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Alanyl-Glutamine and Glutamine Supplementation Improves 5-Fluorouracil-Induced Intestinal Epithelium Damage In Vitro

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Abstract

In this study, we have examined the role of glutamine derivatives in reducing 5-fluorouracil (5-FU)-induced epithelial damage in an undifferentiated crypt intestinal cell line, IEC-6. In this model, we have investigated proliferation indirectly by detecting the enzyme-derived formazan dye from the tetrazolium salt WST-1 in viable cells at 24 and 48 h after 5-FU treatment. Migration was measured at 12 and 24 h after razor scraping of the cell monolayer. Cell death was measured by quantifying the percentage of apoptotic and necrotic figures by flow cytometry at 12 and 24 h following 5-FU challenge. Neither glutamine nor alanyl-glutamine prevented 5-FU-induced apoptosis and necrosis in IEC-6 cells at 12 and 24 h after 5-FU challenge. However, glutamine and alanyl-glutamine enhanced migration and proliferation when compared with 5-FU-treated controls ($P < 0.05$). These new findings support our earlier study on the benefit of oral glutamine in enhancing epithelial recovery after 5-FU challenge.

Keywords

Alanyl-glutamine; Glutamine; 5-fluorouracil; Mucositis; Chemotherapy; Small intestine

Introduction

The small intestinal epithelium is composed of a diverse and dynamic population of cells, which have differential kinetics during intestinal adaptation against mucosal injury [1, 2]. Within the crypts, cells proliferate and migrate towards either the villi or to the bases of the crypts where they differentiate into a variety of cells such as enterocytes, goblet cells, and Paneth cells. Apoptosis is responsible for maintaining the balance between proliferation and elimination of the constantly renewing intestinal epithelial cells, which regenerate every 3 days [3, 4]. A variety of agents, including chemotherapeutic drugs, radiation, and intestinal pathogens are known to disturb the steady intestinal epithelium turnover [5–7]. 5-Fluorouracil (5-FU) is one of the most commonly used adjuvant chemotherapy drugs for colorectal cancer [8], but enteric inflammation accompanied by diarrhea is a commonly reported side-effect, sometimes leading to discontinuation of therapy [9]. Intestinal mucositis with diarrhea is often the dose-limiting adverse effect of 5-FU therapy and is likely accompanied by reduced enterocyte proliferation, migration, and a concomitantly increased apoptotic rate, resulting in intestinal malabsorption and disrupted intestinal barrier function, as reported by Daniele and others [10, 11].

Glutamine is the most abundant amino acid in blood with a concentration of 0.6 mM/l, which is higher than any other amino acid [12, 13]. Glutamine is known to be one of the main sources of energy for the intestinal epithelium by generating adenosine 5'-triphosphate (ATP). It is also the precursor of many other amino acids such as proline, arginine, citrulline, and alanine, which play an important coadjuvant role during gut healing [13, 14]. The intestinal uptake of glutamine is enhanced when the body is subjected to stressful conditions, such as chemotherapy challenge [15, 16]. Glutamine supplementation in rats pretreated with toxic doses of methotrexate enables reduction in the severity of the intestinal mucositis [17] and improves intestinal permeability in patients treated with 5-FU and folic acid [18]. Although glutamine has been shown to reduce diarrhea duration in children [19–21] and is generally recognized to be a conditionally essential amino acid in the critically ill

patient [22], there remains a debate regarding the efficacy of glutamine due to its limited solubility, stimulating the pursuit of more stable glutamine compounds for clinical use [23, 24].

In a previous study, we showed that villus height was blunted due to cell proliferation arrest and that cell apoptosis increased within ileal crypts 24 h following 5-FU treatment in mice [25]. In this model, oral alanyl-glutamine, a more stable glutamine derivative, hastened ileal mucosal recovery following 5-FU challenge, by means of crypt hyperplasia. Although numerous studies have addressed the role of adjuvant glutamine therapy in intestinal mucositis [26], there is a scarcity of in vitro studies addressing the healing role of glutamine derivatives on 5-fluorouracil-induced cell damage.

In order to further investigate intestinal cell dynamics following 5-FU challenge, we have studied cell migration, proliferation, and apoptosis in a crypt intestinal cell line, IEC-6, pretreated with 5-FU, and further evaluated the role of glutamine and alanyl-glutamine supplementation in protecting IEC-6 cells from the 5-FU challenge in vitro.

Methods

Materials

Glutamine (Gln) and alanyl-glutamine (Ala-Gln) were obtained from Sigma (St. Louis, MO). The Annexin V ApopAlert kit was obtained from Clontech (Palo Alto, CA). Tetrazolium salt WST-1 reagent was obtained from Roche (Mannheim, Germany). 5-Fluorouracil was obtained from American Pharmaceutical Partners Inc. (Los Angeles, CA), at a concentration of 50 mg/ml in aqueous solution.

