Glutamine inhibits cytokine-induced apoptosis in human colonic epithelial cells via the pyrimidine pathway

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Evans, Mary E., Dean P. Jones, and Thomas R. Ziegler. Glutamine (Gln) inhibits cytokine-induced apoptosis in human colonic epithelial cells via the pyrimidine pathway. Am J Physiol Gastrointest Liver Physiol 289: G388–G396, 2005. First published May 5, 2005; doi:10.1152/ajpgi.00072.2005.—Glutamine (Gln) prevents apoptosis in intestinal epithelial cells, but the mechanism(s) remain unknown. Gln-derived metabolites include ammonia, glutamate (Glu), glutathione (GSH), and nucleotides. We previously showed that Gln potently inhibited apoptosis in cytokine-treated human colonic HT-29 cells; this effect was specific to Gln, unaffected by Glu, and unrelated to intracellular GSH. The current research examines mechanism(s) for Gln-induced antiapoptotic effects in HT-29 cells treated with TNF-α-related apoptosis-inducing ligand (TRAIL). Proliferating cells were treated with Gln or selected Gln metabolites for 24 h. Cells were then treated with TRAIL and Gln or its downstream metabolites, and apoptosis was assessed at 8 h after treatment. The purine and pyrimidine precursors inosine and orotate inhibited TRAIL-induced apoptosis. However, inhibition of purine synthesis with azaserine did not alter the potent antiapoptotic effect of Gln. In contrast, the pyrimidine synthesis inhibitor, acivicin, completely prevented this response. Supplementation with the pyrimidine uracil or the pyrimidine precursor orotate rescued the acivicin-induced blockade of Gln antiapoptotic action. Removal of bicarbonate, a substrate for pyrimidine synthesis, also inhibited the antiapoptotic effects of Gln. Uracil and thymine alone also significantly decreased TRAIL-induced apoptosis. The antiapoptotic effects of Gln were independent of DNA/RNA synthesis as measured by flow cytometry and bromodeoxyuridine incorporation. In conclusion, Gln prevents TRAIL-induced apoptosis in HT-29 cells through a mechanism involving the pyrimidine pathway. Our data also demonstrate the novel antiapoptotic effects of pyrimidine bases and their precursor orotate in these human intestinal cells.

apoptosis; glutamine; intestine; nucleotides; pyrimidines

INCREASED APOPTOSIS OCCURS in inflamed/ulcerated areas of colonic mucosa in cases of inflammatory bowel disease (IBD) (2) and is associated with impaired mucosal healing (16). Cytokines that induce apoptosis in human intestinal cell lines, such as TNF-α and IFN-γ, are believed to mediate the gut mucosal inflammation in IBD (35, 38). Thus methods to reduce or prevent apoptosis in diseased and/or inflamed intestinal cells during increased cytokine expression may be potentially useful to enhance gut mucosal restitution in IBD and other conditions associated with intestinal inflammation.

Glutamine (Gln) is considered a conditionally essential nutrient during catabolic stress where it is a particularly important nutrient for gut epithelial and immune cells (for review, see Ref. 46). Gln supplementation of the diet and/or parenteral nutrition improves nitrogen balance, immune function, and indices of morbidity in animal models of disease and in critically ill patients (27, 46). Gln has well-described trophic effects in gastrointestinal epithelia, and both proliferative (4, 40) and antiapoptotic effects (3, 6, 9, 28, 29, 43) have been observed. Gln induces proliferation and/or reduces apoptosis in the intestinal mucosa in vivo in weanling piglets (6) and in rat models of short bowel syndrome or bowel transplantation (12, 44). Furthermore, Gln supplementation inhibits gut mucosal inflammation rats with experimentally-induced necrotizing enterocolitis and also maintains gut barrier function in models of intestinal atrophy or injury (5).

In vitro studies demonstrate that Gln inhibits apoptosis in gut epithelial cells. Use of Gln-free medium induces spontaneous apoptosis in rat intestinal epithelial cells, whereas addition of Gln to the culture medium prevents spontaneous apoptosis (28, 29). Gln also prevents oxidant and heat shock-induced cell death in rat small intestine-derived epithelial cells (IEC-6; and IEC-18) (3, 43). We showed that Gln prevents cytokine-induced TNF-α-related apoptosis-inducing ligand (TRAIL; and TNF-α/INF-γ) apoptosis in the human intestinal epithelial cell line HT-29 (9).

