/ml) under the conditions detailed for each experiment.

Materials

L-[U-^{14}C]-Aspartic acid (45 mCi/mmol) and L-[1-^{14}C]-glutamic acid (45 mCi/mmol) were obtained from Amersham Pharmacia Biotech Italia, Milano, Italy. All other chemicals, whenever not indicated otherwise, were from Sigma.

Results

Phenotypic characteristics of L-asparaginase-resistant ARJ cells

Figure 1 shows the effects of ASNase on the parental Jensen cells and the resistant ARJ cells. Proliferation of Jensen cells (Panel A) was abruptly and irreversibly blocked by the enzyme treatment, while ARJ cells, after a transient proliferative slowdown, resumed growth after 2 days of treatment (Panel B). The resistance to the cytotoxic effects of ASNase has been usually attributed to the enhanced activity of the enzyme asparagine synthetase (AS). In Panel C, AS activity was measured in Jensen and ARJ cells. Compared with parental Jensen cells, ARJ cells had a five-fold stimulated AS activity. After a 72h-incubation in the presence of ASNase, AS activity was further stimulated and ARJ cells exhibited a value 7-fold higher than parental, sensitive cells.

ARJ cells maintained the resistant phenotype after a prolonged cultivation (at least 3 months) in the absence of the enzyme and after repeated cycles of freeze-thawing (data not shown).

Intracellular amino acids in Jensen and ARJ cells

The intracellular pools of glutamine and asparagine were measured in Jensen and ARJ cells before and after treatment with ASNase. The results of the representative experiment, reported in Figure 2, indicate that, before

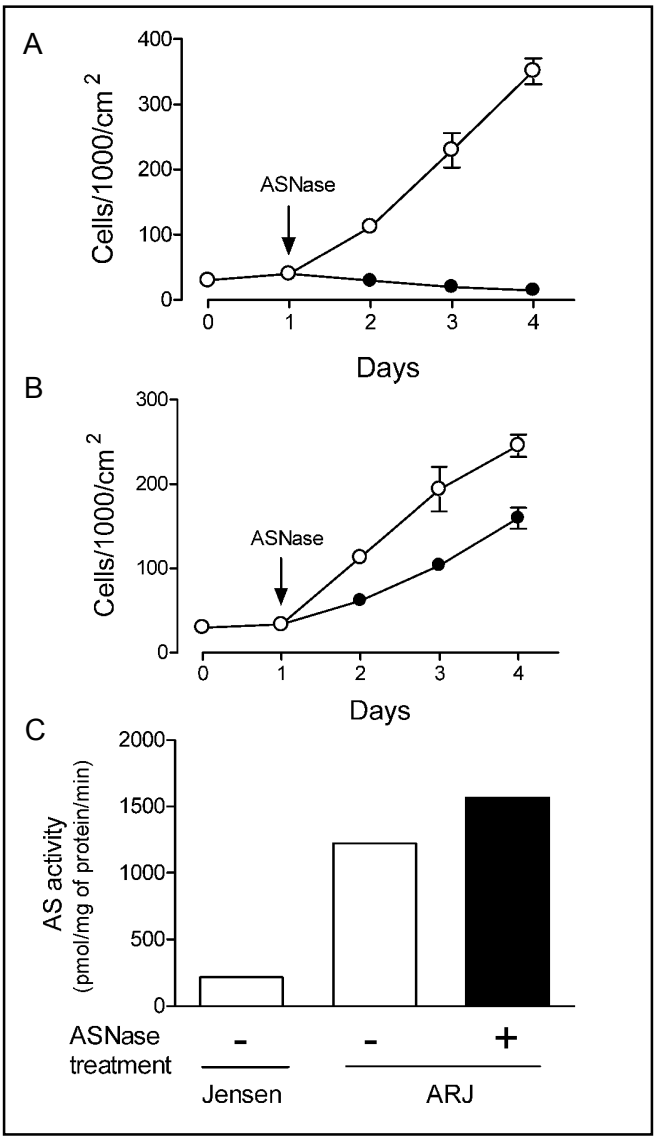

Fig. 1. Characterization of ASNase-resistant phenotype of ARJ cell. Panels A and B. ASNase effect in Jensen (Panel A) and ARJ cells (Panel B). Cells were seeded at 25 10^3 cells /cm^2 (day 0). After 1d, cells were incubated in fresh growth medium in the absence (○) or in the presence of ASNase (●). At the indicated times, cell number was determined. Points represent the mean of four determinations with SD shown. Panel C. Asparagine synthetase (AS) activity in Jensen and ARJ cells. Jensen cells were maintained in the absence of ASNase while ARJ cells were incubated either in the absence or in the presence of ASNase. After 72h cells were lysed and enzyme activity was measured as described in Methods. A representative experiment, repeated three times with similar results, is shown.