Cell Culture

Rat intestinal jejunal crypt cells (IEC-6, passages 20–35) were purchased from American Type Culture Collection (Rockville, MD) and were cultured at 37°C in a 5% CO₂ incubator. The maintenance cell media was Dulbecco's modified eagle media (DMEM; Gibco BRL, Grand Island, NY) supplemented with 5% fetal calf serum (FCS), 5 mg bovine insulin, 50 µg/ml penicillin/streptomycin (DMEM; Gibco BRL, Grand Island, NY), and a final concentration of 1 mM sodium pyruvate. The medium was changed thrice a week, according to standard culture protocols. Dulbecco's modified eagle medium without glutamine (DMEM; Gibco BRL) was used whenever the supplementation effect of Gln or Ala-Gln was evaluated. The cultured cells were trypsinized with 0.25% ethylene-diamine-tetra-acetic acid (EDTA) trypsin when confluence was achieved.

Wounding Assay of IEC-6 Cell Monolayers (Cell Migration)

IEC-6 cells were seeded in six-well plates at a concentration of 2×10^4 cells/well and cultivated in DMEM media with 5% FCS (Gibco BRL, Grand Island, NY). IEC-6 cells were confluent after 4 days following seeding. Wells were then scraped along their diameter and extending 30 mm in length to the right center corner using a sterile razor blade. Prior to scraping, 50% of the medium volume was removed from each study well. The medium was changed and the cells were then incubated for 1 h either with 5-fluorouracil, at

concentrations of 0.3, 1, 3, 10, and 30 mM (selected based on previous study with IEC-6 cells challenged by 5-fluorouracil [27]) or standard Gln-containing media. Afterwards, wells were washed using DMEM. Wells were digitally photographed after 6, 12, and 24 h, and the number of cells in the longest migration line was counted and averaged according to the well surface area (adapted from McCormack and Brito et al. [28]). The digital pictures were captured and opened using Image Pro Plus v. 5.0 software (Media Cybernetics, Silver Spring, MD). A grid (each square = 0.1 mm²) was built, overlaying the digital images of the scraped area. The column of the farthest cell migration was chosen and IEC-6 adherent cells were tracked and identified by traced red dots for counting. The traced red dots within the column were changed to black on a white background and counted digitally by Image Pro Plus software. The resealing speed was also evaluated by averaging the length of the three longest lines of cells in each picture after 24 h.

Cell Proliferation

Cell proliferation was measured indirectly using the tetra-zolium salt WST-1 (4-[3-(4-iodophenyl)-2H-5-tetrazolio]-1-3-benzene disulfonate), according to the manufacturer recommendations. A 96-well plate was seeded with IEC-6 cells in a total concentration of 4×10^3 cells/well in 100 μ l of normal DMEM. Cells were allowed to attach for 48 h, and then the wells were washed with 100 μ l of DMEM without glutamine. The wells were incubated for 1 h with 1 mM 5-FU (because of the greatest efficacy seen in the migration study, as described below at the Results section) diluted in DMEM Gln-free medium or medium alone. Afterwards, the medium was removed and the wells were washed with Gln-free media and then supplemented or not with Gln or Ala-Gln (10 mM in DMEM Gln-free media).

After 24 and 48 h, wells were incubated for 4 h with 10 μ l of the tetrazolium salt and the absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader at 450 nm (reference range 420–480 nm). Tetrazolium salts are cleaved to formazan by mitochondrial enzymes in viable cells. Enhancement of the number of viable cells will result in an increase of the amount of the formazan dye, which is detectable by the ELISA reader. Therefore, this model indirectly measures the cell proliferation rate over time.

Flow Cytometry for Apoptosis and Necrosis

Apoptosis and necrosis were measured by flow cytometry analyses using the ApoAlert Annexin V kit. Annexin V is a molecule that binds to phosphatidylserine (PS) and, when conjugated to a fluorochrome, detects apoptotic cells expressing PS on the reversed membrane surface. For this protocol, propidium iodide (PI) was also used to detect necrotic and late apoptotic cells, which express PI inside the membrane. The cells were seeded on 12-well plates at a concentration of 5×10^5 cells/well. These cells were allowed to attach to the plate surface for 24 h. Afterwards, cells were washed with DMEM Gln-free media and either incubated or not with 5-fluorouracil for 1 h. Wells were then washed again and supplemented with Gln or Ala-Gln (both 10 mM) and then incubated for 6, 12, or 24 h. Cells were trypsinized, centrifuged, and washed with serum-containing media, before incubation with Annexin V. Cells were counted and diluted to 10^5 – 10^6 cells and rinsed with 1 \times binding

buffer, and resuspended in 200 μ l of binding buffer. Five microliters of Annexin V and 10 μ l of PI were added and incubated for 5–15 min in the dark. The samples were then processed at the University of Virginia's Flow Cytometry Core, using a Calibur (Becton Dickinson) dual-laser fluorescence-activated cell sorter (FACS).

