Adipocytes Cause Leukemia Cell Resistance to L-Asparaginase via Release of Glutamine

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Abstract

Obesity is a significant risk factor for cancer. A link between obesity and a childhood cancer has been identified: obese children diagnosed with high-risk acute lymphoblastic leukemia (ALL) had a 50% greater risk of relapse than their lean counterparts. L-asparaginase (ASNase) is a first-line therapy for ALL that breaks down asparagine and glutamine, exploiting the fact that ALL cells are more dependent on these amino acids than other cells. In the present study, we investigated whether adipocytes, which produce significant quantities of glutamine, may counteract the effects of ASNase. In children being treated for high-risk ALL, obesity was not associated with altered plasma levels of asparagine or glutamine. However, glutamine synthetase was markedly increased in bone marrow adipocytes after induction chemotherapy. Obesity substantially impaired ASNase efficacy in mice transplanted with syngeneic ALL cells and, like in humans, without affecting plasma asparagine or glutamine levels. In coculture, adipocytes inhibited leukemic cell cytotoxicity induced by ASNase, and this protection was dependent on glutamine secretion. These findings suggest that adipocytes work in conjunction with other cells of the leukemia microenvironment to protect leukemia cells during ASNase treatment. Cancer Res; 73(10); 2998–3006. ©2013 AACR.

Introduction

Obesity is associated with a substantial increase in cancer incidence and mortality worldwide (1), with an estimated 20% of cancers in the United States due to obesity (2). In addition to increasing cancer incidence, obesity appears to decrease survival from some cancers, including acute lymphoblastic leukemia (ALL; refs. 3, 4). This impaired survival appears to be a direct effect of obesity, and not due to increased risk of treatment complications or toxicities (3). The mechanisms linking obesity to cancer still remain elusive (5). In vivo and in vitro models developed in our laboratory (6) have shown that obesity impairs the efficacy of chemotherapeutics against ALL cells, likely mediated by adipocytes. As leukemia affects 2,000 children (7) and more than 40,000 adults per year in the United States (8), understanding and reversing the associations between obesity and leukemia relapse could prevent significant cancer mortality.

Materials and Methods

Human subjects

Bone marrow biopsy and blood samples were obtained from 19 patients, 10 to 18 years old before and during treatment for high-risk leukemia. Obesity was defined as a BMI greater than or equal to the 95th percentile according to Centers for Disease Control and Prevention guidelines. All patients were treated according to the high-risk CCG/COG protocol, involving a 4-drug induction regimen including 4 weeks of steroids and PEG ASNase (25,000 IU/m², single dose either intramuscularly...
or intravenously). Samples were obtained after written informed consent and assent were obtained, under a protocol approved by the CHLA Committee on Clinical Investigation (Institutional Review Board). Characteristics of the study population are presented in Supplementary Table S1.

Cell lines and culture
The 3T3-L1 cells from the American Type Culture Collection (ATCC) were differentiated into adipocytes as previously described (6), and used for experiments between days +7 and +14 of differentiation. Undifferentiated 3T3-L1 fibroblasts were irradiated and plated at confluence. The bone marrow-derived mesenchymal cell line, OP9, was differentiated into adipocytes in a similar manner.

Murine pre-B ALL cells were previously isolated from a BCR/ABL transgenic mouse (8093 cells; ref. 22). Human leukemia cell lines were obtained from the ATCC and the German Collection of Cell Lines (DSMZ), and included BV173 (Pre B Ph+ ALL), K562 (chronic myelogenous leukemia), Molt-4 (T cell leukemia), Nalm-6 (B cell precursor leukemia), RCH-ACV (pre-B ALL with an E2A-PBX1 fusion protein), RS4;11 (pre-B t(4;11) ALL), SD-1 (pre-B Ph+ ALL), SEM (B cell precursor), and SupB15 (B cell precursor).