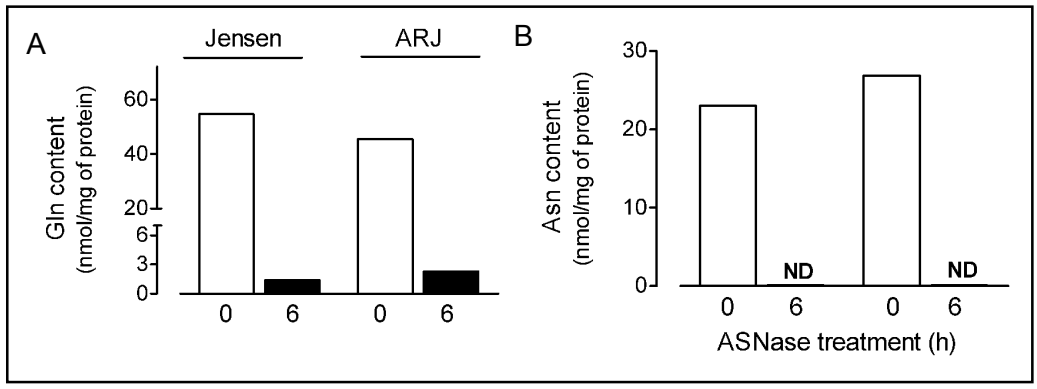

Fig. 2. Cell contents of asparagine and glutamine in Jensen and ARJ cells. Jensen and ARJ cells were incubated for 6h in fresh growth medium in the absence (open bars) or in the presence of ASNase (solid bars). At the end of this period, cells were extracted, and the cell contents of glutamine (Panel A) and asparagine (Panel B) were analyzed by HPLC, as described in Methods. N.D.: not detectable (amino acid content < 0.5 nmol/mg of protein). A representative experiment is shown. Experiment was repeated three times with similar results.

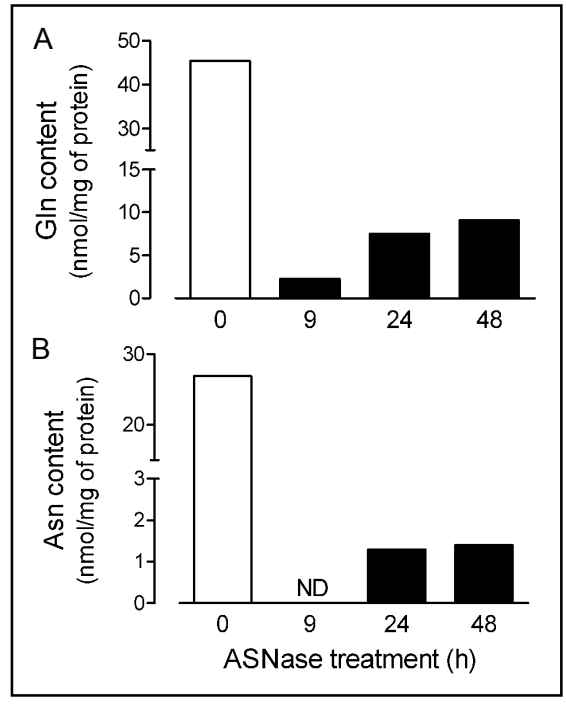

Fig. 3. Restoration of cell asparagine and glutamine in ASNase-treated ARJ cells. ARJ cells were incubated in the presence of ASNase for the times indicated. After the treatment, cells were extracted and the cell content of glutamine (Panel A) or asparagine (Panel B) were analyzed with HPLC. N.D.: not detectable (amino acid content < 0.5 nmol/mg of protein). A representative experiment, repeated three times with similar results, is shown.

ASNase treatment, the intracellular contents of these amino acids were substantially comparable in the two cell types. Upon 6h of incubation in the presence of ASNase, in both lines cell glutamine (Panel A) was severely depleted, with values lowered by more than 95%, while asparagine (Panel B) was no more detectable in the intracellular compartment.

The effect of longer times of incubation with ASNase is shown in Figure 3. The determinations could be done only in ARJ cells since parental Jensen cells had already exhibited severe cytotoxicity and massive cell death at 24 or 48h of incubation. The results indicate that in ARJ cells glutamine content (Panel A) was partially restored with the prolonging of ASNase treatment. Indeed, compared to the level detected after 9h of treatment, intracellular glutamine, measured after 24 or 48h of ASNase treatment, was markedly increased. Consistently, asparagine (Panel B), still undetectable at 9h, became detectable again at 24 and 48h of treatment. At 48h glutamine and asparagine were, respectively, 20% and 5% of the control values.

Glutamine synthetase activity in Jensen and ARJ cells

Figure 4 reports the results of an experiment in which the effects of ASNase treatment on the expression and the activity of glutamine synthetase (GS) were compared in Jensen and ARJ cells. In untreated cultures, GS activity (Panel A) was substantially comparable in Jensen and ARJ cells. Short term ASNase treatment (6h) moderately stimulated GS activity in both cell lines. The expression of GS protein (Panel B), determined on aliquots of the same cell extracts, was barely detectable in both cell types and not substantially changed after 6h of treatment with ASNase.