Gln is a major respiratory fuel for enterocytes through its metabolism by the rate-limiting enzyme glutaminase into its intermediary metabolites, glutamate (Glu) and ammonia (NH₃) for review, see Ref. 26). Glu serves as an energy source through entry into the TCA cycle or biosynthesis into metabolites, including other amino acids such as proline, arginine, and citrulline (23). Glu is also an important precursor for synthesis of the endogenous thiol antioxidant glutathione (GSH), which itself has antiapoptotic properties under conditions of oxidative stress (17). Finally, Gln is essential for the synthesis of purine and pyrimidine nucleotides. Our previous study (9) showed that the ability of Gln to prevent TRAIL-induced apoptosis in HT-29 cells was specific to Gln and could not be replicated with other amino acids including Glu. The results also showed that these antiapoptotic effects required glutaminase and/or amidotransferase activity (both of which are inhibited with diazonorleucine) but were independent of intracellular GSH production, which would depend on glutaminase activity. However, the amidotransferase activity, which appears to be necessary for the antiapoptotic effects of Gln, may be required for metabolic reactions include nucleotide synthesis.

Finally, our previous study found that a dose of 500 μM Gln completely inhibited TRAIL-induced apoptosis (9). This concentration is within the range of Gln concentrations found in normal, healthy human plasma (41) and likely considerably
less than normal dietary consumption, which averages < 10 g/day in healthy, nonvegans (21). However, Gln concentrations in both the blood and tissues decrease dramatically during critical illness or inflammation when its utilization exceeds its supply (22, 42, 45). Furthermore, many enteral diets and total parenteral nutrition solutions used in critically ill/inflamed patients lack sufficient amounts of Gln, which further reduces Gln availability in the intestine. Thus an inhibition of cytokine-induced intestinal cell apoptosis may provide an explanation for the beneficial effects of Gln supplementation in critically ill patients because inflammatory cytokines are often elevated in this patient population.

The purpose of this study was to determine potential mechanism(s) of Gln protection against cytokine-induced apoptosis and specifically whether this prevention involves nucleotide synthesis. For these studies, we used an HT-29 cell model in which we showed that Gln inhibited apoptosis induced with the cytokine TRAIL (9). TRAIL is a member of the TNF-α superfamily and a known inducer of apoptosis in transformed intestinal epithelial cell lines including HT-29 cells (9, 11). Our data demonstrate that Gln prevents TRAIL-induced apoptosis in HT-29 cells through the pyrimidine pathway. Further, we show that the pyrimidine precursor orotate and the pyrimidine bases uracil and thymine have potent antiapoptotic effects in this human gut cell line.

MATERIALS AND METHODS

**Chemicals and biochemicals.** All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

**Cell culture.** HT-29 cells were purchased from American Type Culture Collection (Manassas, VA) and were maintained in DMEM containing 4 g/l glucose (Cellgro, Atlanta, GA) containing 10% FBS, 2.0 mM Gln, and 1% antibiotics (10,000 U/ml penicillin and 100 mg/ml streptomycin-sulfate) at 37°C with 5% CO₂-95% O₂. Fresh medium was added every 2 d, and cultures were passed every 5–7 d to maintain subconfluence. During experimentation, cells were seeded at a density of 8 × 10⁴ cells/cm² in six-well plates (Fisher Scientific, Pittsburgh, PA), unless otherwise indicated, and allowed to adhere using the above medium for 24 h reaching ~60% confluency. During treatment, medium was replaced with serum-free, Gln-free DMEM containing 1% antibiotics. For the bicarbonate starvation experiments, cells were pretreated with 24 h of serum-free DMEM followed by 8 h of treatment with either bicarbonate-containing DMEM or bicarbonate-free DMEM (Cellgro) supplemented with 11 mM HEPES adjusted to a pH = 7.2.