Primary human leukemia cells were passaged in NOD.Cg-Pkd+/m2Il2rg−/−m119b/2SzJ mice (Jackson Laboratories) and harvested from the spleens of these mice and cultured on irradiated OP9 feeder layers for all experiments. These cells were kindly provided by Markus Müschen, Yong-Mi Kim, and Nora Heisterkamp (23). These cells are, hereafter, referred to as human leukemia cells. US7 and US7R were from a Ph-negative patient before and after the patient developed relapse. TXL-2 human leukemia cells. US7 and BLQ-1 ALL cells were Ph-positive and taken from patients patient before and after the patient developed relapse. TXL-2 human leukemia cells. US7 and US7R were from a Ph-negative human leukemia cell line, OP9, was differentiated into adipocytes in a similar manner.

Coculture experiments
Leukemia cells were cultured with fibroblasts, adipocytes, or no feeder layer. In experiments of drug resistance, ASNase was added to achieve an approximate IC50. After 72 hours, cells were counted as above. In additional experiments, 8093 cells were cultured in 0.4-μm pore size TransWells (Corning, Inc.) separated from the feeder layers. To inhibit GS, adipocytes were treated overnight with 1.5 mmol/L methionine sulfone (MSO), and washed 3 times before experiments. Complete GS inhibition was confirmed by HPLC measurement of GLN secretion. Erwinase investigational drug was kindly provided for experimental evaluations by Dr. Paul Plourde (Jazz Pharmaceuticals, Langhorne, Pennsylvania), and used at a dose with equivalent asparagine-deamination activity, as determined by Nessler’s reaction (24). The 8093 cells in TransWells were analyzed for cell cycle and apoptosis after 48 hours in culture by bromodeoxyuridine (BrdUrd) incorporation (BD Biosciences) on a FACSkan (BD Biosciences, CellQuest software).

Amino acid analysis and sample preparation
To measure amino acid secretion, feeder layers were cultured in 24 well plates as described earlier, washed with PBS twice, then cultured in 1 mL per well of AGF media. Media was collected, filtered through 0.45-μm syringe filters, and snap frozen. All samples were stored at -80°C until assay.

Tissue explant amino acid production was measured using fat pads from perfused mice. Fat was cut into approximately 50 mg pieces, washed thoroughly with PBS, and placed in 24-well culture plates with 1 mL AGF media for conditioning.

Blood was sampled from the submandibular plexus of unanesthetized mice into BD EDTA-coated Microtainer tubes, cooled to 4°C to prevent ex vivo deamination, spun at 13,000g, and the plasma obtained was snap frozen.

Murine plasma and conditioned media amino acid measurements were carried out as previously described (25) with slight modifications. Samples were deproteinized using 20% 5-sulfosalicylic acid containing 1.0 mmol/L 1-Norleucine (internal standard, Sigma). Samples were dried in a speedvac, resuspended with a derivatization reagent (methanol, TEA, H2O, and PITC at 7:1:1:1 ratios) and dried again. Samples were measured using a Waters 1525 Binary HPLC pump and absorbance was detected at 254 nm.

Clinical plasma amino acid samples were measured in the clinical laboratory. Briefly, samples were deproteinized with 5-sulfosalicylic acid followed by addition of N3-methylarginine. On-line derivatization was carried out using mixture solution of o-phthaldehyde and 3-mercaptopropionic acid. After derivatization and neutralization, 5 μL was injected to HPLC.
Western blotting

Protein was extracted from leukemia cells, cultured adipocytes, and fat tissue from perfused mice as previously described (6). Cell lysates were separated on Novex Tris-Glycine precast gels (Invitrogen) and transferred to 0.2 μm nitrocellulose membranes (Invitrogen). Membranes were then incubated with a mouse anti-GS monoclonal antibody (Abcam), a rabbit anti-ASNS polyclonal antibody (Abcam), or rabbit anti-GAPDH antibody (Cell Signaling Technologies), with appropriate horseradish peroxidase-conjugated secondary antibody from Cell Signaling Technologies. Membranes were developed using the HyGLO HRP detection kit (Denville). To allow intergel comparison of fat-pad Western blots, K562 cell lysates (positive for ASNS, GS, and GAPDH) were run on all gels and used to correct for exposure time and run variances. Band intensity for ASNS, GS, and GAPDH) were run on all gels and used to correct for exposure time and run variances. Band intensity was quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

