Stressing Out Over Survival: Glutamine as an Apoptotic Modulator

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Introduction. **The amino acid glutamine (GLN) has received considerable attention as a potential therapeutic adjuvant in critical illness and in improving postoperative clinical outcomes. Most studies on the role of GLN in cellular physiology have historically focused on its anabolic roles in specific cell types and its contribution to growth in cancer cells. However, an emerging body of work that examines the consequences of GLN deprivation on cellular survival and gene expression has constructed a new paradigm for this amino acid, namely, that limited extracellular GLN supplies modulate stress and apoptotic responses.**

Methods. **A survey of the scientific literature was conducted on GLN in cell survival signaling and apoptosis. Work from our laboratory in liver cancer cells also was included in this review.**

Results. **Most studies on this topic have used mammalian cell lines derived from the gut, immune system (including hybridomas), and various cancers. GLN limitation, even in the presence of an adequate glucose supply, impacts stress-related gene expression, differentially modulates receptor-mediated apoptosis, and directly elicits apoptosis through signaling mechanisms and caspase cascades that are specific to cell type. To date, GLN transporters, cellular hydration, glutaminyl-tRNA synthetase, ATP levels, mRNA stability, and glutathione economy have been variably implicated in GLN-dependent survival signaling.**

Conclusion. **The cell type-specific mechanisms underlying the regulatory role of GLN in cell survival continue to unfold at a steady pace through *in vitro* studies. These results have collectively provided testable hypotheses for further *in vivo* studies into their physiological relevance during GLN "nutritional pharmacology."** © 2005 Elsevier Inc. All rights reserved.

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INTRODUCTION

In the world of academic surgery, a desire to improve postoperative patient care and outcomes drives much of the research efforts. A major theme that has developed in this clinical realm is the concept of "nutritional pharmacology," where specific nutrients are added in excess to patient nutritional regimens in an effort to enhance convalescence and reduce morbidity, mortality, and hospitalization time during critical illness. One of the more highly studied nutrients for this purpose is the amino acid glutamine (GLN). Classified as a "nonessential" amino acid by most biochemistry texts because of the ability of most cells to produce it, GLN has been reclassified as "conditionally essential" during the last decade because physiological demand often exceeds the cellular capacity to produce it endogenously during critical illness. The resulting GLN deficit can adversely affect cells that rely heavily on this amino acid for normal function, such as those of the gastrointestinal tract and immune system. The merits of GLN-supplemented nutritional regimens in improving outcomes has been evaluated and roundly debated. Indeed, most roundtable discussions on this topic end with the consensus that "we need more randomized prospective clinical trials" [1, 2]. Evidence for the clinical efficacy of GLN in critical illness will not be further discussed here; for very good recent reviews on this topic, the reader is referred to other sources [3–6]. Instead, GLN supplementation in patient care was broached to provide context for a more academic topic: What are the cellular mechanisms by which GLN may potentially impact clinical outcomes? For clinicians, demonstrated efficacy of "glutamine therapy" would be sufficient, but as scientists, this is a topic that we are compelled to pursue.

The study of GLN in cellular physiology historically has focused on its anabolic effects, namely, its role as a metabolic precursor and physiological regulator of DNA and protein synthesis in cellular growth. GLN is particularly important for the growth, survival, and physiological health of actively dividing cells in the body such as fibroblasts [7, 8], enterocytes (intestinal epithelia) [9, 10], and lymphocytes [11, 12]. Indeed, most of these cell types have been shown to possess a GLN-intensive metabolic profile, almost to the point of auxotrophy. Not surprisingly, clinical conditions for which GLN therapy has been proposed and tested, such as after bone marrow transplant, maintenance of a "healthy bowel" after radiation therapy or resection, after burn injury and during chemotherapy, involve these cell types. Cancer cells also are avid GLN consumers [13–17]. In an effort to better understand the impact of GLN provision on energy metabolism, a recent study examined the effects of GLN depletion and subsequent repletion on metabolic and gene expression profiles in mouse hepatoma cells via microarray analysis and found that GLN depletion globally downregulated metabolism [18], which is not surprising.

Just as cancer biologists have refocused their attention from growth (oncogenes) to evasion of programmed cell death (apoptosis and tumor suppressor genes) and the integration of the two processes during the last decade, recent studies have suggested that GLN may act not only to promote growth but also to suppress apoptosis and to evoke and modulate stress responses. This review therefore focuses on a new twist to an old theme, namely, the merits of GLN in supporting cellular physiology, not from an anabolic perspective, but rather as a survival factor. Hereafter, GLN metabolism and apoptosis are briefly reviewed, followed by a retrospective analysis of studies in specific cell types (enterocytes, cells of the immune system and cancer cells) showing a role for GLN in cell survival and in eliciting and modulating cellular stress responses, including implicated mechanisms for GLN effects.

GLN METABOLISM

As the most abundant amino acid in the plasma at levels around 0.6 mM, GLN exhibits the most rapid intracellular turnover rate of all amino acids [19]. Because of its abundance and rapid metabolism, GLN has been described as the major intercellular nontoxic ammonia shuttle in the body. GLN also serves as a metabolic intermediate contributing carbon and nitrogen for the synthesis of other amino acids, nucleic acids, fatty acids and proteins [20, 21]. Because GLN is a major source for cellular glutamate, it can serve as a rate-limiting step in the synthesis of glutathione (GSH), a tripeptide consisting of glutamate, glycine, and cysteine, that serves to protect cells from oxidative stress [22, 23]. GLN is also an important osmolyte for cell volume control [24] and has been shown to increase hepatocyte cell volume, eliciting anabolic processes like DNA, RNA, and protein synthesis [25]. GLN homeostasis in the body is largely maintained by the enzymes glutamine synthetase (glutamate + NH_3 + ATP → GLN) and glutaminase (GLN → glutamate + NH_3) [26]. Historically, cells propagated in culture by growth and serial passage were shown to require GLN at concentrations higher than any other amino acid, as originally shown by Harry Eagle in the 1950s [27–29]. As we approach the 50th anniversary of his seminal work, the cellular and molecular events that underlie these time-tested observations on GLN reliance in cultured cells continue to take shape, but are by no means settled.

APOPTOSIS

Programmed cell death (PCD) is an evolutionarily conserved biochemical pathway resulting in a characteristic morphological cell death termed apoptosis [30]. Hallmarks of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, and endonucleolytic cleavage of DNA [31]. Unlike necrosis, apoptosis is an energy (ATP)-dependent process that is highly regulated and avoids eliciting an inflammatory response from cell death. Apoptosis is required for successful organogenesis during embryonic development and to maintain cellular homeostasis throughout life to prevent cancer and autoimmunity [32]. Because apoptosis is a conserved evolutionary process, it can be identified by its biochemical and morphological events [33].