**Cell treatments.** All experiments were conducted with HT-29 cells, treated, harvested, and assessed for percent apoptotic cells using flow cytometric analyses after 8 h of TRAIL (100 ng/ml) treatment according to methods outlined previously (9). As was shown previously, 500 μM Gln was the minimal concentration of Gln necessary to completely prevent TRAIL-induced apoptosis in HT-29 cells (9). Thus all Gln-supplemented cells were treated with 500 μM Gln in the present study. Non-TRAIL-treated cells in Gln-free culture medium served as controls. In our previous studies, 500 μM Gln specifically and completely inhibited the TRAIL-induced increase in the percentage of cells in the sub-G₁ peak, caspase-8 and -3 activation, and nuclear condensation (9) as well as cytochrome c release and caspase-9 activation (8). Figure 1 shows the pathways of Gln metabolism and the major downstream metabolites investigated in this and previous research (8) of the anti-apoptotic actions of Gln.

To determine whether the antiapoptotic effects of Gln were the result of Gln-derived ammonia production, cells were pretreated with 250 or 500 μM NH₄Cl (a concentration half of or equivalent to the amount of NH₃ derived from 500 μM Gln metabolism by glutaminase, respectively) for 24 h and then treated with 100 ng/ml TRAIL, with or without 250 or 500 μM NH₄Cl for 8 h. To evaluate the role of de novo synthesis of purine or pyrimidine nucleotides in the antiapoptotic effects of Gln, cells were treated with 1.25, 2.5, or 5 mM inosine or orotate, respectively, or a combination of 5 mM inosine and orotate and cultured as above. Such concentrations were selected because a mixture of purines and pyrimidines in this concentration range was previously shown to stimulate proliferation of intestinal cells in vitro (40). To assess whether inhibition of purine synthesis prevented the antiapoptotic abilities of Gln, cells were treated with azaserine (25 to 500 μM, a concentration, which has been shown to inhibit purine synthesis in human cells in culture) (10). To evaluate whether inhibition of pyrimidine synthesis prevented the antiapoptotic abilities of Gln, cells (± Gln) were treated with 1 μM acivicin, a known inhibitor of carbamoyl phosphate synthetase II, a component of the rate-limiting multidomain enzyme protein carbamoyl phosphate synthetase II aspartate transcarbamylase-dihydroorotase; DON, diazo-6-norleucine.

![Fig. 1. Pathways of glutamine (Gln) metabolism. This figure shows the major downstream metabolites investigated in this and previous research (9) of the antiapoptotic actions of Gln. Inhibitors are shown in bold underlined type with a blunt arrow that indicates the metabolic step inhibited. Enzymes are highlighted in a gray box. Metabolites previously shown not to be involved in the antiapoptotic abilities of Gln are indicated with an X. Glnase, glutaminase; CAD, carbamoyl phosphate synthetase II-aspartate transcarbamylase-dihydroorotase; DON, diazo-6-norleucine.](http://ajpgi.physiology.org/10.220.33.2)
acid (DNA/RNA) synthesis in the ability of Gln to prevent apoptosis was assessed with both flow cytometry and bromodeoxyuridine (BrdU) incorporation as described below. Finally, the effect of Gln on uridine 5'-diphosphate (UDP)-hexose concentrations was determined after 24 h of pretreatment with Gln for 1 h after serum-starvation for 24 h. UDP-hexose concentrations were determined by HPLC as outlined below.

**Flow cytometric assessment of apoptosis.** The percentage of cells in the sub-G1 peak was determined by flow cytometry and used as a measure of apoptosis (9). Our previous research into TRAIL-induced apoptosis in this model demonstrated a marked increase in percentage of cells in the sub-G1 peak as well as a concomitant induction of nuclear condensation, activation of caspase-8 and -9, increased caspase-3 activity and cytochrome c release (8, 9). Thus the current research used flow cytometry to assess apoptosis. Furthermore, a dose of 500 μM Gln was used because it was able to completely inhibit TRAIL-induced apoptosis as measured by all the above apoptotic indicators (9). A total of 10,000 cells were analyzed with a Becton, Dickinson FACSort flow cytometer. Data were analyzed with CellQuest software (Becton, Dickinson, Franklin Lakes, NJ).