Immunohistochemistry

Paraformaldehyde-fixed bone marrow samples were embedded with paraffin, sliced, and mounted by the CHLA Pathology Core. Sections were subjected to antigen retrieval with Tris-EDTA, pH 8.0, with steam for 30 minutes. Endogenous peroxidases were inactivated with 3% H₂O₂. Nonspecific staining was blocked with 2.5% normal goat serum before staining with rabbit anti-mouse GS or ASNS (Abcam), and detected with the ImmPRESS reagent (Vector Laboratories Inc.) containing polymerized peroxidase labeled goat anti-rabbit immunoglobulin (mouse adsorbed). The reaction was detected with ImmPACT DAB (Vector Laboratories Inc.) and counterstained with Mayer’s hematoxylin. Images were acquired on a Zeiss Axiosoplan Microscope (×40/1.3) with a SPOT QE Color Digital Camera.

Statistical analysis

Body weights were compared with unpaired, 2-sided t tests. Survival curves were generated by Kaplan–Meier Life Tables, and compared using Cox Proportional Hazards. Each coculture experiment was conducted on different days or using different cell thaws, and the averages of 3 triplicate wells for each condition in each experiment were calculated. Paired t tests were used to compare number of viable leukemia cells over the various feeder layers. A P-value of less than 0.05 was considered significant.

Results

Adipocytes in the leukemia microenvironmnet produce glutamine

We and others have previously found that obesity worsens treatment outcome in adolescents with high-risk ALL (3, 4). To test whether obesity might impair ASNase efficacy, we measured plasma levels of amino acids in adolescents before and after induction chemotherapy for high-risk ALL, which included a single dose of PEG-ASNase. There were no significant differences in amino acid levels between obese and lean subjects, with ASN being fully suppressed by ASNase, and GLN largely unaffected in both groups (Fig. 1A).

Because plasma amino acid levels might not reflect conditions in the leukemia microenvironment, we examined bone marrow biopsy specimens from 4 obese and 4 lean adolescent leukemia patients for expression of ASNS and GS, the rate-limiting steps for ASN and GLN production. Cells positive for ASNS were found throughout the marrow, and expression appeared unaltered after treatment (Fig. 1B). Before treatment, GS expression was low and appeared to be localized in scattered adipocytes. After treatment, there was a large increase in the area occupied by adipocytes, as has been previously shown (26), together with an apparent increase of GS in these cells.

Obesity impairs L-asparaginase efficacy in mice

To test whether obesity per se can cause ASNase resistance, we implanted diet-induced obese (DIO; 41.5 ± 4.4 g) and nonobese (30.4 ± 2.0 g; P < 0.001) male mice with syngeneic leukemia cells at 20 ± 2 weeks of age (6). ASNase, administered proportional to body weight, prolonged survival in nonobese mice over vehicle (33.4 ± 12.0 vs. 26.6 ± 5.6 days, P < 0.01), but yielded no detectible survival benefit to obese mice (26.4 ± 7.5 days, P < 0.0001 vs. non-obese, P = n.s. vs. vehicle; Fig. 2A). There was no difference in survival between vehicle-treated nonobese and obese mice (28 ± 4.5 vs. 26 ± 4.2, data not shown). Obesity similarly decreased survival after treatment with the more stable pegylated form of ASNase (P < 0.05 obese vs. nonobese; Fig. 2B). Plasma amino acid levels showed a similar pattern to that of humans, with no differences between diet groups (Fig. 2C). Nor was there any significant difference between plasma ASNase activity following a single dose of E. coli ASNase between diet groups, although obese mice tended to have higher levels than nonobese mice (Fig. 2D). Thus, similar to humans, obese mice exhibited impaired leukemia outcome with no significant differences in plasma ASN or GLN.