Results

Effects of 5-Fluorouracil and Ala-Gln and Gln on IEC-6 Cell Migration

Pretreatment with 5-FU (for 1 h, diluted in standard media) inhibited cell migration in a dose-dependent fashion at 24 h (Fig. 1). The strongest migration inhibition was seen with 30 mM at 24 h (inhibition of 65.6% versus control). The dose of 1 mM of 5-FU blunted migration significantly at 24 h (inhibition of 47.1% versus control); hence, this dose was used to evaluate the effects of Ala-Gln and Gln supplementation on 5-FU-induced epithelial cell damage. Migration was inhibited by 1 mM of 5-FU by 55.5% compared to the control when Gln-free media was used after 24 h. The resealing speed, decreased significantly by 14.3% after 5-FU treatment: $413 \pm 10.9 \mu\text{m}/24 \text{ h}$, $N = 15$, versus $354 \pm 18.2 \mu\text{m}/24 \text{ h}$, $N = 18$ ($P = 0.007$, by unpaired Student *t*-test), an average speed reduction of 2.45 $\mu\text{m}/\text{h}$, as compared to IEC cells seeded under Gln-free media.

Both Ala-Gln and Gln supplementation (10 mM) significantly improved migration after 5-FU treatment ($P < 0.001$), by 83.1% and 49.7%, respectively, at 24 h (Figs. 2 and 3) and resealing speed by 25.4% and 15%, respectively, for Ala-Gln and Gln at 24 h ($P < 0.05$, by unpaired Student's *t*-test). Ala-Gln and Gln increased three- and twofold the average IEC-6 migration speed (μ/h), respectively, away from the scraping line, as compared to 5-FU. From the four supplementation doses tested for each nutrient, the peak dose of 10 mM was chosen, at both 12 and 24 h, for the remaining studies.

Effect of 5-Fluorouracil and Ala-Gln and Gln on Cell Proliferation

5-FU significantly reduced proliferation at 24 and 48 h in previously exposed cells compared to the control group without glutamine (18.9% at 24 h and 38.6% at 48 h; $P < 0.05$). Ala-Gln and Gln, at a dose of 10 mM, enhanced cell proliferation compared to the control cells seeded in glutamine-free medium at both 24 and 48 h (55.9% and 91%, respectively, for Ala-Gln, and 60.8 and 91.3%, respectively, for Gln); see Fig. 4. Ala-Gln (10 mM) increased cell proliferation following 5-FU pretreatment at a rate of 23.2% and 38.6% after 24 and 48 h, respectively. Supplementation with 10 mM of Gln increased cell proliferation at a rate of 25.1% and 29.2%, after 24 and 48 h, respectively, as detected by the WST-1 technique. At 50 mM concentration, both Ala-Gln and Gln did not increase cell proliferation at 12- and 24-h incubations.

Lack of Effect of Ala-Gln and Gln on 5-FU-Induced Apoptosis and Necrosis

Triplicate flow cytometry analyses demonstrated a significant increase in apoptosis in IEC-6 cells after 1 h of 1 mM 5-FU exposure. The apoptotic rate peaked at 12 h (35.6%), $P = 0.01$ versus either the untreated control containing Gln or Ala-Gln-enriched media. No further increase was observed at 24 h, following 5-FU treatment. Additionally, we could not find a significant difference in apoptotic rates before 12 h following 5-FU exposure (data not

shown) as compared to the respective controls. The lack of glutamine in the medium for 12 and 24 h increased significantly the apoptosis rates to 28.8% and 22.1%, respectively, when compared with Gln-enriched medium (10 mM of Gln), which showed an apoptosis rate of 2.9% and 8% at 12 and 24 h, respectively. There was no significant difference between the group without glutamine, and therefore under stressful conditions, and the group treated with 1 mM of 5-FU after 1 h (Figs. 5 and 6). Apoptotic cells were positive for annexin V labeled with fluorescein isothiocyanate (V-FITC) only (lower right quadrant) and cells that were both necrotic and apoptotic were both positive for annexin V-FITC and propidium iodide (upper right quadrant). The viable cells did not stain for annexin V-FITC or with propidium iodide. Gln (10 mM) and Ala-Gln (10 mM) failed to reduce significantly the apoptotic cell death due to 5-FU pretreatment (Gln: 21.9% and Ala-Gln: 30.08% at 12 h; Gln: 23.2% and Ala-Gln: 21.3% at 24 h), although a trend was observed toward apoptotic and necrotic decline, especially at 12 h (Figs. 5 and 6). The apoptosis rate of 8% observed at 24 h in the Gln-enriched media group (10 mM of Gln) showed a significant increase when compared with the apoptosis rate of 2.9% at 12 h. There was no significant difference in apoptosis in the rate of the Ala-Gln-enriched group (10 mM of Ala-Gln) at 12 and 24 h.

Discussion

In this study we have shown that IEC-6 cells, which represent a rat crypt undifferentiated cell line, when previously exposed to 5-FU treatment, exhibited mitotic arrest, increased apoptotic rates, and migration inhibition in a dose-dependent manner, corroborating clinical findings on intestinal barrier impairment seen with 5-FU chemotherapy [29, 30]. Cell proliferation, migration, and apoptosis at the crypt level are critically important in modulating dynamics of enterocyte turnover within the mucosa, especially following severe catabolic states, in order to repave the epithelial lining layer lost due to cell shedding at the villus level [31].