The balance between apoptosis and survival is largely maintained within the cell by the Bcl-2 protein family. This family is further divided into three subfamilies: Bcl-2 (including Bcl-2 and Bcl-x$_L$), Bax (including Bax and Bak), and "BH3-only" proteins (including Bad and Bid) [34]. Members of the Bcl-2 subfamily promote cell survival whereas the Bax and BH3-only subfamily members are proapoptotic [35]. The interaction between these family members is complex, cell type-specific, and determines the balance of cellular life and death largely through the maintenance or compromise of mitochondrial integrity, but signaling from the endoplasmic reticulum may also be involved [32, 36]. During the initial stages of apoptosis, phosphatidylserine (PS) is translocated from the inner plasma membrane leaflet to the outer as a marker for phagocytosis [37]. Annexin V staining has thus been used as an apoptotic assay since it preferentially binds PS on the outer leaflet and shows minimal binding to phosphatidylcholine and sphingomyelin which are normally present in the outer leaflet [38]. Another early event of apoptosis is a decrease in the mitochondrial

transmembrane potential (MTP, or $\Delta\psi_m$) [39, 40], elicited largely by mitochondrial outer membrane permeability (MOMP) [41].

The main enzymatic components of the apoptotic pathway are the caspases, a family of cysteine proteases that cleave after aspartic acid residues [42]. Caspases are expressed as proenzymes that are activated after proteolytic processing into heterodimers. Caspases can be broadly classified as initiators, which respond to proapoptotic signals and initiate cell disassembly, and effectors, which carry out the death mechanism [43]. Two well-characterized caspase cascades include the cell surface death receptor (extrinsic) pathway and the mitochondrial initiated (intrinsic) pathway [44]. The extrinsic pathway involves death domain receptors like CD95/Fas/Apo1 and tumor necrosis factor receptor-1 (TNFR1) [45]. Binding of the CD95 ligand (CD95L or FasL) to its receptor causes clustering of the CD95 death domains eliciting binding of the adaptor protein FADD (Fas-associated death domain). FADD recruits pro-caspase-8, which undergoes oligomerization and subsequent self-cleavage [46]. Active caspase-8 can directly cleave effector pro-caspases-3, -6, and -7 or cleave the proapoptotic Bcl-2 member Bid to its truncated form (tBid), which initiates cytochrome c release from the intermembrane space (IMS) of the mitochondria [47]. The intrinsic pathway involves the formation of the heptameric apoptosome [48]. Once cytochrome c is released from the mitochondria, it can bind Apaf-1 via its WD-40 domains, together with dATP/ATP and recruit and activate pro-caspase-9 [49]. Active caspase-9, similar to caspase-8, cleaves the effector pro-caspase-3, initiating the late morphological changes of apoptosis. The intrinsic and extrinsic apoptotic pathways may operate in parallel, or synergize through considerable crosstalk to elicit cellular death [50].

Once activated by an initiator caspase, caspase-3 cleaves Acinus resulting in chromatin condensation without DNA fragmentation [51]. Caspase-3 also cleaves the inhibitor of CAD, ICAD/DFF45, releasing caspase-activated DNase (CAD)/DNA fragmentation factor 40 (DFF40) resulting in DNA fragmentation [52, 53]. This process is responsible for the 180 bp internucleosomal cleavage of DNA apparent as the hallmark ladder pattern after gel electrophoresis [54]. Further, caspase-3 cleaves and inactivates the nuclear DNA repair enzyme, poly(ADP-ribose) polymerase (PARP) [55].

Apoptosis can be induced by a number of conditions, such as irradiation, heat shock, oxidative stress, hypoxia, death domain receptor binding, and nutrient deprivation, including GLN. Surprisingly, the antiapoptotic nature of GLN often appears to have little to do with its role as a source of cellular energy [56, 57]. This review will focus on other possible mechanisms by which GLN may act as a cellular survival factor. We will concentrate on three cell types: enterocytes, immune system-derived cells, and cancer cells, where much of the work on this topic has been done.

ENTEROCYTES

The importance of GLN in maintaining gut homeostasis and health has long been established [58]. GLN is the major oxidative energy source for intestinal epithelial cells [59, 60]. Animal studies have shown the necessity of GLN for the synthesis of enterocyte nucleotides [61] and maintenance of intestinal glutathione levels [62]. Prolonged total parenteral nutrition (TPN) without GLN is known to result in whole-body GLN depletion and gut mucosal atrophy which can be ameliorated with GLN supplementation [9, 10, 63]. The gut mucosal epithelium renews itself every 2 to 8 days by balancing growth in the crypts of Lieberkuhn with apoptosis in the crypt and villus compartments [64]. The apoptotic suppressive qualities and indispensable nature of GLN for enterocyte proliferation [65, 66] could collectively explain why it is necessary for gut mucosal homeostasis. Studies to date have shown that overt GLN starvation leads to intrinsic enterocyte apoptosis, and that ambient GLN levels modulate stress responses and cytokine-induced cell death in this cell type.

Using a nontransformed rat intestinal epithelial cell line (RIE-1) as a model, Papaconstantinou *et al.* have shown that GLN starvation for 24 h causes a 60% reduction in cell number due to increase rate of apoptosis as assessed by Annexin V staining, DNA laddering and nuclear condensation. Cell number decreased after GLN deprivation in a dose-dependent fashion and apoptosis as assessed by DNA laddering was observed only at GLN concentrations below 0.2 mmol/L [67]. Later studies by this same group determined that the mechanism of apoptosis after GLN starvation in RIE-1 cells involves the activation of caspase-3 after 10 h followed by DNA laddering at 12 h with maximal induction of both at 18 h. Caspase-2 also was activated at 18 h. Pro-caspases-1, and -8 were expressed in RIE-1 cells but did not respond to GLN deprivation up to 24 h [68]. Interestingly, treatment of GLN-deprived RIE-1 cells with Z-VAD-FMK (80 μmol/L), a broad-scope caspase inhibitor [69], could prevent caspase activation, DNA fragmentation, and nuclear condensation but had no effect on cellular loss [68]. These findings correlate well with results obtained using Z-VAD-FMK under other apoptotic conditions as reviewed by Denecker *et al.* [47]. Z-VAD-FMK was shown to inhibit pro-caspase activation and DNA fragmentation in response to Bax or Bak overexpression but not nuclear condensation, cell shrinkage, membrane blebbing and loss of cell membrane integrity. Z-VAD-FMK also inhibits DNA fragmentation and chromatin condensa-

tion in response to etoposide, staurosporine, actinomy-cin D, and dexamethasone. In all these cases, cell death occurred after cytoplasmic vacuolization suggesting a role for necrosis [47]. Thus, GLN deprivation may lead to necrosis as a parallel death pathway if the apoptotic caspase cascade is blocked. However, the possible role of other caspases not significantly inhibited by Z-VAD-FMK (as discussed later) cannot be discounted.