The activity and the expression of GS were determined after prolonged incubation in the presence of ASNase only in ARJ cells (Fig. 5), due to the massive cell death occurring at these experimental times in Jensen cultures. After 24h of ASNase treatment of resistant cells both GS activity (Fig. 5, Panel A) and protein (Panels B and C) were greatly enhanced compared to control,

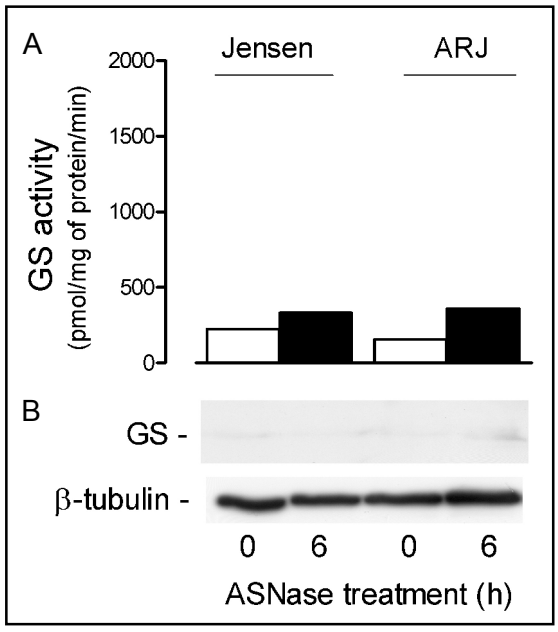

Fig. 4. Activity and expression of glutamine synthetase in Jensen and ARJ cells. Effect of short-term ASNase treatment. Jensen and ARJ cells were seeded in 10-cm dishes in complete growth medium. After 1d, cells were incubated in fresh growth medium in the absence or in the presence of ASNase. The activity (Panel A) and the expression (Panel B) of GS were determined in parallel cultures as detailed in Materials and Methods. For GS expression, cells were lysed and equal amounts of whole cell lysates employed for Western Blot analysis with a GS monoclonal antibody (upper) or β-tubulin polyclonal antiserum (lower). For all the Panels data have been obtained in a representative experiment repeated twice with similar results.

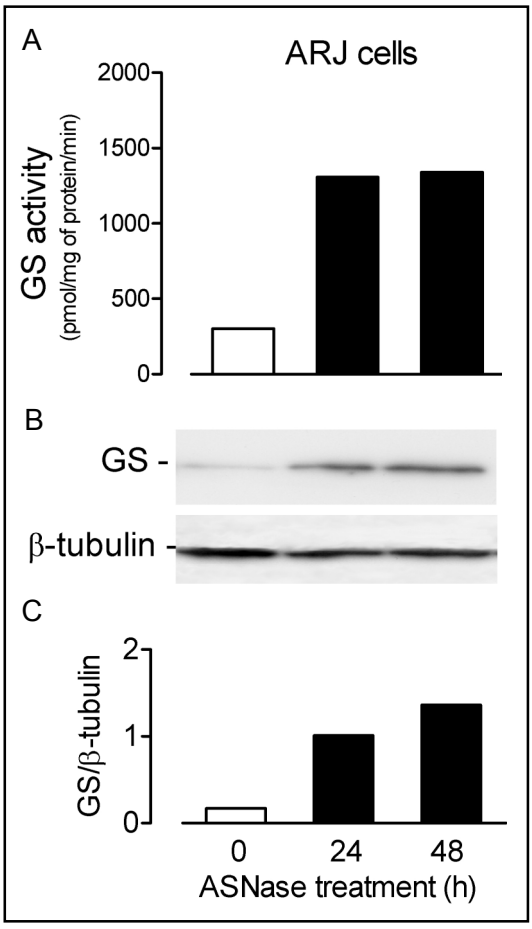

Fig. 5. Induction of expression and activity of glutamine synthetase in ARJ cells by chronic exposure to ASNase. ARJ cells were seeded in 10-cm dishes in complete growth medium. After 1d, cells were incubated in fresh growth medium in the presence of ASNase. The activity (Panel A) and the expression (Panel B) of GS were determined in parallel cultures before and after 24 or 48h of ASNase treatment (see Methods). For GS expression, Western Blot analysis was performed with a GS monoclonal antibody (upper) or β-tubulin polyclonal antiserum (lower). Panel C shows the densitometric analysis of GS expression, reported as GS/tubulin ratio. For all the Panels data have been obtained in a representative experiment repeated twice with similar results.