Unlike in humans, we did not observe a change in bone marrow GS expression in mice treated with ASNase (Supplementary Fig. S1). Likewise, although GS was dramatically higher in 3T3-L1 adipocytes than in undifferentiated 3T3-L1 cells, as has been previously shown (27), expression of ASNS and GS appeared to decrease following 72 hours of culture in ASN- and GLN-depleted (AGF) media (Fig. 2E). We, therefore, considered whether the increase in GS found in human samples could be caused by another chemotherapy given during induction. Indeed, dexamethasone increased 3T3-L1 adipocyte GS levels approximately 2-fold, as has been shown in other studies (28).

As we have shown that ALL cells infiltrate adipose tissue during treatment (6), we next investigated GS expression in adipose tissue. Mouse adipose tissue expressed detectible GS, but not ASNS, by Western blot analysis (Fig. 2F). Furthermore, fat tissue explants from wild-type C57 mice secreted GLN...
(105.69 ± 53.00 nmol/100 mg tissue per 24 hours) but not ASN, into the media (Table 1). Dosing obese mice with ASNase daily for 5 days resulted in an approximately 50% increase in GS expression in subcutaneous fat (Fig. 2F), but no overall effect in other fat pads (Supplementary Fig. S2). We observed no significant differences between GS expression in fat pads between obese and lean mice (Supplementary Fig. S2). ASNase dosing also did not lead to detectible ASNS protein expression in fat pads (not shown).

In vitro, 3T3-L1 adipocytes secreted a small amount of ASN. Supplementing the media with the required substrates for ASN synthesis, aspartic acid and GLN, together with the GLN precursor glutamic acid, increased ASN secretion by adipocytes (Table 1). Adipocytes secreted a substantial amount of

Figure 1. Effect of obesity on plasma and bone marrow asparagine and glutamine in patients following L-asparaginase treatment. A, plasma amino acid measurements of ASN (left) and GLN (right) in lean and obese patients during induction chemotherapy for newly diagnosed high-risk ALL, treated on CCG1961, including a single dose of 2,500 IU/m² of pegylated L-asparaginase. B, ASN synthetase (ASNS, left) and GLN synthetase (GS, right) staining of bone marrow taken from 4 lean (Pt1-4) and 4 obese (Pt5-8) children before and after induction chemotherapy. Images were acquired on a Zeiss Axioplan Microscope (×40/1.3) with a SPOT QE Color Digital Camera. Calibration bar (top right image) is 50 μm.
GLN, approximately 18-fold more than undifferentiated 3T3-L1 cells.

Adipocytes protect leukemia cells from ASNase via GLN production

To determine whether adipocytes could protect ALL cells from ASNase, we cultured 8093 murine ALL cells over irradiated 3T3-L1 fibroblast-like cells or differentiated 3T3-L1 adipocytes, in media with 1 IU/mL ASNase. The 3T3-L1 adipocytes protected ALL cells from ASNase both with and without direct contact (Fig. 3A). A similar pattern was observed with adipocytes differentiated from OP9 bone marrow mesenchymal cells (Fig. 3B). Adipocyte protection was associated with decreased apoptosis and increased cell cycling during ASNase exposure (Fig. 3C; Supplementary Fig. S3).

As adipocytes produce both ASN and GLN, we next tested whether either of these amino acids were responsible for adipocyte protection of ALL cells from ASNase. Twice-daily addition of ASN had no effect on ASNase cytotoxicity (Fig. 4A), whereas GLN supplementation partially blocked ASNase cytotoxicity (Fig. 4B). Pretreatment with MSO, an irreversible inhibitor of GS, rendered adipocytes unable to protect ALL cells from ASNase (Fig. 4C). Similarly, use of Erwinase, a form of asparaginase with 5-fold greater glutaminase activity than *E. coli* ASNase (12), was able to inhibit the protective effect of adipocytes (Fig. 4D).

Adipocytes also protected human leukemia cell lines from both ASNase (not shown), and media lacking ASN and GLN (AGF media; Fig. 4E). To determine which amino acids human leukemia cells were sensitive to, we cultured 10 leukemia cell lines in media lacking ASN, GLN, or both (Fig. 5A). Only 3 of 10 human leukemia cell lines were sensitive to removal of ASN.