In addition to directly eliciting intrinsic apoptosis in enterocytes, GLN limitation has been shown to modulate apoptosis via the extrinsic (death receptor) pathway. Ziegler's laboratory used the human colon carcinoma cell line HT-29, which retains many colonic epithelial properties, to study the role of GLN in cytokine-induced apoptosis [70]. GLN inhibited apoptosis induced by *t*umor necrosis factor-α-*r*elated *a*poptosis-*i*nducing *l*igand (TRAIL, 100 μg/L) in a dose-dependent manner from 0 to 500 μmol/L (i.e., in the physiological range). GLN prevented nuclear condensation and activation of caspases-3 and -8 in response to TRAIL treatment. These effects required GLN metabolism as 6-diazo-5-oxo-l;-norleucine (DON), a non-metabolizable GLN analog that inhibits glutaminolysis, did not induce apoptosis alone, but prevented the antiapoptotic effects observed with GLN supplementation, presumably acting as a GLN analog or antimetabolite. The antiapoptotic nature of GLN was found to be independent of the intracellular glutathione (GSH) redox status, so it is presently unclear which arm of the GLN metabolic profile is required for survival signaling in this cell line.

Cellular survival relies upon the ability to endure physiological stresses and mount responses such as thermotolerance, which necessarily involves the induction of heat shock proteins. GLN has been shown to modulate this stress response pathway in the rat intestinal epithelial cell line IEC-18, where GLN alone was sufficient to increase expression of the chaperone heat-shock protein 70 (HSP70) without previous heat-shock treatment [71]. HSP70 induction in response to GLN and the ability of GLN to protect cells from lethal heat (49°C) and oxidant injury from NH_2Cl was concentration-dependent peaking at 10–15 mM, an order of magnitude greater than physiological plasma values, but one achievable via enteral nutrition on the apical surface of these cells. In contrast to its inhibitory effects in TRAIL-induced apoptosis, DON mimicked the protective effects of GLN; however, GLN effects were diminished by treatment with quercetin, a bioflavinoid that inhibits HSP70 production. These results suggest that GLN-induced protection is not solely related to its role as a metabolic intermediate and relies heavily on HSP70 induction. Similarly, GLN also increased HSP70 expression and enhanced heat-shock (43°C) cell survival in a dose-dependent fashion up to 8 mm in the rat intestinal IEC-6 cell line [72]. Studies by
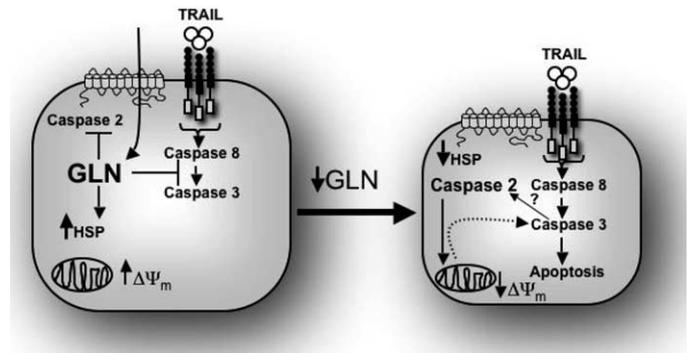

FIG. 1. Effect of GLN deprivation on apoptotic signaling in enterocytes. GLN is supplied to enterocytes via amino acid transporters on the apical and basolateral surfaces. Reduced extracellular GLN supply enables TRAIL-induced death receptor-mediated apoptosis and induces caspase-2 activity. Caspase-2 in turn promotes the release of proapoptotic intermembrane space proteins, associated with a reduced mitochondrial membrane potential ($\Delta\Psi$m). Heat shock protein levels are compromised in GLN-deprived cells, rendering them more susceptible to environmentally induced death as well.

Chang and colleagues further showed that an intravenous GLN bolus induced HSP25 and HSP72 expression in rodent tissues, including colon and ileum, and protected them from endotoxin-induced injury [73]. Moreover, enteral [74] or parenteral [75] GLN administration was shown to induce the expression of heme oxygenase-1 (HO-1), otherwise known as HSP32, in duodenal mucosa. HO-1 is a highly inducible 32-kDa ER protein that utilizes NADPH and O_2 to break down potentially toxic heme to biliverdin, iron and carbon monoxide, and protects cells from a variety of pathological insults like inflammation. Thus, GLN directly influences apoptotic signaling and reductions in its extracellular concentrations compromise stress responses critical for enterocyte survival (Fig. 1).

IMMUNE SYSTEM-DERIVED CELLS

The importance of GLN to cells of the immune system is well established [76, 77]. For example, GLN is required for the late events of T-cell activation, lymphocyte progression through the cell cycle [78], and protection of activated human T cells from apoptosis [79]. To determine the role of GLN in activation-induced T-cell death, Chang *et al.* stimulated Jurkat T cells, a CD4$^+$ human lymphoblastoid cell line, with phorbol myristate acetate (PMA, 20 ng/mL), a protein kinase C activator, and ionomycin (1 μM), a Ca^{+2}-ionophore [79]. Jurkat T cells stimulated with PMA + ionomycin demonstrated IL-2 production which was enhanced by GLN in a dose-dependent manner from 0 to 2 mM. GLN also increased cell proliferation and viability of stimulated T cells and decreased apoptosis in both stimulated and unstimulated cells as assessed by subdiploid DNA content with flow cytometry. Jurkat T cells stimulated with PMA + ionomycin in the
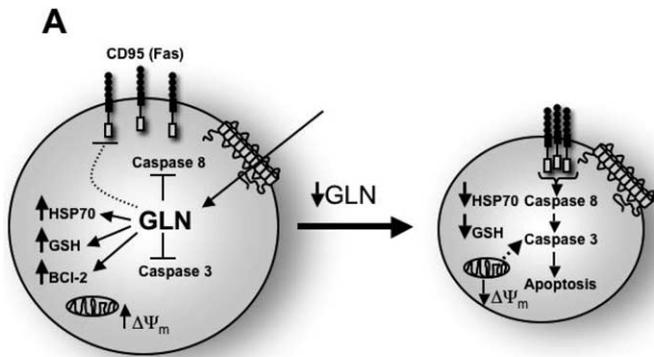
FIG 2. Effect of GLN deprivation on apoptotic signaling in immune system-derived cells. GLN limitation results in depressed heat shock protein and glutathione (GSH) levels, and induces shrinkage in some cells. The reduced cell volume appears to induce a death receptor (CD95)-mediated apoptotic cascade, perhaps via receptor clustering and activation. Reduced mitochondrial membrane potential ($\Delta\Psi$m) further exacerbates the apoptotic cascade, and is enabled in part by a depressed anti-apoptotic Bcl-2 level in the cells.

presence of GLN exhibited decreased reactive oxygen species (ROS) and increased intracellular GSH. The protective effects of GLN could be inhibited by DL-buthionine-[S, R]-sulfoximine (BSO, 100 μM), an inhibitor of GSH synthetase, and addition of exogenous GSH (5 mM) decreased apoptosis of stimulated T cells. GLN supplementation down-regulated expression of CD95 (Fas) and CD95L but up-regulated Bcl-2 in stimulated T cells, effectively raising the "apoptotic threshold" of these cells. Binding of CD95L to its death receptor CD95 initiates the extrinsic apoptotic pathway. Consistent with this model, GLN inhibited the activation of caspases-3 and -8 observed in Jurkat T cells stimulated with PMA + ionomycin. Thus, GLN appears to protect activated human T cells from apoptosis by enhancing GSH and Bcl-2 levels and inhibiting the extrinsic apoptotic pathway (Fig. 2).