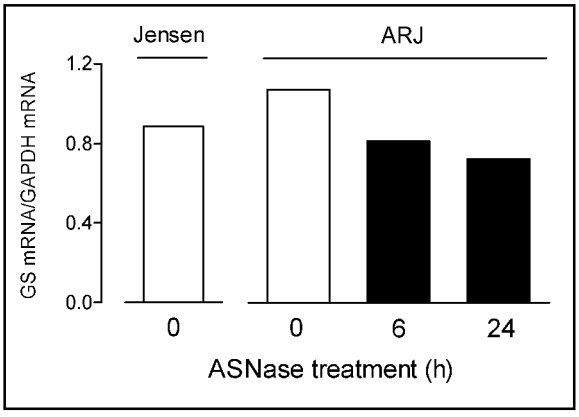

Fig. 6. Glutamine synthetase mRNA in Jensen and ARJ cells. Jensen cells, cultured in the absence of ASNase (empty bar), and ARJ cells, cultured either in the absence (empty bar) or in the presence of ASNase (solid bars) for 6 or 24h, were lysed and employed for quantitative RT-PCR to assess GS mRNA levels, as described in Materials and Methods. A representative experiment, repeated twice with comparable results, is shown.

untreated cells. While GS activity was stimulated by more than 400% (Panel A), GS protein was 6-fold more abundant in lysates of ASNase-treated cells than in untreated controls (Panel C). Enhanced GS expression and activity were detected also in ARJ cells maintained for 48h in the presence of ASNase. These findings suggest that ASNase-induced expression of GS protein and activity requires prolonged times of incubation with the antitumor enzyme.

The different levels of expression and activity of GS were not related to GS mRNA levels. Indeed, the determination of GS mRNA levels in Jensen cells, maintained in the absence of ASNase, and in ARJ cells, incubated either in the absence or in the presence of

Table 1. The effects of MSO and ASNase on the intracellular amino acid pool. N.D.: not detectable (amino acid content < 0.5 nmol/mg of protein).

	Amino acid content (nmol/mg of protein)							
	24h treatment				48h treatment			
	Asp	Asn	Glu	Gln	Asp	Asn	Glu	Gln
Control	49.4	42.2	237.4	65.5	42.5	47.2	188.8	51.5
MSO	42.8	37.6	232.2	55.4	46.5	51.4	197.5	51.4
ASNase	37.5	0.50	143.9	6.9	39.6	1.20	163.7	14.3
ASNase+MSO	33.2	N.D	228.0	N.D	34.5	N.D	219.1	N.D

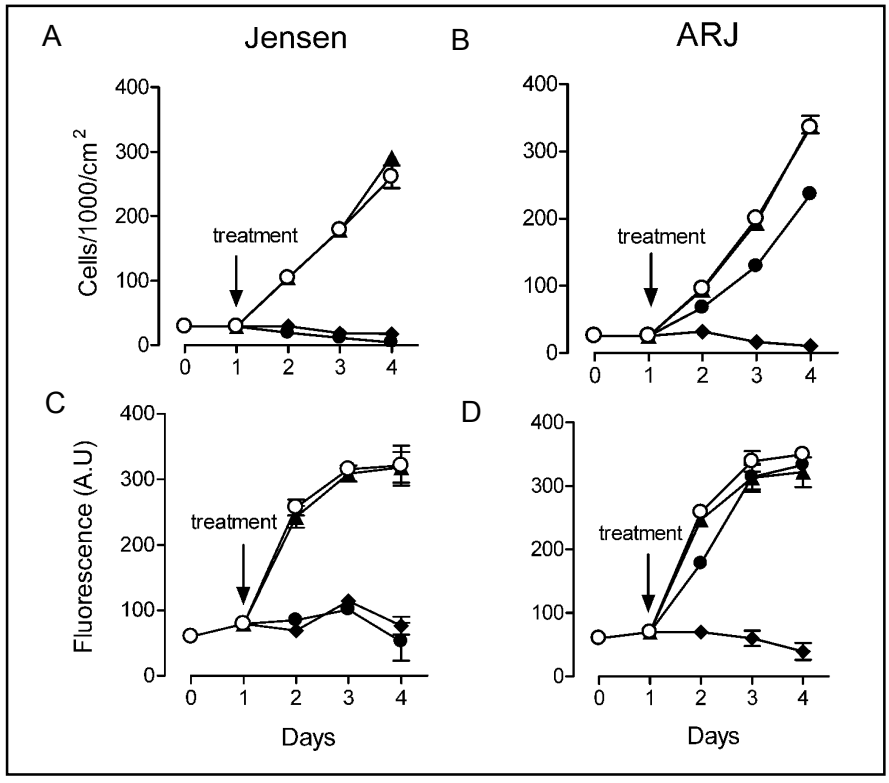

Fig. 7. Effects of L-methionine-sulfoximine (MSO) on the growth of Jensen and ARJ cells. One day after seeding (day 0), Jensen (Panels A and C) and ARJ (Panels B and D) cells were incubated in fresh growth medium in the absence (○) or in the presence of ASNase (●), 1 mM MSO (▲), or both (◆). At the indicated times cell number (Panels A and B) or cell viability (Panels C and D) were determined in parallel cultures as described under Methods. Points are means of three independent determinations with SD shown.