Table 1. ASN and GLN secreted by cells over 72 hours

<table>
<thead>
<tr>
<th>Cell type</th>
<th>ASN, nmol/mL</th>
<th>GLN, nmol/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3-L1 Fibro</td>
<td>&lt;0.005</td>
<td>23 ± 27</td>
</tr>
<tr>
<td>3T3-L1 Adipo (AGF)</td>
<td>23 ± 13</td>
<td>417 ± 176</td>
</tr>
<tr>
<td>3T3-L1 Adipo (MSO Treated)</td>
<td>87 ± 15</td>
<td>-</td>
</tr>
<tr>
<td>(+ ASP, GLU, GLN) a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 Adipo (MSO Treated)</td>
<td>1.6 ± 2.6</td>
<td>56 ± 50</td>
</tr>
<tr>
<td>Fat Explant (100 mg)</td>
<td>&lt;0.005</td>
<td>247 ± 43</td>
</tr>
</tbody>
</table>

*a400 μmol/L aspartic acid, 400 μmol/L glutamic acid, and 2,000 μmol/L glutamine supplemented.*
Adipocytes Cause Leukemia L-Asparaginase Resistance

Discussion

Although obesity has been recognized as a major factor in leukemia progression and relapse, the precise mechanism(s) by which obesity impairs treatment outcome remains unclear. In order to elucidate the role of obesity in leukemia treatment, we have investigated the use of the front-line chemotherapy L-asparaginase, which despite its use clinically for over 50 years, is still being studied to determine ideal treatment strategies. Several studies have shown improved patient outcome with more intense or longer treatment with ASNs (30, 31), while insufficient drug exposure, as in the case of silent hypersensitivity, is associated with higher risk of relapse (32). ASNs cytotoxicity relies on its ability to deplete ASN and GLN from plasma. This effectively starves lymphoid cells, which unlike most other cells are unable to sustain themselves through de novo production (11). Effective use of ASNase has traditionally been measured by the depletion of plasma ASN and GLN, or its surrogate, plasma asparaginase activity (33).

In our murine leukemia model, ASNs treatment was less effective in obese mice than nonobese mice. Notably, there was no significant difference in plasma amino acid levels between obese and nonobese mice at any timepoint, despite the dramatic difference in survival. Regardless of diet group, plasma ASN remained suppressed, while GLN began to recover within 12 hours. Similarly, in patient samples, GLN did not appear suppressed, although early timepoints were not sampled in this study. Although a rebound effect was found in other studies (34), its mechanism is unknown and may be the result of an increase in endogenous GLN synthesis or release of tissue GLN during cachexia. We documented a dramatic increase in GS in bone marrow collected from ALL patients at the end of induction, but not in adipose tissue or bone marrow following ASNs treatment in mice. Although it is possible that this difference results from species-specific response to ASN and/or GLN depletion in the plasma, it is more likely that it results from the use of combination chemotherapy in human ALL. In particular, glucocorticoids have been shown to induce GS in some tissues (28), and we found that dexamethasone treatment induced an increase in GS protein levels in 3T3-L1 adipocytes. The potential for glucocorticoid induction of GS to impair ASNs efficacy, particularly in the context of the tumor microenvironment, may be the target of future studies.

GLN is the most abundant amino acid in plasma, and necessary for nucleotide and amino acid synthesis. Although a nonessential amino acid, a variety of human cancer cell lines, including pancreatic cancer, colon cancer, small cell lung cancer, and leukemia have been shown to be highly dependent on GLN for proliferation and survival (35). GLN depletion can induce apoptosis in cancer cells, with a higher selectivity than glucose depletion (36). In addition, leukemia cells adapt to ASNs treatment by increasing synthesis and transport of GLN, and inhibition of GS has been shown to resensitize resistant leukemia lines to ASNs (37). These studies highlight the possibility of targeting GLN metabolism to combat ASNs resistance. However, studies aimed at systemic inhibition of GLN metabolism have been limited due to toxicity (38, 39).