In isolated rat neutrophils, GLN repletion enhanced phagocytic capacity, while deprivation caused apoptosis as assessed by chromatin condensation after 24 h and Annexin V staining after 3 h [80]. Similar results were obtained in human neutrophils where GLN supplementation decreased DNA fragmentation in a dose-dependent manner from 0 to 2 mM. GLN deprivation for 3 h caused a loss of the mitochondrial transmembrane potential ($\Delta\Psi$m) in both human and rat neutrophils, and conversely, GLN metabolism was necessary to maintain the $\Delta\Psi$m, as DON blunted the protective effects of this amino acid. Similar to studies in enterocytes discussed earlier, GLN may also modulate stress responses in neutrophils. Although the data are presently correlative, neutrophils from critically ill patients with depressed plasma GLN concentrations exhibit lower HSP70 levels compared to those from healthy patients, suggesting that GLN may modulate expression of this important mediator of stress *in vivo* [81].

Work by Guidotti's group demonstrated that human leukemia/lymphoma cell lines CEM (lymphoblastic leukemia), HL-60 (promyelocytic leukemia), Namalwa (Burkitt lymphoma), and U937 (histiocytic lymphoma) experience cellular loss after a few days in culture when GLN and glucose have been depleted from the growth medium [57]. When grown in GLN-free medium, these cell lines undergo apoptosis as assessed by DNA laddering after 24 h. Studies with CEM cells indicated that the onset of apoptosis correlated with GLN concentration in the medium. Apoptosis occurred at 48 h when the initial GLN concentration was 0.25–0.5 mM, at 72 h when the concentration was 0.75–1 mM and after 96 h in control media with 2 mM GLN. CEM cells grown in its absence could be rescued by adding back 2 mM GLN after the first 24 h. Substantial decreases in intracellular ATP levels were not detected in GLN-deprived CEM cells suggesting that apoptosis does not result from a loss of cellular energy.

Further studies by this same group showed that GLN deprivation of CEM lymphoblastic leukemia cells elicited a more than 2-fold increase in caspase-8 activity by 3 h, which increased to more than 3-fold by 12 h. Annexin V staining was observed after 6 h and DNA laddering and cellular blebbing was apparent after 24 h. DNA fragmentation as assessed by a photometric enzyme immunoassay steadily increased over time from 6 to 24 h and could be blocked by treatment with Z-IETD or Z-DEVD (both at 40 μM) [82], strong caspase-8 and caspase-3 and -8 inhibitors, respectively [69]. DNA fragmentation after GLN deprivation was also inhibited by Z-IETD and Z-DEVD in HL-60 cells. Thus, in contrast to induction of the intrinsic pathway in enterocytes, GLN deprivation apparently leads to apoptotic cell death by the extrinsic pathway in these leukemic cell lines.

Lending credence to its link to death domain receptor signaling, GLN deprivation did not elicit apoptosis in CD95-negative murine lymphoma L1210 cells, although CD95L-CD95 interaction appeared not to play a role in GLN deprivation-induced apoptosis of CEM cells [82]. However, GLN deprivation caused a loss of intracellular water that resulted in a 15% decrease in cell volume after 60 min, progressively decreasing to 60% at 12 h as measured by labeled 3-O-methyl-D-glucose distribution. Similar cell shrinkage was observed after GLN deprivation for 6 h in HL-60 and L1210 cells (26 and 38% decrease in cell volume, respectively), but shrinkage of L1210 cells in response to GLN deprivation did not induce caspase-8 activity or DNA fragmentation as mentioned earlier. In CEM cells, loss of cell volume after GLN deprivation preceded caspase-8 activation and DNA fragmentation and could not be prevented by Z-IETD. Furthermore, supplementation with 6 mM D-GLN, 10 mM betaine, a compatible organic os-

molyte, or 5 mM 2-methylaminoisobutyric acid, a non-metabolizable amino acid analog, could significantly prevent cell shrinkage and apoptosis after GLN deprivation, suggesting the effects were independent of metabolism. Complete amino acid deprivation also caused decreases in cell volume, caspase-8 activation and DNA fragmentation in these cells. Interestingly, these effects could be prevented by the addition of 2 mM GLN alone and by expression of the v-Flip viral proteins MC159 and E8 [82]. MC159 binds to FADD and inhibits death receptor signaling while E8 binds the caspase-8 prodomain blocks its recruitment to FADD [83]. The authors concluded that cell shrinkage could reduce the tension of the cell surface causing CD95-receptor aggregation and multimerization resulting in recruitment of FADD and subsequent caspase-3 activation, ultimately resulting in apoptosis [82]. The hydrating effects of GLN uptake normally offset these events, invoking a physiological role for extracellular GLN in addition to its well-established metabolic roles.

Directly addressing the role of GLN in leukemia cell survival, these authors also showed that treatment with asparaginase (2 U/mL) lowered GLN concentrations in the medium within 24 h causing apoptosis of CEM and HL60 cells that was independent of asparagine depletion. No apoptosis was observed in L1210 cells under the same conditions [82], suggesting again that ambient GLN dynamics impinge upon CD95 in lymphocyte survival. Asparaginase treatment has also been shown to deplete intracellular levels of GLN and glutamate in NIH3T3 fibroblasts, decreasing DNA and protein synthesis followed by growth arrest by 48 h [56]. Within 12 h, the nucleus and cytoplasm shrink suggesting a role for apoptosis. Asparaginase treatment had no effect on intracellular ATP pools, the Na^+ transmembrane gradient or active transport of amino acids. These results collectively imply that extracellular GLN levels *per se* regulate lymphocytic and mesenchymal cell survival.

Other studies with human lymphoma U937 cells have shown that treatment with Fas ligand (CD95L, 2 μL/mL), TNF-α (10^4 IU), and heat shock (45°C) all for 20 min increased apoptosis within 4 h as assessed by Annexin V staining [84]. Apoptosis was 50 to 60% higher if the cells were grown in GLN-free media. U937 cells exposed to UV irradiation (5 exposures of 2000 kJ/cm^2 UV-C and UV-B) showed high levels of apoptosis after 24 h, but there was no difference between cells grown in the absence or presence of GLN. Further studies showed that GLN also helps prevent nuclear condensation after treatment with FasL, TNF-α and heat shock, similar to the results obtained in enterocytes described earlier. All four treatments caused caspase-3 cleavage independent of GLN concentration in the medium suggesting GLN exerts its anti-apoptotic effects downstream of caspase-3. The authors

concluded that one possibility for such a downstream effector could be HSP70. Although HSP70, whose levels are directly affected by GLN, previously has been shown to act upstream of caspase-3 in U937 cells [85], others have shown—albeit in ME-180 cervix carcinoma cells and WEHI-S fibrosarcoma cells—that HSP70 prevents apoptosis downstream of caspase-3-like proteases by inhibiting the function of caspase-activated cytosolic phospholipase A_2 ($cPLA_2$) and the $\Delta\psi_m$ collapse [86].