ASNase (Fig. 6), indicated that no significant induction of GS mRNA was observed during ASNase treatment.

Effects of GS inhibition on cell growth and viability

In the representative experiment shown in Figure 7, the effects of L-methionine sulfoximine (MSO), an irreversible inhibitor of GS, were tested in both Jensen (Panels A and C) and ARJ cells (Panels B and D), in the absence or in the presence of ASNase. As expected, in Jensen cells, but not in ARJ cells, ASNase had severe cytotoxic effects, either in terms of cell number (Panels A and B) or of cell viability, assessed with the resazurin assay (Panels C and D). When also MSO was added in the presence of ASNase, a complete proliferative block and a decrease in cell viability was observed in both cell types. Dose response analysis indicated that EC_{50} of MSO on cell viability after a 48h-treatment was 8.04 ± 0.580 μM (not shown). When added in the absence of the antitumor enzyme, MSO did not affect appreciably the proliferation or the viability of either cell population even after several days of treatment.

The effects of GS inhibition on the intracellular amino acid pool were determined after 24 or 48h of cell treatment with MSO. The results, presented in Table 1, indicate that no restoration of the cell contents of glutamine and asparagine was observed in cells incubated in the presence of the GS inhibitor. Interestingly, a decrease in glutamate content was observed in ASNase treated cells. The decrease was completely reversed if MSO was present during ASNase treatment.

The images reported in Figure 8 demonstrate that the inhibitor was without evident effects on either Jensen or ARJ cells in the absence of ASNase (Panels A and C), while it caused evident cell loss in the presence of the antitumor enzyme (Panels B and D). A morphological analysis of the cytotoxicity induced by MSO was performed with CLSM in ARJ cells preloaded with

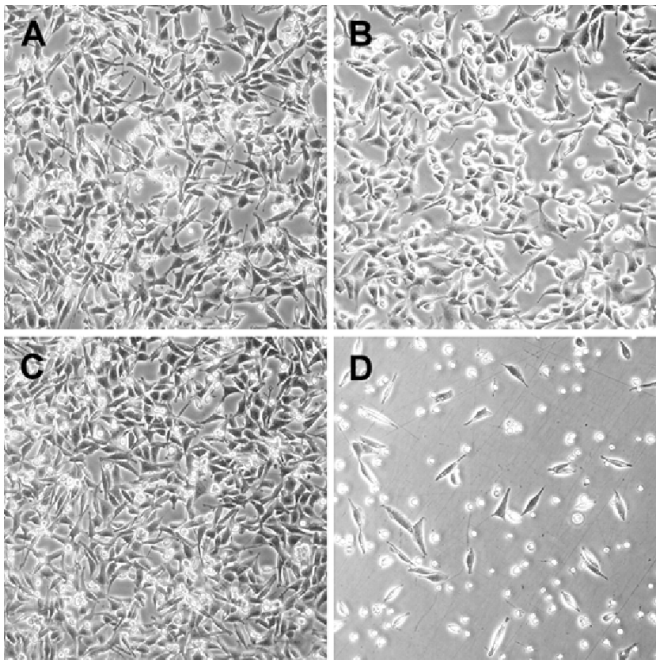

Fig. 8. Effects of L-methionine-sulfoximine (MSO) on ARJ cells. Images of ARJ cells were taken after a 48h-incubation in the absence (Panel A) or in the presence of 5 IU/ml ASNase (Panel B), 1 mM MSO (Panel C), or both drugs (Panel D). x120.