In our model, glutamine and alanyl-glutamine supplementation improved cell proliferation and migration in IEC-6 cells previously treated with 5-FU. Glutamine cooperates with distinct growth factors within the injured mucosa to enhance crypt turnover activity and to be involved in mitotic signaling pathways, ultimately leading to intestinal barrier healing by shifting the balance toward more proliferative instead of cell death programming responses [32, 33]. In addition, critical growth factors, such as the insulin-like growth factor-1 (IGF-1), are involved in intestinal mucosal healing following injury, and have now been associated with increased amino acid transporters in the enterocyte membrane [34, 35]. Our findings further support the strategy of exogenous glutamine administration in an attempt to supply adequate fuel to the rapidly proliferating immune and intestinal cells, following endogenous glutamine tissue deprivation in the severely ill, as observed in successful clinical trials with glutamine-enriched oral hydration therapy and parenteral supplementation [16, 21, 36, 37]. Furthermore, the observed beneficial effects in 5-FU-injured intestinal cells in this study suggests a possible protective or reparative role of glutamine or alanyl-glutamine supplementation during chemotherapy in clinical situations.

Our novel findings with alanyl-glutamine supplementation *in vitro* further support our previous study, in which we confirmed the benefit of the more stable alanyl-glutamine on

intestinal recovery following a single dose of 5-FU (150 mg/kg) treatment in mice, mostly associated with increased mitotic index and crypt hyperplasia. However, in contrast to our in vitro data, glutamine failed to accelerate mucosal healing in our murine model, which may suggest the superiority of the dipeptide over the more poorly soluble compound, glutamine, in vivo [25]. Interestingly, both in vivo and in vitro studies did not find a role of glutamine or alanyl-glutamine in preventing 5-FU-induced apoptosis in crypt cells 24 h after the challenge, at the peak time of the apoptotic curve, which is believed to precede chemotherapy-induced crypt hypoplasia [38]. Other studies have also described a possible lack of effect of glutamine supplementation in preventing apoptosis in vitro during chemotherapy treatment with methotrexate [39], supporting our findings, and suggesting that glutamine may act through different pathways on preventing cell death. Glutamine modulation of cell apoptosis appears to recruit cell-type-specific signaling, involving the intrinsic and extrinsic pathways [40, 41] and its deprivation was shown to induce apoptosis in vitro [42–44]. The role of glutamine deprivation in the significant increase in apoptotic rates is corroborated by the fact that IEC-6 cells seeded for 24 h in a glutamine-enriched media, without media change, show a higher apoptotic cell death as compared to 12 h, suggesting that a high metabolic consumption of glutamine by the cells rapidly depletes this conditionally required nutrient from the media. Nonetheless, the rate of apoptosis was unchanged in IEC-6 seeded in alanyl-glutamine enriched media, under the same conditions, suggesting better bio-availability of this dipeptide as a source of glutamine [45]. Although a trend toward reduction of apoptosis was seen, we could not show a significant glutamine effect in our in vitro model. Other studies, including our own [46, 47], have found a protective role of glutamine in preventing cell apoptosis seen with toxins, atrophy or cytokines [47–49] in other more differentiated intestinal cell lines. However, we postulate that apoptosis at the level of undifferentiated stem cells is critical to the effects of 5-FU in preventing the emergence of mutant cell lines in the crypts, which would prevent further neoplastic growth. Nevertheless, glutamine may still protect the intestinal mucosa even during chemotherapy, since it is not believed to negatively affect tumor growth [50].

In this current study, we have addressed the role of glutamine derivatives in IEC-6 cells following 5-FU challenge, evaluating cell migration, proliferation, and apoptosis using a specific undifferentiated intestinal cell line. This study further clarifies crypt cell dynamics following 5-FU-induced epithelial cell damage seen in vivo. The optimal dose of 10 mM of glutamine and alanyl-glutamine show enhanced crypt cell proliferation following 5-FU challenge. The same concentration proved to be beneficial in our previous studies [47, 51] with *Clostridium difficile* toxin A-induced epithelial injury. This concentration used in the medium far exceeds the physiological serum concentration, confirming the high requirement for these peptides for cell growth and adaptation following severe epithelial injury.

Further studies are needed to define the molecular mechanisms involved in intestinal cell viability and injury repair, including studies of the WNT signaling pathway and R-spondin-1 [52], promitotic factors which may interact with glutamine to hasten intestinal recovery. Additionally, the interaction with other critical guttrophic nutrients such as zinc and arginine are critical targets for developing more efficient strategies to alleviate malabsorption and

diarrhea due to undesirable intestinal mucositis, and to rebuild the small intestinal barrier, work which is currently underway in our laboratory.