Some of the results in cell lines have been corroborated in studies with isolated human lymphocytes and monocytes. Oehler *et al.* showed that reduction of GLN levels from 0.5 mM to 0.125 mM decreased HSP70 induction by 40% in heat-shocked (42°C) primary human lymphocytes [87], and likewise, reduction in ambient GLN from 2.0 mM to 0.06 mM dramatically reduced cell viability and HSP70 expression in isolated human monocytes chronically heat-shocked in the physiological range (41°C) [88]. Moreover, this same group has recently shown that GLN-starved peripheral blood monocytes exhibit defective ubiquitin-mediated proteasomal turnover of key cellular proteins [89]. In contrast to results from other cell types, GLN starvation led to ATP depletion in monocytes, invoking energy deficit as part of the underlying mechanism. Thus, despite cell type-specific differences, it is apparent that GLN levels modulate cytokine- and stress-induced cell death by mechanisms that remain to be elucidated.

HYBRIDOMA CELLS

Fusion of immortalized myeloma cells with spleen-derived lymphocytes to create monoclonal antibody–producing hybridoma cells has been standard practice and a watershed to the biotechnology industry since this innovative technique was conceived in the 1970s [90]. The exhaustion of nutrients, especially GLN, leads to apoptotic cell death during large-scale mammalian cell culture in bioreactors [91, 92]. Apoptosis can have devastating effects on the production of biopharmaceuticals like monoclonal antibodies [93]; as such, it has been long established that batch culture survival can be extended with additional feeding of amino acids and glucose. Studies with the murine hybridoma TB/C3 have shown that deprivation of any single amino acid leads to apoptosis; however, aspartate, serine, glutamate, asparagine, glycine and proline had minimal effects. Over-expression of Bcl-2 could restore viability of the culture to greater than 70% during deprivation of any single amino acid except threonine (42%) and GLN (55%) [94].

GLN deprivation of KB26.5 murine hybridomas induced apoptosis as assessed by Annexin V staining, DNA laddering, TUNEL staining, and cytochrome c release [95]. Peptide inhibitors (as listed $K_i < 50$ nm), Z-VAD-FMK (caspases-1,-3,-5,-7,-8 and -9), Ac-DEVD-

CHO (caspases-3,-7 and -8), and Z-YVAD-CMK (caspase-1) [69, 96] were used to determine which caspases were involved in the apoptotic mechanism. KB26.5 murine hybridomas grown in the absence of GLN and treated with Z-VAD-FMK (50–100 μM) delayed apoptosis as indicated by a 75% decrease in Annexin V staining, lack of DNA laddering and extended viability up to 36 h. The protective effects of Z-VAD-FMK were lost after 60 h. Ac-DEVD-CHO (100 μM) only slightly delayed apoptosis and Z-YVAD-CMK had little effect even at concentrations of 200–300 μM [95]. Given the relatively specific role of caspase-8 in death receptor-initiated apoptosis [44], the apparent lack of a murine caspase-5 homologue, the ineffectiveness of Z-YVAD-CMK (caspase-1) and the observed cytochrome c release, these authors conclude that caspases-3, -7, and -9 are the principal caspases involved in GLN-deprivation-induced cellular death of hybridomas [95], implying the intrinsic apoptotic pathway.

Another possibility for a mediator of GLN deprivation-induced apoptosis could be caspase-2, as its activity is not significantly blocked by any of the peptide inhibitors used in the aforementioned study [95]. At least two other reports in nonimmune cells have shown that caspase-2 activation occurs relatively late, after caspase-3 activity, in response to GLN deprivation [68, 97]. Although caspase-2 can be cleaved by caspase-3 [98], the exact activation mechanism of caspase-2 is still unclear. Recent evidence argues that it is an initiator caspase [99], and acts to amplify the apoptotic response by inducing release of proapoptotic proteins from the mitochondrial IMS [100, 101]. It is possible that under GLN limiting conditions in some cell types, caspase-2 is activated independent of caspase-3. This could explain why Z-VAD-FMK could only prevent apoptosis for 60 h in KB26.5 murine hybridomas, and has no effect on cellular loss in rat intestinal (RIE-1) [68] and human hepatoma (SK-Hep1) cells after GLN starvation (Bode, unpublished data). Nonetheless, it appears that GLN deprivation induces hybridoma apoptosis via the intrinsic pathway.

In the Sp2/0 murine hybridoma, 60% of cells were committed to undergo apoptosis after 2 h of GLN deprivation and this effect was greater than for any other amino acid [102]. Rapid intrinsic apoptotic events centering on mitochondrial dysfunction were observed. Release of the IMS proteins cytochrome c and SMAC/DIABLO into the cytosol occurred within 30 min, presumably as a result of the observed Bax translocation to the mitochondria. Caspase-9 activity was increased within 1 h, which led to significant caspase-3 activation, PARP cleavage and DNA fragmentation at the same time point. Peptide inhibitors Z-VAD-FMK and DEVD-FMK (both at 10 μM) could protect the hybridoma cells from apoptosis. The rapid induction of apoptosis in the Sp2/0 hybridomas was attributed to low

levels of glutamine synthetase, Bcl-2, and Bcl-x$_L$ expression and to the constitutive expression of c-myc [102], a proto-oncogene that paradoxically leads to cellular death in the absence of adequate growth factor and nutrient supply [103, 104].

Franek et al. have studied starvation-induced apoptosis with the mouse B-lymphocyte hybridoma PVA-187. Eight amino acids added individually could protect the cells from apoptosis: glycine, L-alanine, L-serine, L-threonine, L-proline, L-asparagine, L-glutamine and L-histidine [105]. Additional studies by this group with the human T-lymphoblastic leukemia cell line MOLT-4 grown in diluted media yielded an almost identical panel of anti-apoptotic amino acids with GLN being the strongest [106]. Surprisingly, these amino acids were reported to decrease the number of cell divisions per day, so the authors concluded that they act as survival factors by delaying apoptosis and not by promoting cell growth.

The data in neutrophils, lymphocytic, and hybridoma cells collectively point to an apoptotic suppressive role for GLN, and conversely, an apoptotic response if adequate extracellular GLN levels are insufficient. The premise for such a model involves the concept of an extracellular "GLN threshold" that would vary according to cell type and impact stress responses and apoptotic signaling pathways, once exceeded. Catabolic stress from major surgery, radiation or chemotherapy, burn injury, and sepsis, all can lead to a depressed systemic GLN economy [107], which may result in impaired immune responses or cellular death in monocytes, neutrophils and lymphocytes if their physiological "GLN threshold" is sufficiently breached. The apoptosis-suppressing effects of GLN in cells of the immune system could also explain the documented benefits of giving bone marrow transplant (BMT) patients GLN-supplemented TPN, effectively reducing the incidence of infection and enhancing convalescence [108]. It should be noted that relative GLN deficit in vivo might not always manifest in depressed plasma GLN levels. For example, in the well-cited study above [108], BMT patients exhibited normal plasma GLN levels around 625 μm, which were boosted to 925 μm with TPN supplemented with 0.57 g L-GLN/kg/day. In such a scenario, it may be that exogenous GLN provides cells of the immune system with adequate levels of this amino acid for normal cellular function and survival, and alleviates the systemic energy investment otherwise needed to maintain plasma GLN at normal values. Subsequently, the spared ATP can be diverted to enhance immune function such as cytokine and immunoglobulin production, phagocytic capacity, diapedesis and cell proliferation. Elevated plasma GLN levels may also help prevent cytokine-induced apoptotic responses and bolster intracellular HSP70 levels necessary for lymphocyte survival, as discussed earlier (Fig. 2).