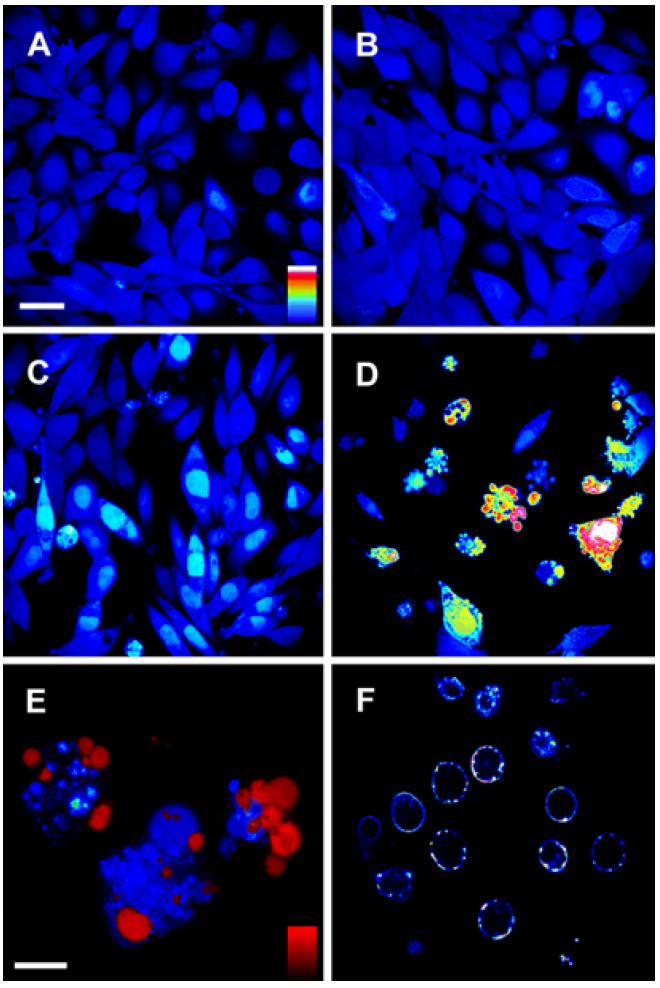

Fig. 9. Cytotoxic effects of MSO in ASNase-treated ARJ cells. ARJ cells were seeded on four-chamber coverslips. Panels A-E. After 24h medium was changed with fresh growth medium (Panel A) or with fresh growth medium supplemented with 1 mM of MSO (Panel B), 5 IU/ml ASNase (Panel C), or both (Panels D and E), and incubated for supplemental 48h. Before image acquisition cells were loaded for 10 min with calcein-AM and propidium iodide (see Methods). In Panel D morphological features corresponding to different steps through the apoptotic process, such as blebbing and apoptotic bodies, are shown. Panel E: Higher magnification of a late apoptotic stage with nuclear fragmentation. Loss of membrane competence is evidenced by chromatin positivity to propidium iodide (red) and vanishing calcein signal (blue). Panel F: Cells were incubated for 24h in the presence of both 5 IU/ml of ASNase and 1 mM MSO. Before image acquisition membranes were tagged for 5 min with annexin-V (see Methods). In the acquired images, the intensity of calcein and annexin signals is rendered in discrete pseudocolor scales (range 0-255) of iris (palette included in Panel A). The intensity of propidium signal is rendered in a scale of reds (palette included in Panel E). Scale bar: Panels A-D and F=20 μm; Panel E = 5 μm.

calcein-AM (Fig. 9). Compared with untreated controls (Panel A), cells exhibited only minor changes upon 48h-incubation with either MSO (Panel B) or ASNase (Panel C). In contrast, when the two agents were both present (Panel D), impressive changes were detected such as marked enhancement of calcein signal in the nuclear zone, cell shrinkage and rounding, fragmentation and production of intensely positive, round bodies. However, most cells were calcein-positive, thus pointing to the substantial preservation of membrane integrity. At the same time point, positivity to propidium iodide was detected only in some rounded cell fragments (Panel E). Interestingly, after 24h of treatment with the two drugs, many cells were clearly annexin V-positive (Panel F). These results indicate that the combined treatment with ASNase and MSO produced typical apoptotic changes in ARJ cells.

Effects of MSO on the cell content of glutathione

MSO may affect the cell content of glutathione (GSH) through the reversible inhibition of γ-glutamylcysteine synthetase (γ-GS), the rate limiting enzyme for GSH synthesis [31]. To ascertain if the effect of the inhibitor on the viability of ARJ cells was referable

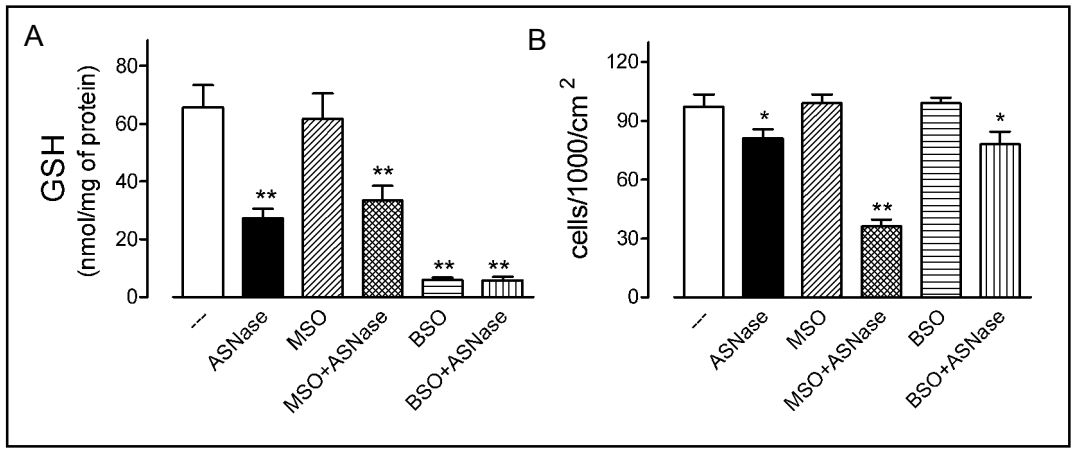

Fig. 10. Effects of MSO on the cell content of glutathione. ARJ cells were incubated for 24h in the absence or in the presence of ASNase (5 IU/ml), MSO (1 mM), MSO + ASNase, buthionine L-sulfoximine (BSO, 100 μM), or BSO + ASNase, as indicated. The cell content of glutathione (Panel A) and cell number (Panel B) were then determined as described in Methods. Bars are means of 6 independent determinations with SD indicated. The experiment was repeated twice with similar results. * $p < 0.05$; ** $p < 0.01$ vs. control (no treatment).