Using our in vitro system we found that adipocytes protect leukemia cells both from L-asparaginase and from ASN/GLN starvation, primarily through secretion of GLN. As adipose tissue secretes significant amounts of GLN into interstitial fluid (40), and, as we have previously shown, leukemia cells from the media, while 8 of 10 were sensitive to GLN removal. All lines tested were most sensitive to removal of both amino acids. In similar tests with 4 human leukemia cells (BLQ1, TdT2, U87, and USTR; ref. 23), 1 line was sensitive to ASN removal, 3 were sensitive to GLN removal, and all 4 were sensitive to removal of both amino acids (Fig. 5B). Sensitivity to either amino acid could not be explained by ASNS or GS expression (Fig. 5C; ref. 29).

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infiltrate adipose tissue (6), it is possible that adipose tissue is a sanctuary where ALL cells are protected from ASNase activity. This may also happen in the bone marrow after initiation of chemotherapy, when the number of adipocytes and their expression of GS both increase dramatically. As obese patients have large amounts of body fat, it might increase the probability that the adipocyte-mediated protection from ASNase may become clinically relevant, and be one of the factors leading to increased relapse rate in obese ALL patients.

Our results complement the recent finding that bone marrow–derived mesenchymal cells (MSC) protect leukemia from ASNase treatment through ASN secretion (19–21). Laranjeira and colleagues showed that leukemia-cell secretion of IGFBP-7 increased ASN synthesis by stromal cells (21). Interestingly, this effect was further increased by the addition of insulin and IGF-1, both of which are elevated in obesity (41).

Two recent publications have questioned the role of bone marrow MSCs as a source of ASN during ASNase treatment in patients (42, 43). These studies found that, upon treatment, the extent of depletion of ASN and GLN were similar in the bone marrow and the peripheral blood. Interestingly, both studies showed higher aspartic acid concentrations in the marrow than in the peripheral blood, possibly indicating either high rates of de novo aspartic acid production or a greater turnover of ASN in the microenvironment. Further investigation into the extent to which known sanctuary sites may counteract the depletion of ASN and GLN from blood should be carried out. In particular, it is possible that tissues with poor capillarization, such as adipose tissue in obese patients (44), may provide an environment more removed from ASNase treatment.

Several groups have been developing alternative ASNase preparations with lower glutaminase activity (45–47). The goal is minimizing the side effects associated with GLN starvation such as immunosuppression and pancreatitis. One study looked into the possibility of supplementing the diet of rats treated with ASNase with alanyl-glutamine to counteract the immunosuppressive effects of GLN depletion (34). In our study, 8 out of 9 commercial cell lines and 4 out of 4 human leukemia cells were more sensitive to GLN than ASN starvation and, in nearly all cases, depletion of both amino acids had a stronger effect than either amino acid individually. These results are in line with a study conducted by Offman (47), who showed that, in their recombinant ASNase, some cell lines responded better to wild-type ASNase than an asparaginase with decreased...
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Figure 5. Leukemia cells show greater dependence on glutamine than asparagine. A, proliferation of 1 murine and 9 human leukemia cell lines when cultured in asparagine-free media (white bars), glutamine-free media (horizontally lined bars), or media lacking both amino acids (hatched black bars) compared with complete media (vertically lined bars, n = 4). B, proliferation of 4 human leukemia cells in direct coculture with OP9 feeder layers in media as in A (n = 4). C, representative Western blot of ASNS and GS protein expression before and after an EC50 dose of ASNase in 4 human cell lines. ', P < 0.05

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: E. Ehsanipour, J.W. Behan, A. Butturini, V.I. Avramis, S.D. Mittelman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Ehsanipour, A. Butturini, V.I. Avramis, S.D. Mittelman
Writing, review, and/or revision of the manuscript: E. Ehsanipour, X. Wang, V.I. Avramis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Ehsanipour, X. Wang, V.I. Avramis
Other (provided the E. coli Asparaginase and Erwinase drugs, the HPLC instruments, oversight of quality control of methodology for the AA assays, review and correction of article draft, and shared grant funding as well as new applications on asparaginase sensitivity and resistance in ALL): V.I. Avramis

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