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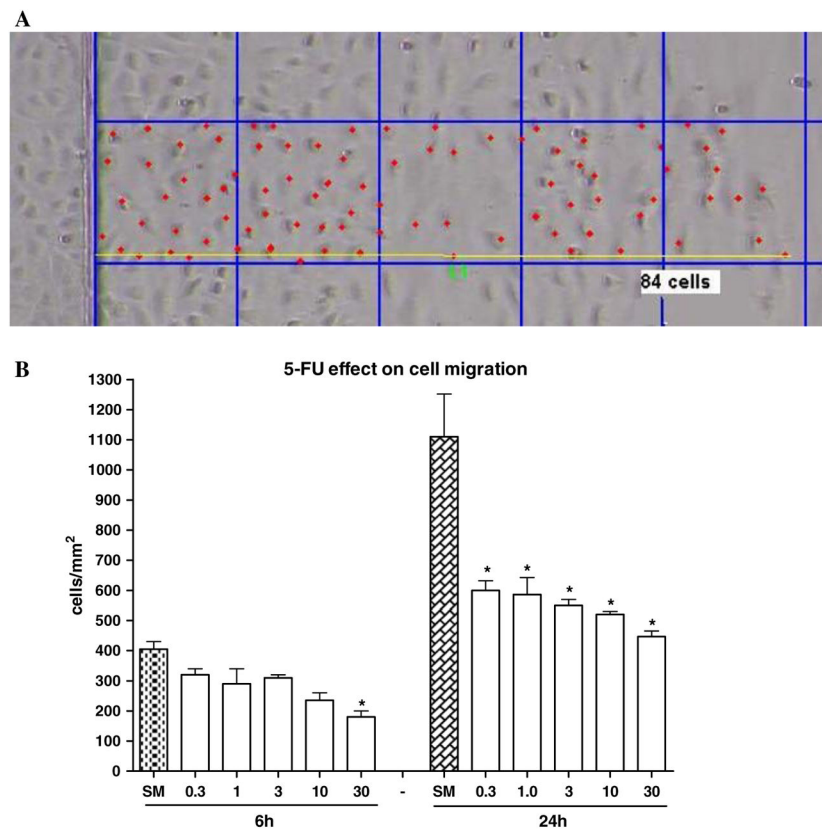


Fig. 1.

(a) Diagram showing a representative digital image of the scraping area with superimposed grid (each square = 0.1 mm²), overlapping the column of farthest cell migration. IEC-6 adherent cells were tracked by traced red dots for counting. The traced red dots within the column were changed to black on a white background and counted digitally by Image Pro Plus software. A yellow line was drawn to depict the migration distance from the scraping point. (b) Dose-response and time-effect graphs of 5-fluorouracil (5-FU) at 6 and 24 h, showing inhibition of cell migration in vitro. IEC-6 cell monolayers were scraped to induce migration, and incubated for 1 h with 5-FU immediately after standard medium replacement, diluted in standard media at doses of 0.3, 1, 3, 10, or 30 mM. The bars represent means \pm standard error (SE) for the number of migrating cells per square millimeter of scraped area. *Statistically significant difference ($P < 0.05$) compared to migrated cells in standard medium without 5-FU treatment (SM), determined by analysis of variance (ANOVA) and Bonferroni's test