**UDP-sugar analysis.** Because de novo synthesized pyrimidines contribute primarily to UDP-sugar and cytidine diphosphate (CDP)-phospholipid synthesis (37), the effect of Gln on UDP-sugar content was assessed as an indicator of increased de novo pyrimidine synthesis in HT-29 cells. To assess the effect of Gln supplementation on UDP-sugar content, cells were plated and allowed to adhere for 24 h; medium was then removed and replaced with serum-free medium for 24 h. Next, cells were treated or without 500 μM Gln for 1 h to determine the role of Gln supplementation on UDP-sugar concentrations. Following treatment, cells were washed with ice-cold PBS, and small metabolites, including UDP-sugars, were extracted using a variation of the procedure of Ross et al. (34). Briefly, cells were harvested in 11 ml PBS. An aliquot was removed for protein quantification and the remaining cells were quenched with 20 ml 60% methanol. Cells were pelleted by centrifugation, resuspended in 2:1:4 parts 100% methanol/H2O/chloroform, and vigorously agitated for 45 min at 4°C. The aqueous phase was collected, reextracted with 125 μl MeOH and 125 μl H2O, and the aqueous phase was combined with that from the first extraction. The aqueous layers were vacuum-dried for 24 h. UDP-sugar concentrations were determined by HPLC as previously described (34).

**Nucleic acid synthesis.** Nucleic acid (DNA/RNA) synthesis was determined by using 5-BrdU incorporation per cell number. Briefly, 10 × 10^4 HT-29 cells were seeded, allowed to adhere for 24 h, and treated with TRAIL ± Gln and acivicin or azaserine as above in a 96-well plate for 24 h. Medium was removed and BrdU was added for 2 h. BrdU incorporation was detected spectrophotometrically per the manufacturer’s directions with anti-BrdU (Sigma). Concurrent plates were treated similarly and cell number was determined manually at the same time point. BrdU incorporation is reported as BrdU incorporation/cell number as a percentage of controls.

**Statistical analyses.** Differences between endpoints were compared across groups by one-way ANOVA. Specific treatment doses were compared post hoc using the Fisher’s paired least significant difference test (SPSS for Windows; SPSS, Chicago, IL).

**RESULTS**

**Ammonia treatment does not prevent apoptosis in TRAIL-treated HT-29 cells.** Treatment with TRAIL alone markedly increased apoptosis, whereas 500 μM Gln completely prevented this response (Fig. 2). Addition of 250 or 500 μM NH4Cl alone without TRAIL had no effect on apoptosis. With TRAIL, apoptosis decreased marginally with 250 μM NH4Cl and there was no protection with 500 μM NH4Cl.

**Orotate and inosine treatment decrease TRAIL-induced apoptosis.** Supplementation of cell culture medium with 1.25–5 mM the pyrimidine precursor orotate or the pyrimidine precursor inosine resulted in a dose-dependent decrease in TRAIL-induced apoptosis (Fig. 3). Orotate decreased apoptosis to a far greater extent than did inosine. Combined inosine and orotate was less effective in preventing TRAIL-induced apoptosis, suggesting an inhibitory effect of purines in the presence of orotate. Supplementation with either 5.0 mM uracil or thymine in the absence of Gln markedly decreased TRAIL-induced apoptosis (by 79 or 84%, respectively) (data not shown). Cytosine supplementation was substantially less effective than uracil or thymine, but modestly decreased TRAIL-induced apoptosis compared with unsupplemented TRAIL-treated cells.

**Inhibition of pyrimidine synthesis prevents antiapoptotic effects of Gln.** Increasing doses of azaserine (25–500 μM) in the presence of Gln without TRAIL had no effect on apoptosis (Fig. 4). Azaserine treatment (25 μM) slightly increased apoptosis in the TRAIL + Gln-treated cells; however, the ability of Gln to block TRAIL-induced apoptosis was unaffected by increasing doses of azaserine, indicating that Gln does not block apoptosis through this pathway of nucleotide synthesis.

**Inhibition of pyrimidine synthesis prevents antiapoptotic effects of Gln.** Concurrent treatment of HT-29 cells with 1–100 μM acivicin and Gln in the absence of TRAIL had no effect on apoptosis compared with controls (Fig. 5A). In contrast, addition of 1–100 μM acivicin to TRAIL + Gln-treated cells resulted in a dose-dependent decrease of the antiapoptotic effects of Gln such that treatment with 100 μM acivicin...
completely prevented the antiapoptotic effects of Gln. Removal of bicarbonate (an essential component for pyrimidine synthesis) in the absence of TRAIL did not affect the percentage of apoptosis compared with bicarbonate-supplemented controls (Fig. 5B). TRAIL-treated cells starved of both Gln and bicarbonate exhibited a marked increase in apoptosis. However, bicarbonate-starved, Gln-supplemented cells exhibited a moderate decrease in apoptosis, whereas Gln/bicarbonate supplementation completely prevented TRAIL-induced apoptosis. Taken together, these two experiments strongly suggest that pyrimidine synthesis is a mechanism for antiapoptotic effects of Gln.