to changes in the cell content of the tripeptide, we have compared cell glutathione (Fig. 10, Panel A) and cell number (Panel B) after a 24h-incubation with MSO, ASNase, or both drugs. The effect of L-buthionine-sulfoximine (BSO), which is a 100-fold more potent inhibitor of γ-GS than MSO [31, 32], was also determined. A marked fall in the cell content of GSH and a slight decrease in cell number were detected upon ASNase treatment. MSO had no significant effect on cell GSH when employed alone. Moreover, it did not produce a further GSH decrease when employed together with ASNase, although, as expected, a massive cell loss was detected under this condition. BSO produced a very marked drop in GSH content that decreased by more than 90% either in the absence or in the presence of ASNase. However, these dramatic effects on cell GSH were not associated to changes in cell viability. Indeed, neither BSO significantly lowered cell number, when employed alone, nor it exerted any additive effect on cell viability when used together with ASNase.

Discussion

The results presented in this contribution demonstrate that, in the presence of asparaginase, survival and proliferation of ARJ cells, an ASNase-resistant line derived from Jensen sarcoma cells, strictly depend on glutamine synthetase (GS) activity. This conclusion is based on the following evidences: i) upon chronic incubation in the presence of ASNase, resistant cells exhibit progressively higher GS protein expression and activity compared to untreated controls; ii) the simultaneous treatment with ASNase and MSO, a GS inhibitor, overcomes the resistance to the antitumor enzyme and rapidly triggers a typical apoptosis. However, MSO has no effect on cell viability when employed alone. Thus, the inhibitor does not exert a cytotoxic effect per se but synergizes the adverse metabolic effects of ASNase.

In the absence of ASNase, sensitive Jensen cells and resistant ARJ counterparts exhibit comparable GS activity and expression. Thus, GS overexpression does not seem a marker of ASNase resistance but, rather, it represents an adaptation to ASNase treatment. It is noteworthy that a significant induction of GS activity in ASNase-resistant cells requires such a long exposure to ASNase to render unfeasible parallel determinations in ASNase-sensitive cell populations which, at that time, already present unequivocal signs of massive apoptosis. These findings suggest that, besides AS overexpression, ASNase-resistant cells are also endowed with anomalies that slow down cell proliferation or hinder the efficiency of apoptotic pathways, thus allowing the full development of proper metabolic adaptations to the metabolic stress imposed by ASNase. Interestingly, a recent analysis performed in B-lineage ALL has indicated that the expression of at least 54 genes is significantly discriminated between ASNase-resistant and sensitive cells [11]. Of these, while overexpressed genes are involved in protein metabolism, several genes involved in cell death or cell proliferation are down regulated [11].

In mammalian tissues, GS is highly regulated not only at transcriptional level [33, 34], where glucocorticoids constitute major inducers , but also at translational [36] and protein levels [37]. Recently, the induction of GS gene