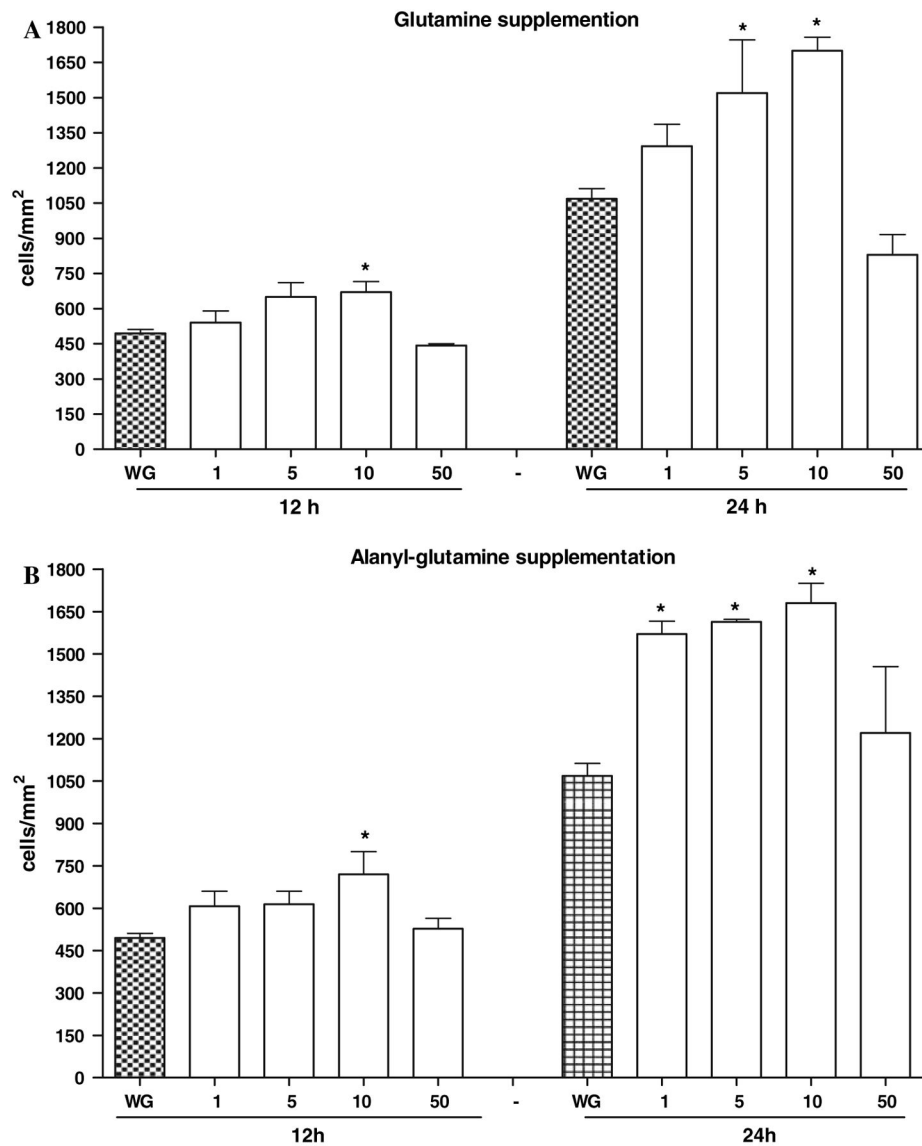
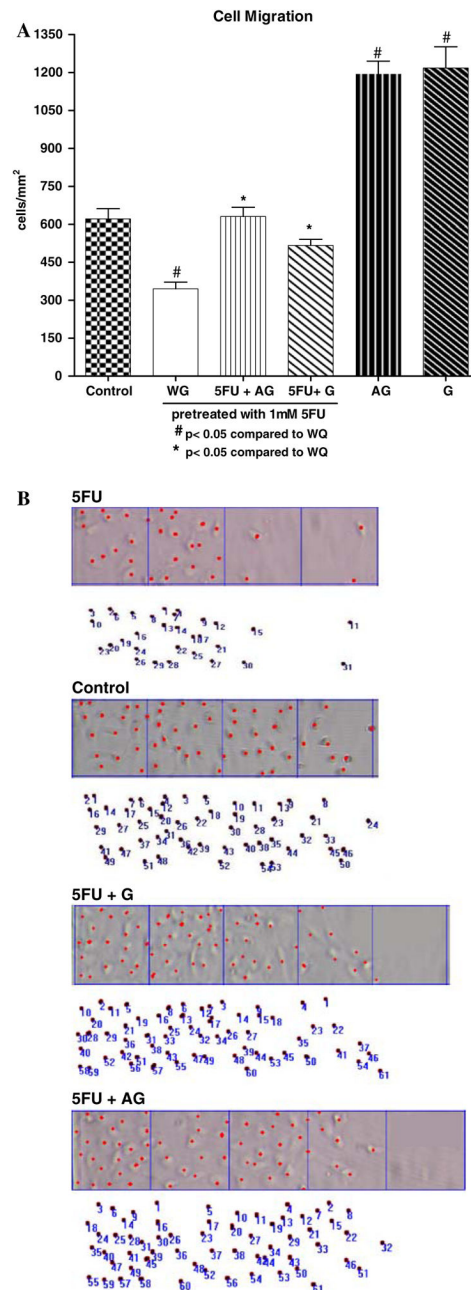


Fig. 2.

(a) Dose-response and time-effect graphs of glutamine (A) and alanyl-glutamine (B) at 12 and 24 h, in enhancing cell migration in vitro. IEC-cell monolayers were scraped to induce migration, and incubated with glutamine and alanyl-glutamine (1, 5, 10, and 50 mM) diluted in glutamine-free media, immediately after standard medium replacement. The bars represent means \pm SE for the number of migrating cells per square millimeter of scraped area. *Statistically significant difference ($P < 0.05$) compared to migrated cells in medium without glutamine (WG), determined by ANOVA and Bonferroni's test

**Fig. 3.**

(a) Protective effect of glutamine (G) and alanyl-glutamine (AG) (10 mM) in cell migration following 24 h of 5-FU challenge, in enhancing cell migration in vitro. IEC-6 cell monolayers were scraped to induce migration, and incubated with glutamine and alanyl-glutamine diluted in glutamine-free media, immediately after standard medium replacement. The bars represent means \pm SE for the number of migrating cells per square millimeter of scraped area. * $P < 0.05$ compared to migrated cells in glutamine-free media without treatment (WG), # $P < 0.05$, compared to migrated cells in standard media pretreated with 1 mM of 5-FU, by ANOVA and Bonferroni's test. (B) Representative migration columns of adherent IEC-6 cells from the experimental groups 24 h following the 5-FU challenge. The control group is shown at the top, representing migrating cells not challenged with 5-FU embedded in standard media