Orotate and uracil rescue the effects of acivicin. TRAIL-treated cells supplemented with 5 mM orotate or uracil alone demonstrated a marked inhibition of apoptosis; however, neither agent was as effective as 500 μM Gln supplementation (Fig. 5C). Supplementation with 100 μM acivicin completely prevented antiapoptotic effects of Gln. In contrast, when acivicin-treated cells were supplemented with 5 mM orotate or uracil there was a significant decrease in TRAIL-induced apoptosis. These results show that supplementation with the pyrimidine base uracil or pyrimidine precursor orotate rescues the acivicin-induced blockade of antiapoptotic effects of Gln in TRAIL-treated cells.

Uracil decreases apoptosis in a dose-dependent manner. Supplementation of TRAIL-treated cells with increasing doses of uracil (0.25–1.0 mM) resulted in a dose-dependent reduction in the percentage of apoptotic cells; however, uracil concentrations greater than 1.0 mM provided no additional protection against TRAIL-induced apoptosis (Fig. 6A).

Gln and uracil additively inhibit apoptosis. Supplementation of TRAIL-treated cells with either 50 μM Gln or 500 μM uracil reduced TRAIL-induced apoptosis to a similar degree (56 and 61%, respectively) (Fig. 6B). However, concurrent supplementation with 50 μM Gln and 500 μM uracil significantly decreased TRAIL-induced apoptosis to a greater extent than either of these agents alone at these concentrations. These data indicate that Gln and uracil additively inhibit TRAIL-induced apoptosis in HT-29 cells.

Gln supplementation increases UDP-hexose concentrations. Evidence suggests that pyrimidines synthesized de novo are preferentially utilized to synthesize UDP sugars and CDP phospholipids (37). In HT-29 cells, supplementation with 500 μM Gln for 1 h increased UDP glucose from 74 ± 29 pmol/mg protein to 144 ± 40 pmol/mg protein (a 96% increase; P = 0.007) and UDP galactose was increased from 20 ± 9 to 31 ± 6 pmol/mg protein (a 55% increase; P = 0.005). These data indicate that Gln supplementation increases the production of UDP hexoses, a known pyrimidine downstream product.

Gln prevents TRAIL-induced apoptosis by a mechanism independent of nucleic acid (DNA/RNA) synthesis. TRAIL treatment resulted in a marked increase in the number of apoptotic cells (Fig. 7, Aa). Addition of Gln to TRAIL-treated cells completely prevented the accumulation of cells in the sub-G1 peak (Fig. 7, Ab). Concurrent supplementation with Gln and either azaserine (Fig. 7, Ac) or acivicin (Fig. 7, Ae) without TRAIL resulted in S-phase arrest (indicating an inhibition of DNA/RNA synthesis). However, neither treatment resulted in an increase in cells in the sub-G1 peak. Cells treated with TRAIL + Gln + azaserine (Fig. 7, Ad) exhibited S-phase arrest, but azaserine did not prevent antiapoptotic effects of Gln (no increase in the number of cells in the sub-G1 peak),
whereas TRAIL + Gln + acivicin (Fig. 7, A) induced S-phase arrest and prevented the antiapoptotic effects of Gln.

BrdU incorporation per number of cells with TRAIL was similar to controls, whereas BrdU incorporation increased markedly with Gln + TRAIL supplementation (Fig. 7B). Both acivicin and azaserine substantially inhibited Gln’s induction of nucleic acid synthesis, indicating that the antiapoptotic effects of Gln are not the result of increased DNA/RNA synthesis.

DISCUSSION

Our study demonstrates for the first time that the Gln-derived pyrimidine pathway is a mechanism for Gln’s prevention of cytokine-induced apoptosis in human intestinal epithelial cells. Pyrimidines (including cytosine, thymine, and uracil) are synthesized de novo from Gln, ATP, and bicarbonate through the action of CAD—a single multidomain protein with multiple enzymatic activities, including carbamoyl phosphate synthetase II (CPS II)-aspartate transcarbamoylase-dihydroorotase. Orotate, an intermediate of the pyrimidine pathway, is generated early in pyrimidine metabolism through CAD action and serves as a precursor for uracil, cytosine, and thymine (for review, see Ref. 14).