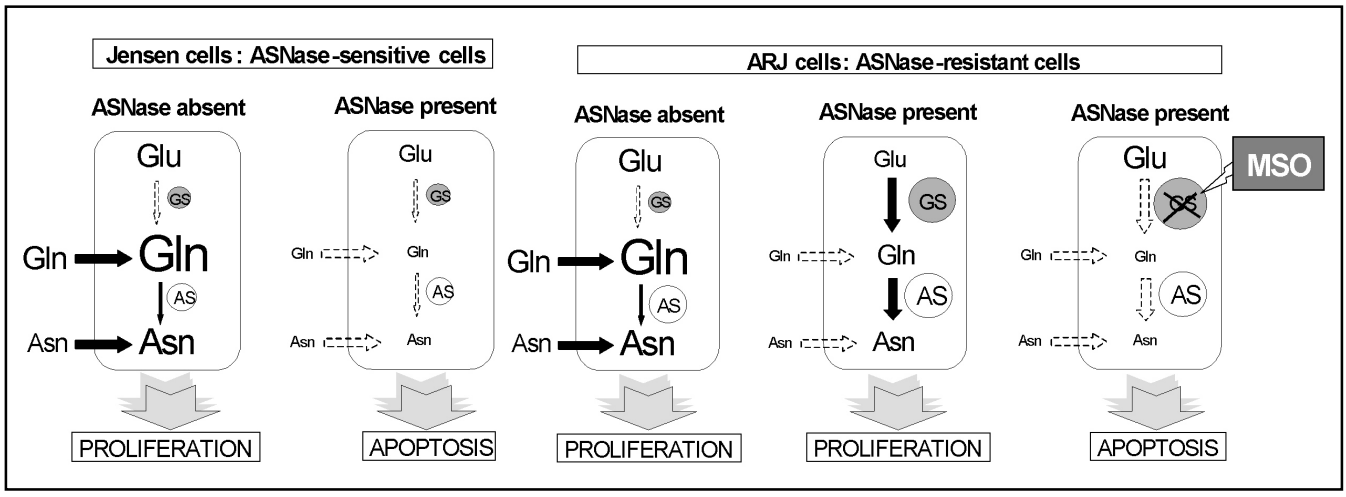

Fig. 11. Glutamine synthetase activity is required for cell survival in the presence of ASNase. See text for explanation.

has been also shown to be a target of the Wnt/ β-catenin signaling system [38], a pathway that is involved in stem cell self-renewal [39] and activated in several types of hematopoietic neoplasias [40-42]. However, Aslanian et al. [21] have showed that in MOLT-4 leukemia cells ASNase-resistant cells have higher GS activity but not increased GS mRNA abundance. In the present contribution, no significant difference of GS mRNA abundance is detected with qRT-PCR during ASNase treatment of resistant cells, when GS protein and activity are progressively increased. These data are not consistent with the hypothesis that ASNase treatment induces GS gene. Rather, they suggest that, as observed in examples of ASNase-independent glutamine starvation [43-45], the maintenance of low intracellular levels of glutamine by ASNase may prolong the shelf life of GS protein, thus leading to a slow, progressive overexpression of GS protein and to a stimulation of GS activity.

The possibility that the dramatic effects of MSO on the viability of ASNase-resistant cells are due to mechanisms other than GS inhibition appears unlikely. Although MSO is an inhibitor of γ-glutamylcysteine synthetase [31], the decrease of cell glutathione, observed upon the highly toxic, combined treatment with ASNase and MSO, is not different from that caused by ASNase alone, a condition that affects cell viability only modestly (see Fig. 10). Moreover, the cytotoxic effects of MSO on ARJ cells were not referable to interference on AS activity. Indeed, while 1 mM MSO abolished GS activity either if directly added to the cell extracts during the enzyme assay or added for 15h to the culture medium before the preparation of cell extracts, AS activity was completely unaffected by the inhibitor with both approaches (J. Uggeri and B.M. Rotoli, results not shown). Thus, the dramatic effect of MSO on ARJ cell survival implies that GS activity is of pivotal relevance for cell metabolism if ASNase is present in the extracellular medium.

A model to explain this critical dependence is offered in Figure 11. In the absence of ASNase (Figure 11, left), extracellular glutamine and asparagine satisfy cell metabolic requirements through membrane transport in both sensitive Jensen cells and resistant ARJ cells. The antitumor enzyme completely hydrolyzes extracellular glutamine and asparagine, thus nullifying the metabolic relevance of adaptive changes in membrane transport that, however, are fully comparable in sensitive and resistant cells (B.M. Rotoli and O. Bussolati, unpublished results). As a consequence, in the first hours of treatment with ASNase, cell glutamine and asparagine fall at very low values (Fig. 11, center). The depletion of cell asparagine occurs both in parental Jensen cells and in ARJ cells, although the latter cell line is endowed with a higher activity of asparagine synthetase (AS). To understand this apparent paradox, it should be considered that, under normal conditions, the intracellular concentration of glutamine largely exceeds the K_m of AS for the amino acid, which ranges from 1 to 2 mM [5]. In contrast, in ASNase-treated cells, cell glutamine may be rate limiting for asparagine synthesis. Indeed, considering the volume of cells treated with ASNase for 9h (6.9 ± 0.89 μl/mg of protein (B.M. Rotoli, unpublished results)), the intracellular concentration of glutamine would be much less than the K_m value of AS for the amino acid (see Fig.

2). With the prolongation of ASNase treatment, while sensitive parental cells have undergone apoptotic death, the expression and the activity of GS are raised in ARJ cells (see Fig. 5). As a consequence, a progressive, although partial, restoration of cell glutamine occurs. Under these conditions, cell asparagine becomes detectable again, although ASNase is still present in the extracellular compartment, and the cell population resumes an intense proliferative activity. The addition of MSO (Fig. 11, lower right) suppresses GS activity, blocks this adaptive mechanism and activates the apoptotic pathway, as expected for a severe glutamine depletion [46].

ASNase resistance is frequently observed in refractory ALL patients and significantly correlates with a poor clinical outcome both in newly diagnosed [47, 48] and in relapsed patients [49]. The results presented here should, therefore, prompt investigations aimed to assess if GS inhibition may be employed to induce apoptosis in ASNase-resistant cells.

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