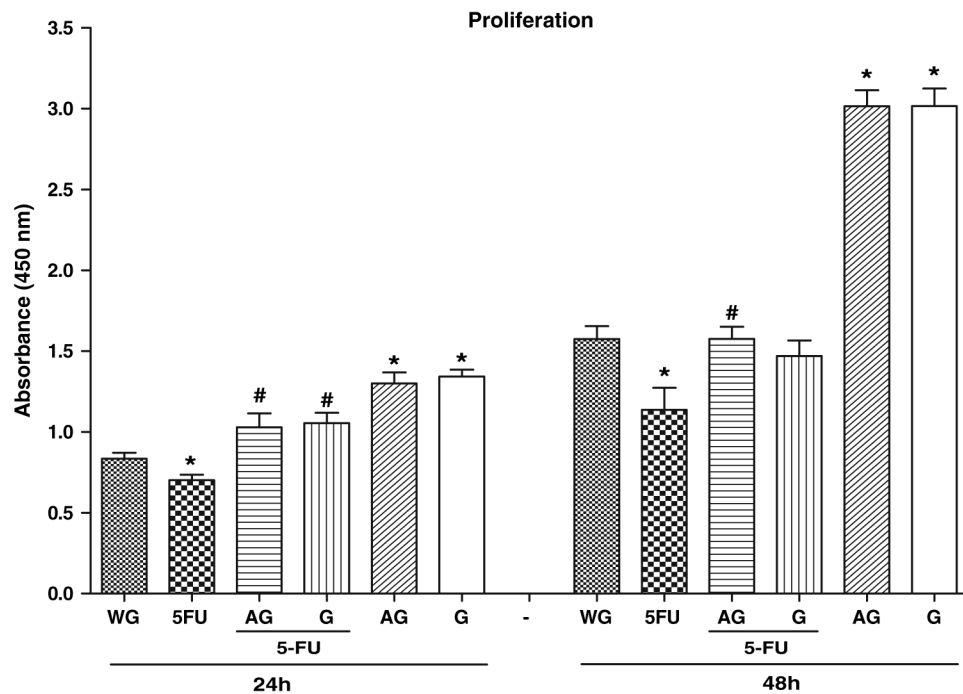


Fig. 4.

Effect of glutamine (G) and alanyl-glutamine (AG) on cell proliferation assay by detected absorbance using an ELISA microplate reader at 450 nm in 96-well seeded IEC-6 cells, following 1 h of 5-fluorouracil (5-FU) exposure (1 mM). 5-FU was diluted in DMEM Gln-free media. After 24 and 48 h, wells were incubated for 4 h with 10 μ l of the tetrazolium salt and the absorbance was measured. * $P < 0.05$ compared to untreated control in glutamine-free media (WG) at 24 and 48 h, # $P < 0.05$, compared to standard media pretreated with 1 mM of 5-FU at 24 and 48 h, by Student's t test and ANOVA and Bonferroni's test, respectively