Evidence for a pyrimidine pathway mechanism as explanation for the antiapoptotic effects of Gln in this study includes 1) complete inhibition of the antiapoptotic effects of Gln with acivicin (an inhibitor of CPS II); 2) prevention of the effects of Gln with bicarbonate starvation (an essential precursor for pyrimidine synthesis); and 3) antiapoptotic effects of treatment with the pyrimidine bases uracil and thymine and the pyrimidine precursor orotate in the absence of Gln. Furthermore, this antiapoptotic effect was isolated to the pyrimidines uracil and thymine, was not the result of an increase in nucleic acid, (DNA/RNA) synthesis, and was moderately inhibited with the addition of the purine precursor inosine. Our data also show that Gln prevention of apoptosis is not the result of N production (as ammonia) nor the purine pathway. Thus these results suggest a novel mechanistic pathway for the previously identified antiapoptotic effects of Gln in HT-29 cells (9). In addition, this study shows for the first time that the pyrimidine precursor orotate and the pyrimidine bases (uracil and thymine) have antiapoptotic effects in cytokine-treated human gut epithelial cells.

Glutaminase activity is necessary for the release of the amido nitrogen from Gln for pyrimidine synthesis. In our previous studies, glutaminase inhibition with diazo-6-norleucine (DON) prevented the antiapoptotic effects of Gln (9), but supplementation with the glutaminase products Glu or NH3 did not prevent TRAIL-induced apoptosis. DON also functions as an amidotransferase inhibitor, and thus can prevent the transfer of the amido N group of Gln in the first step of pyrimidine synthesis. Taken together, these findings support the importance of CAD action, Gln’s amido N, and thus pyrimidine synthesis, as a mechanism for the antiapoptotic effects of Gln in HT-29 cells. Purine synthesis likewise depends on Gln for its amido nitrogen source; however, in our in vitro cell model, the purine pathway did not appear to be involved in Gln’s prevention of TRAIL-induced apoptosis.

The specific physiological mechanism(s) through which pyrimidines inhibits TRAIL-induced apoptosis in HT-29 cells...
remains unclear. However, we have shown that this antiapoptotic effect is not the result of overall de novo nucleic acid synthesis. It is possible that pyrimidines or their downstream metabolites may be affecting apoptotic signaling processes, including recruitment of death domains or downstream caspases (for review, see Ref. 20). Moreover, pyrimidines or their subsequent metabolites may induce activation and/or production of specific inhibitory proteins against TRAIL-dependent signaling events (for review, see Ref. 33). Finally, underlying mechanisms for the more potent antiapoptotic effect of thymine and uracil compared with cytosine remain to be determined.

Gln is a critically important amino acid for normal gastrointestinal function. Several lines of evidence suggest beneficial effects of Gln supplementation on the morbidity, mortality, immune function, and gastrointestinal function of critically ill patients requiring parenteral and/or enteral nutrition support (for review, see Refs. 24 and 46). Animal studies of Gln supplementation also suggest gut mucosal cytoprotective effects of this amino acid in chemically-induced IBD, which is associated with increased local cytokine production and (15, 18) and accelerated epithelial cell apoptosis (16). Few studies have examined the impact of Gln-derived nucleotides and/or their derivatives themselves on the prevention of intestinal epithelial cell apoptosis. In piglets, dietary supplementation with a combination of nucleotides, nucleosides, and nucleobases for 28 days prevented weaning-associated apoptosis of enterocytes in the distal ileum (6), although these investigators did not distinguish specific purine vs. pyrimidine effects. That study also suggested that combined pyrimidine and Gln supplementation was additive in inhibiting apoptosis. In the current in vitro study, we similarly found a significantly greater antiapoptotic effect with combined Gln and uracil compared with identical concentrations of Gln or uracil alone. Uridine, but not thymidine, has also been shown to prevent p53-dependent apoptosis in small intestine and colon induced by the pyrimidine antimetabolite 5-fluorouracil in BDF1 mice (32). Finally, nucleotide supplementation, specifically using the purine AMP and the pyrimidine cytidine monophosphate (CMP) in developing primary human fetal small intestinal cells, had differing effects on apoptosis (39). AMP supplementation for 8–24 h was associated with an increase in apoptosis, whereas CMP supplementation did not induce apoptosis compared with controls after 12 or 24 h of culture (39).