CANCER CELLS

Some of the studies discussed earlier regarding "enterocytes" and "immune system-derived cells" used cancerous cell lines (HT-29, lymphoma, leukemia, myeloma), so those results may also be interpreted as cancer-related responses to GLN deprivation in addition to the underlying premise that they retain properties of the normal parent tissue. The propensity of cancerous cells to exhibit heightened GLN consumption has been long established, and has led to reluctance to use GLN-supplemented nutrition in oncology patients. GLN has been reported as the major oxidizable substrate for tumor cells [109, 110]. In fact, the frequent observation that cancer cells consume GLN at rates that exceed their metabolic needs have led to their designation as "nitrogen traps" [15, 16]. Viable theories to explain this appetite for GLN have been proffered [13, 111, 112], but the issue remains open to debate. For example, hepatocellular carcinoma (HCC) is characterized by decreased glutamine synthetase activity and increased glutaminase activity [113] in the tumor compared to normal liver tissue, which, when coupled with accelerated GLN uptake [114], are probably responsible for the observed decrease in plasma GLN concentrations in patients with HCC [115, 116]. However, several subsequent studies have focused on the corollary to this issue: What happens when cancer cells are deprived of an adequate GLN supply? Results from breast, liver and cervical cancer cells, and immortalized kidney cells have begun to shed some light on this issue, and have indicated that stress response, angiogenic and apoptotic pathways are activated, as might be expected. These additional studies also have indicated cell type-specific roles for amino acid transporters, glutathione, and glutaminyl tRNA synthetase in apoptotic signaling.

Abcouwer *et al.* have used normal and cancerous breast cell lines as a model to study GLN dependence and utilization [117]. Additional studies by this group have assessed stimulated stress response gene expression after GLN deprivation [118, 119]. Their work has focused mainly on the growth arrest and DNA damage-inducible genes [120] GADD45 [121] and GADD153/CHOP (C/EBP-homologous protein) [122], the endoplasmic reticulum stress-response (ERSR) gene GRP78/BiP (glucose-regulated protein of 78 kDa/immunoglobulin binding protein) [123] and the proangiogenic cytokines interleukin-8/CXCL8 (IL-8) [124] and VEGF (vascular endothelial growth factor) [125]. The GADD gene products are involved in stress-induced cellular apoptosis [126, 127]. GLN starvation elevated GADD45 and GADD153 mRNA levels within 1.5 h in subconfluent cultures of the human breast cell lines HBL100 and TSE. In HBL100 cells, the GAPDH-normalized levels of GADD45 peaked at 20-fold after 12 h, while GADD153 peaked at 12-fold after 6 h.

Likewise, GRP78 mRNA levels also peaked at 1.8-fold after 6 h of GLN starvation. In TSE cells, all three genes increased linearly over the course of time, with GADD45 and GADD153 expression increased at least 50-fold at 24 h whereas GRP78 was only elevated 3.5-fold at this same time point. Serial dilutions of GLN in the media were made to determine the dose-response of induction. Maximum induction of GADD45, GADD153 and GRP78 occurred at GLN concentrations of 0.5 mM in HBL100 cells and 0.06 mm in TSE cells [118]. Since both HBL100 and TSE cells are highly dependent on GLN for growth and viability [117], other breast cell lines (T47D, SKBR3 and BT483) with varying dependence on GLN were used to determine their response to GLN deprivation. A direct relationship was found where cell lines that are less sensitive to GLN show smaller inductions of GADD45 and GADD153 that occur at lower GLN concentrations. Nuclear run-on assays and mRNA decay studies determined that GRP78 induction in response to GLN deprivation was equally attributable to transcription and post-transcriptional mechanisms. However, the response of the GADD genes was mainly caused by increased mRNA stabilization and only small increases between 2–5 fold were seen in GADD45 and GADD153 transcription [118]. Thus, GLN deprivation induces the expression of stress-related genes in some situations, in contrast to its repressive impact on others (e.g., HSPs).

Using HeLa cervical carcinoma cells, Ko *et al.* have shown that GLN suppresses apoptosis initiated by Fas ligation [128], similar to the results in lymphocytic cells and TRAIL-induced apoptosis in enterocytes discussed earlier. Treatment with an activating anti-Fas antibody elicited apoptosis as assessed by cellular blebbing, DNA laddering and increased caspase-3 activity when the cells were grown in GLN-free media. Under these conditions, the activity of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and apoptosis signal-regulating kinase 1 (ASK1) increased after 10 min. Further studies in the human embryonic kidney cell line HEK-293 demonstrated that in the presence of GLN, the glutaminyl-tRNA synthetase (QRS) associates with ASK1. Cells grown in the absence of GLN underwent apoptosis when transfected with ASK1, a response that could be attenuated by co-expression of QRS. To our knowledge, this is the only study showing a role for a tRNA synthetase in mediating the apoptotic suppressive effects of GLN in mammalian cells.

GLN or ASCT2 in Apoptotic Repression?

Studies in our laboratory have focused on the role that GLN and amino acid transporter ASCT2 play in the growth and survival of liver cancer cells using an aggressive human hepatoma cell line SK-Hep1. ASCT2 mediates the majority of GLN uptake in several human

liver cancer cell lines [129], and is up-regulated in a number of human cancers [130]. To test its utility in hepatoma growth and survival, an inducible ASCT2 antisense RNA expression system was developed; upon induction an 85% and 73% decrease in ASCT2 mRNA levels and 49% and 65% reductions in ASCT2-mediated GLN transport were observed after 14 and 24 h, respectively [97]. Notably, a dramatic cell loss of 98% was observed after 48 h. In contrast, overt GLN starvation elicited a more delayed cellular loss, requiring 72 h to achieve the same level of cell death caused by ASCT2 silencing. In both cases, cell death occurred by apoptosis based on enzymatic and morphological analyses. Induction of antisense ASCT2 also led to a 3.3-fold increase in caspase-3 activity and a 2-fold increase in PARP cleavage after 24 h. Similarly, GLN deprivation led to increased caspase-3 activity over time from 1.6-fold at 24 h to 4.1-fold at 48 h, where a 5.3-fold increase in PARP cleavage was detected. Induction of antisense ASCT2 led to significant activation of caspase-9 activity (2.5- and 1.6-fold) and caspase-2 activity (3.2- and 2.7-fold) after 14 and 24 h, respectively, as well as small increases in caspase-8 activity. Surprisingly, GLN deprivation failed to stimulate caspase-8 or -9 activities, but induced caspase-2 activity by 72% after 48 h. Thus, caspase-2 activation followed that of caspase-3 in response to GLN deprivation, but preceded it upon ASCT2 silencing, suggesting that the apoptotic signal(s) generated is (are) distinct between transporter loss and substrate deprivation. The exact nature of caspase-2 (initiator *versus* effector) is still unresolved as discussed earlier [131]. Could caspase-2 be an initiator after one stimulus (ASCT2 silencing) but an effector after another (GLN deprivation)? Nonetheless, the results from the ASCT2 silencing studies clearly point to a role for this transporter in cell survival signaling that transcends its role as the major route of GLN delivery. A composite summary of some of the demonstrated responses of cancer cells to GLN deprivation and ASCT2 silencing is depicted in Fig. 3.