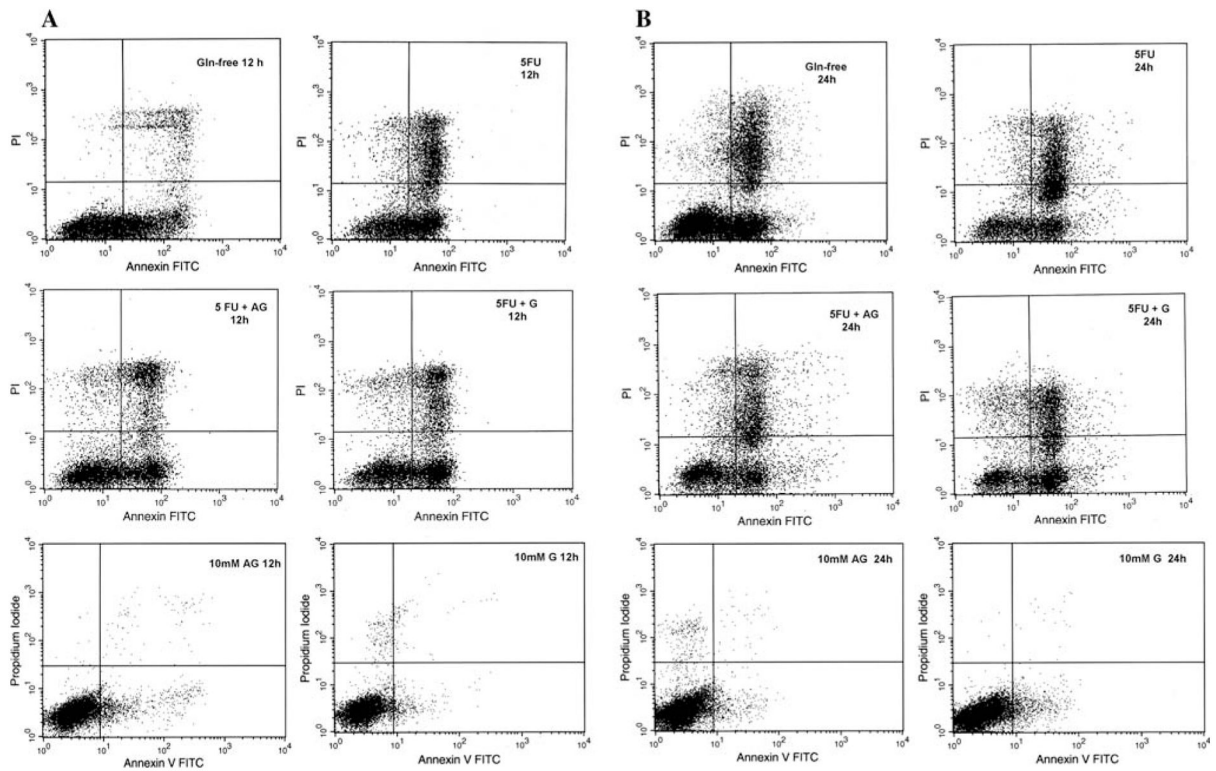
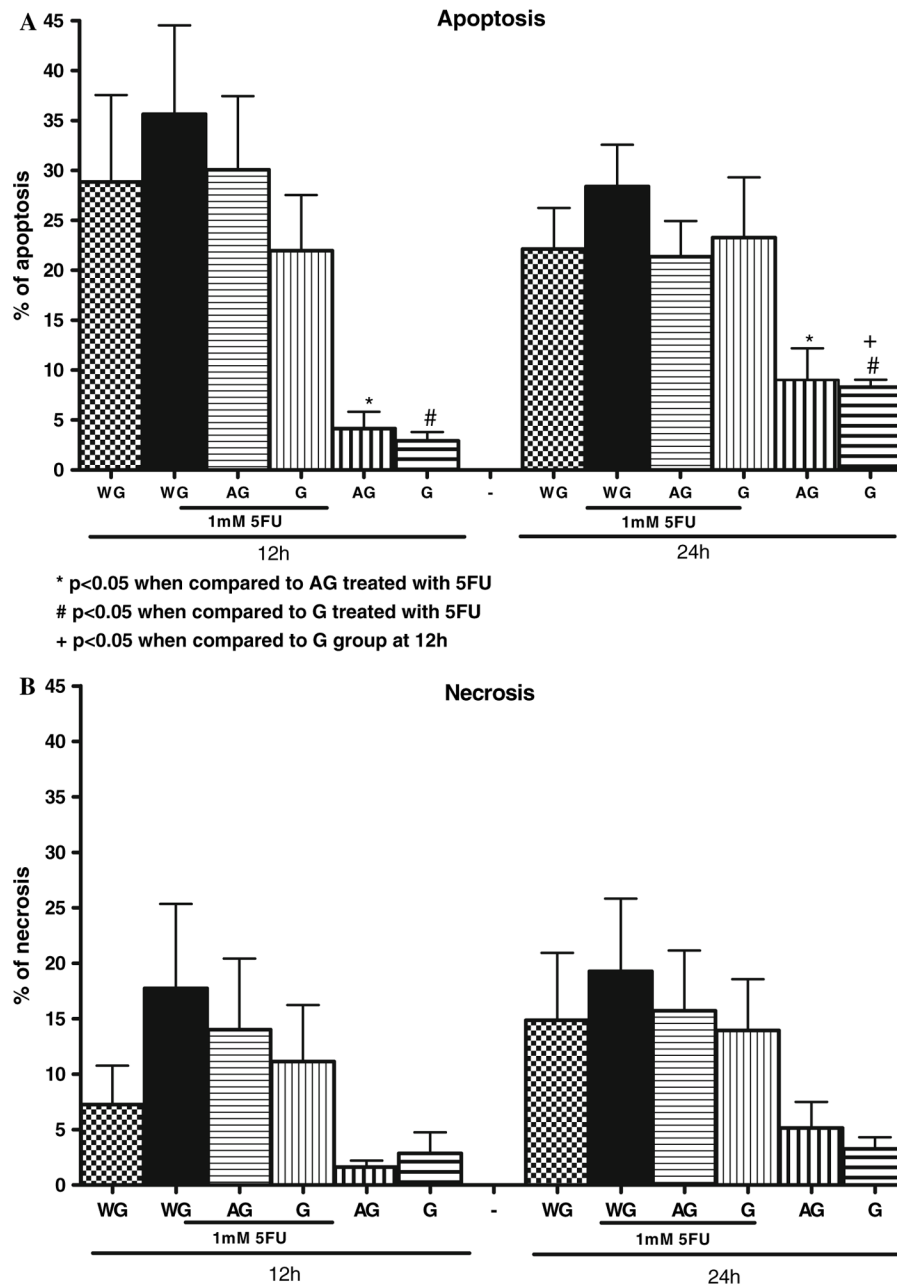


Fig. 5.

Apoptosis and necrosis induced by 1 h 5-fluorouracil (5-FU) pretreatment (at 1 mM, diluted in glutamine-free media) were not inhibited by alanyl-glutamine (AG) and glutamine (G) at 12 (**a**) and 24 h (**b**) in IEC-6 cells. Cells were stained with FITC-conjugated annexin V and propidium iodide and analyzed by flow cytometry. Results are shown as density plots with propidium iodide versus annexin V-FITC. Viable cells have low annexin-V-FITC and low propidium iodide staining (lower left quadrant), apoptotic cells have high annexin V-FITC and low propidium iodide staining (lower right quadrant), and necrotic cells have high propidium iodide and annexin V-FITC staining (upper right quadrant)

**Fig. 6.**

Effect of glutamine (G) and alanyl-glutamine (AG) on apoptosis (a) and necrosis (b) by flow cytometry in IEC-6 cells, following 1 h of 5-fluorouracil (5-FU) exposure (1 mM). 5-FU was diluted in DMEM Gln-free media. * $P < 0.05$ compared to media enriched with 10 mM of AG, pretreated with 1 mM of 5-FU at 12 and 24 h, # $P < 0.05$, compared to media enriched with 10 mM of G, pretreated with 1 mM of 5-FU at 12 and 24 h, + $P < 0.05$ compared to media enriched with 10 mM of G at 12 h, by Student's t test