It is interesting to note that higher concentrations of the pyrimidine precursor orotate were necessary to achieve inhibition of apoptosis in our model of cytokine-induced apoptosis compared with Gln. However, the concentration of uracil that substantially inhibited TRAIL-induced apoptosis (1.0 mM) was much lower and closer to that of Gln. Both the concentrations of orotate and uracil used in this study are higher than what is present in the normal diet. For example, skim milk has been found to contain up to 0.783 mM orotate (25), whereas infant formulas may contain up to 0.66 mM orotate (7). Mixed nucleotide-containing formula diets contain a mixture of nucleotides in the \( \mu \text{M} \) range (31); however, studies on humans have been conducted with orotate at concentrations up to 6 \( \text{g/day} \) (19). Thus concentrations similar to that of our study might be possible with supplementation.

One explanation for the observed concentration differences in our model may be that nucleotide synthesis through the
Fig. 7. A: azaserine and acivicin induce S-phase arrest. Cells were treated with or without Gln and azaserine or acivicin for 24 h. TRAIL was then added for 8 h and flow cytometry was performed. Cell treatments consisted of 100 ng/ml TRAIL (a), 500 μM Gln + 100 ng/ml TRAIL (b), 500 μM Gln + 500 μM azaserine (c), 500 μM Gln + 100 μM acivicin (d), 500 μM Gln + 100 μM azaserine + 100 ng/ml TRAIL (e), or 500 μM Gln + 100 μM acivicin + 100 ng/ml TRAIL (f). Representative flow histograms for DNA were stained with propidium iodide. B: Gln’s effect on BrdU incorporation is inhibited with both azaserine and acivicin. Cells were pre-treated with 500 μM azaserine or 100 μM acivicin with or without 500 μM Gln for 24 h followed by the same treatments plus 100 ng/ml TRAIL for 8 h and BrdU, and the number of cells was then determined. Control cells were treated with serum-free medium without Gln. Bars not sharing the same letters are significantly different (P < 0.05).
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Gln-dependent de novo pathway is more efficient than nucleotide synthesis through the salvage pathway in HT-29 cells (for review, see Ref. 14). Transformed human colonic Caco-2 cells depend to a greater extent on de novo synthesis of nucleotides than nontransformed rat small IEC-6 cells, as evidenced by larger cellular nucleotide pools in IEC-6 cells (13). Furthermore, in that study, nucleotide supplementation further induced proliferation in IEC-6 cells beyond that of nonsupplemented cells when Gln was present, whereas nucleotide supplementation of Caco-2 cells did not increase proliferation in the presence of Gln. Tuhacek et al. (40) likewise found that a mixture of nucleotides induced proliferation to a similar extent as Gln in IEC-6 cells when grown under normal proliferation conditions. In addition, pyrimidine nucleotides may be of increased relevance in vivo because the gastrointestinal tract depends largely upon the salvage pathway for nucleotide synthesis (23). Thus pyrimidines may exert antiapoptotic effects at lower concentrations in both normal intestinal epithelial cells in culture and in gastrointestinal cells in vivo, a point that requires further investigation. It is also possible that the effective concentration difference between Gln and the pyrimidines in our study are due to different transport, uptake and/or metabolism of Gln compared with orotate or uracil. Whether there is a difference in this model between cellular pyrimidine and Gln uptake and/or metabolism, and thus altered intracellular concentrations of these metabolites, warrants further investigation. Moreover, the effect of TRAIL + Gln supplementation on Gln metabolism and bioavailability remains to be determined.

In conclusion, our data show that Gln prevents cytokine-induced apoptosis in proliferating HT-29 cells via the pyrimidine pathway. The results provide an important clarification of the mechanism by which Gln may protect intestinal epithelial cells from cytokine-induced injury. Our results also show for the first time that supplementation with orotate or pyrimidine bases prevent cytokine-induced apoptosis in human intestinal cells. Together, these observations may have translational significance for in vivo studies in models of cytokine-induced gut mucosal cell death.

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