A Reductionist View: The GLN–Glutathione Link in Cancer Cells

It is well established that GLN is an essential component of glutathione homeostasis, and that reduced glutathione (GSH) is the primary intracellular antioxidant, scavenging free radicals, peroxides and other reactive oxygen species [23, 132]. The human hepatoma cell line HuH-7 stably transfected with a zinc-inducible sense c-myc expression vector undergoes apoptosis when cultured in serum-free media plus 37.5 μM zinc [133]. Apoptosis induced by serum deprivation is dependent on c-myc expression as HuH-7 cells stably transfected with an antisense c-myc expression vector were protected; as mentioned earlier, activated c-myc
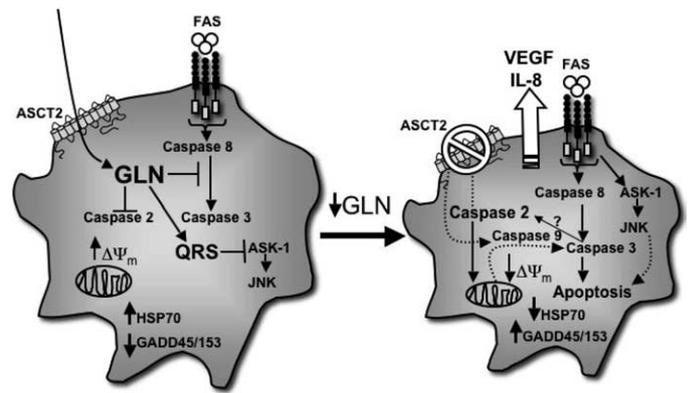

FIG 3. Effect of GLN deprivation on apoptotic signaling in cancer cells. Under conditions of an adequate GLN supply, glutaminyl tRNA synthetase (QRS) associates with apoptosis signal-regulating kinase (ASK-1) in HeLa cells and prevents the activation of the proapoptotic jun N-terminal kinase (JNK). In the absence of an adequate GLN supply, death receptor (Fas)-mediated stimulation of ASK-1 and caspase 8-mediated apoptosis occurs. GLN deprivation in breast cancer cells induces GADD45/153 and angiogenic (VEGF and IL-8) gene expression while it depresses heat shock protein expression in other cell types. Prolonged GLN deprivation results in decreased mitochondrial membrane potential ($\Delta\Psi$m) and caspase 2-mediated apoptosis. Targeted suppression of amino acid transporter ASCT2, the major conduit for GLN delivery, elicits rapid apoptosis of human liver cancer cells via an intrinsic (caspase-2 and -9-mediated) apoptotic pathway that is distinct from GLN deprivation alone.

is known to cause cellular apoptosis in the absence of adequate "social survival signals" [103, 104]. In this study, apoptosis was induced by GLN deprivation in wild-type HuH-7 and the two stable transfectants, even in the presence of adequate growth factors. Myc expressing HuH-7 cells cultured in serum free media plus zinc in the absence of GLN for 4 h exhibited a 41% decrease in cellular glutathione levels and enhanced H_2O_2 content as compared to cells cultured in the presence of GLN. The authors concluded that apoptosis under these conditions is caused by myc-induced oxidative stress [134], linking GLN deprivation to glutathione economy. This study also suggests that an adequate GLN supply may be required for survival of oncogene-accelerated oxidative metabolism of cancer cells. A similar finding of enhanced reactive oxygen species upon GLN deprivation was reported in DU-145 human prostate cancer cells grown as multicellular spheroids [135].

A recent body of work has emerged that examines the effects of dietary GLN supplementation on tumor growth *in vivo*, and points to mechanisms based on cellular glutathione dynamics. For example, Klimberg's group reported that methotrexate therapy was enhanced in fibrosarcoma-bearing rats consuming GLN-enhanced diets due to a decrease in tumor glutathione content [136]. More recently, this group has examined the effects of dietary GLN on GSH levels and apoptotic protein expression in chemically induced

breast tumors in rats [137]. They found that a GLN enriched diet reduced tumor GSH-to-oxidized glutathione (GSSG) ratios, up-regulated Bax and caspase-3 expression and reduced Bcl-2 levels, essentially reducing the "apoptotic threshold" of the tumor. This same group also reported that GLN-enriched diets depressed insulin-like growth factor-1 (IGF1), its receptor (IGF1R) and PKB/Akt protein levels and enhanced Bad expression in breast tumors more than in normal tissues, again depressing survival signaling in cancerous tissue [138]. The mechanism by which GSH levels and anti-apoptotic signaling are selectively repressed by GLN-enriched diets in chemically induced fibrosarcoma and breast tumors is unclear, but the implication from this research thus far is that the reluctance to use GLN-enriched nutritional regimens in cancer patients may be unfounded.

The Ehrlich Ascites Tumor (EAT) model in mice has been used extensively to study tumor-host metabolic relationships over the past several decades, and these cells that grow in the peritoneal cavity are avid GLN consumers, eventually causing depressed plasma GLN levels in the host animal [15, 16]. Similar to the results from the c-myc HuH-7 GLN deprivation study, 40% lower intracellular GSH levels and 35% higher reactive oxygen species were found in EAT cells stably expressing an antisense glutaminase mRNA (termed 0.28AS-2) compared to the normal parent cell line [139]. 0.28AS-2 cells were more susceptible to methotrexate- and H_2O_2-induced apoptosis, and exhibited higher percentages of endogenous apoptosis than the parent line. This study in EAT cells in conjunction with the GLN deprivation studies in HuH-7 and DU-145 cells mentioned earlier collectively point to the importance of an adequate extracellular GLN supply and subsequent metabolism in providing glutamate for maintenance of intracellular GSH levels. Given this relatively simplistic model for cellular redox economy and its intimate link to cell survival, how are the results reconciled from *in vivo* studies where GLN-enriched diets enhanced tumor death and diminished GSH levels?

The answer may reside in a series of studies that show GLN accelerates oxidative metabolism, increases formation of reactive oxygen intermediates (ROI), and lowers mitochondrial GSH (mtGSH) in some tumor cells. GSH is synthesized in the cytoplasm and must be transported into mitochondria, where it comprises at most 5% of total cellular GSH, but is vital in protecting this organelle from ROI that are generated as a result of oxidative metabolism and inflammatory mediators. Studies by Estrela and colleagues have shown in EAT cell-bearing mice that GLN-enriched diets selectively increase glutaminase activity, decrease glutamine synthetase activity and reduce mtGSH by over 40% in EAT cells [140]. The mechanism for these effects seems

to reside in an inhibition of GSH transport into mitochondria by elevated cytosolic glutamate, formed via GLN-enhanced glutaminase activity. Notably, the reduced mtGSH content renders the cancer cells more susceptible to TNF-α induced formation of ROI, eventually leading to cell death via loss of $\Delta\psi_m$, followed by MOMP and release of proapoptotic proteins from the intermembrane space [141]. Thus, the dichotomy in the GLN–GSH relationship can be explained by two separate mechanisms: inhibition of GLN metabolism (or supply) leads to decreased intracellular glutamate for GSH biosynthesis in many cell types, while GLN-induced oxidative metabolism results in glutamate-dependent inhibition of mitochondrial GSH uptake, lowering mtGSH and rendering this organelle more susceptible to apoptotic stimuli. Again, cancer cells appear to be more susceptible to the latter form of mtGSH depression, as host tissues did not display the same drop in mtGSH as the EAT cells in response to GLN-enriched diets [140]. It is interesting that GLN appears to be required for TNF-α toxicity in certain cancer cells such as EAT and L929 fibrosarcoma [142], an observation that directly contrasts with GLN-mediated repression of TRAIL, TNF-α and CD95 death receptor signaling in enterocyte and lymphocytic cell lines discussed earlier. The reason for this disparity is currently unclear, but may reside in the cell type-specific effects of GLN on mitochondrial ROI production and subsequent glutamate disposition.

In summary, an adequate supply GLN is required for maintenance of GSH economy in several cell types and ironically may help to lower the apoptotic threshold to death receptor signaling in some cancerous cells via mtGSH depression (Fig. 4) [143]. GLN should be further explored as a potential adjuvant to established or exploratory therapies for specific cancers.

CONCLUSIONS AND FUTURE DIRECTIONS

In summary, overt GLN deprivation ultimately elicits apoptosis by intrinsic and/or extrinsic pathways, depending on cell type. Conversely, an enhanced GLN supply curbs death receptor-mediated apoptosis in certain cell types, but may actually enhance it in some cancer cells. Prior to the onset of apoptosis, GLN limitation promotes adaptive stress response pathways that aid in survival such as cell cycle arrest, ER stress (also known as the unfolded protein response) and angiogenesis, again depending upon cellular context, but subverts other stress response pathways necessary for cell survival such as heat shock. Apoptotic signaling mechanisms implicated in response to GLN deprivation are also cell type-specific. We must emphasize, however, that the studies reviewed here were largely performed in cell lines, and that the mechanisms and consequences of GLN deprivation elucidated thus far await validation *in vivo*.
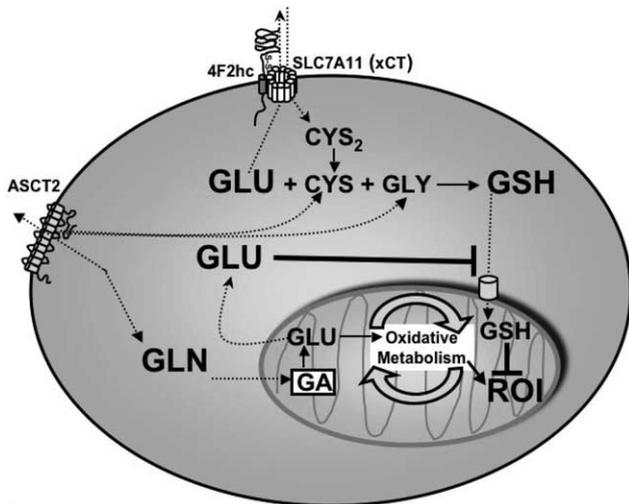
FIG 4. The glutamine paradox. In many cell types, limiting extracellular GLN supply or inhibiting GLN metabolism via glutaminase (GA) reduces the supply of intracellular glutamate (GLU) available for glutathione (GSH) biosynthesis. Intracellular GLU is also used to drive the uptake of cystine (CYS_2) via a counter-exchange mechanism through the 4F2 heavy chain-complexed heterodimeric transporter system Xc^- (SLC7A11; xCT) [143] a major source of cysteine (CYS) for GSH biosynthesis. Depressed intracellular GLU therefore compromises cytosolic GSH biosynthesis. Amino acid transporters such as ASCT2 supply the cell with the GSH precursors GLN, CYS and glycine (GLY) as well. Conversely, *in vivo* studies have shown that GLN feeding actually reduces mitochondrial GSH levels in some cancer cells. This apparent contradiction appears to be the collective result of enhanced reactive oxygen intermediate (ROI) generation spurred by oxidative GLN metabolism, which depletes mtGSH, as well as GLU inhibition of mitochondrial GSH transport (uptake). Depressed mtGSH in turn renders the cancer cells more susceptible to TNF-α-induced cell death (not shown).

As we come full circle, the age-old question is begged: Are *in vitro* results relevant to *in vivo* physiology? Certainly cells in the body are not subjected to overt GLN starvation; but the underlying assumption is that the tractability of cultured cells and associated ability to induce maximum responses over short time periods *in vitro* may faithfully reflect similar yet more subtle consequences on a longer time scale *in vivo*. Even so, it is unlikely that physiological nadirs in ambient GLN levels are alone sufficient to elicit apoptosis directly. Plasma GLN levels *per se* are maintained within a fairly narrow range, but dynamics in delivery (perfusion) coupled with accelerated consumption of GLN may combine to cause localized changes in ambient GLN concentrations that are sufficient to elicit some of the cellular responses reported in this review. It is likely that GLN exerts most of its influence on cell survival via modulation of stress response and survival signaling protein levels that collectively determine the apoptotic threshold of a cell to subsequent insults such as inflammation. The potential influence of GLN on the apoptotic threshold of specific cell types adds to the collective roles of this "conditionally essential" amino acid in human physiology.

Scientifically, future *in vitro* studies into the underlying mechanisms of GLN effects on survival signaling should harness and integrate the power of proteomics, microarrays and physiological genomics and be carried out using physiologically relevant ranges of this amino acid. Combined with classic molecular signaling investigations, these studies will yield a comprehensive assessment of potential mechanisms for GLN-modulated survival signaling *in vivo*. Clinically, more large-scale randomized prospective clinical trials are indeed required to determine the utility of GLN supplementation in specific situations—as an adjuvant to the chemotherapy of individual cancers or as a prophylactic/palliative supplement to at-risk patients. Until then, the *in vitro* results will continue to provide novel endpoints for testable hypothesis-driven studies *in vivo*, or ultimately, clinically, and help to advance this field into true translational research.

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