

## Pathways from glutamine to apoptosis

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## 1. ABSTRACT

It is fascinating that a relatively simple amino acid like glutamine is involved in such large variety of cellular reactions. Glutamine is required for nitrogen-stimulated proliferation in many cells, but glutamine stimulates not only the growth of cells but also the expression of surface antigens, the formation of cytokines, the synthesis of heat shock proteins and many more vital events. Among all of them, apoptosis is a recent but outstanding incorporation to the whole of phenomena regulated by this peculiar amino acid. Apoptosis is an important process in a wide number of biologic systems. Apoptotic signalling mechanisms implicated in response to glutamine deprivation are cell type-specific. In any case, new findings indicate that glutamine availability is strongly related to the induction of apoptosis, working both as a nutrient and as a signalling molecule, acting directly or indirectly on the pathways leading to programmed cell death. Following, we will describe as glutamine and the related species glutamate, glutathione and glucosamine can play important roles in the pathways leading to apoptosis.

## 2. INTRODUCTION

Glutamine is a multifaceted amino acid used for hepatic urea synthesis, renal ammoniogenesis, and gluconeogenesis in both liver and kidney (1). Decreased glutamine concentrations are found during catabolic stress (2). Glutamine is the most abundant free amino acid in the body and is known to play a regulatory role in several cell specific processes including apoptosis (3). Glutamine is considered to be the main energy source for the cells (4). It is also a lipogenic precursor, and is involved in cell proliferation, protein synthesis, and protein degradation (5). Glutamine also plays important functions in the regulation of insulin resistance, insulin secretion, contractile protein mass, extracellular matrix synthesis, respiratory burst, and redox potential (6). Thus, glutamine has been shown to regulate the expression of many genes related to metabolism, signal transduction, cell defense and repair, and to activate intracellular signalling pathways (1). In fact, glutamine it is also important for cell defence from sub-lethal concentrations of toxic agents (7). In conclusion, the function of glutamine goes beyond that of a simple metabolic fuel or protein precursor as previously assumed.

## Glutamine and apoptosis

The amino acid glutamine plays a central role in nitrogen transport and is a fuel for rapidly dividing cells and proliferation. Suppression of proliferation and apoptosis are strongly related (8). Plasma glutamine levels decline during critical illness and catabolic stress. Under these conditions, cells suffer from glutamine starvation leading to an energy depletion that is associated with a reduced responsiveness to exogenous stimuli and to susceptibility to infections. Additionally, glutamine-starving cells show a reduced expression of the heat shock proteins of 70 kDa (HSP70), which is an important factor for cell survival, and contain a reduced level of the antioxidant glutathione (GSH). Recent findings show that the extracellular glutamine level affects the susceptibility of cells to different apoptosis triggers: whereas glutamine-starving cells are more sensitive to Fas ligand-mediated apoptosis, they are desensitized against the cytotoxic effects of tumor necrosis factor-alpha (TNF-alpha). Molecular mechanisms of glutamine sensing includes AMP-activated protein kinase, the cellular redox state, osmosignalling, the regulation of translation, and amino acyl transfer RNA synthetases (9).

### 3. THE IMPORTANCE OF A NON-ESSENTIAL AMINO ACID

Glutamine via glutamate, is one of the precursors for the synthesis of glutathione, the major endogenous antioxidant in mammalian cells, which protects them from oxidative injury and has antiapoptotic effects. Cancer cells have higher GSH levels than the surrounding normal cells, which attributes to a higher rate of cell proliferation and resistance to chemotherapy. Therefore, selective tumor depletion of GSH presents a promising strategy in cancer treatment. Experimental studies have associated decreased GSH levels with inhibition of proliferation and stimulation of apoptosis. Glutamine supplementation causes a significant decrease in the tumor GSH levels and the ratio GSH/oxidized GSH (10).

Glutamine itself significantly enhanced IL-2 production, cell proliferation, and cell viability of Jurkat T cells. Glutamine also decreased the number of apoptotic cells stimulated with phorbol myristate acetate (PMA) plus ionomycin, and significantly decreased caspase-3 and caspase-8 activities in activated T cells. In addition, glutamine significantly increased GSH, but decreased reactive oxygen species levels in activated T cells. Blockade of intracellular GSH formation enhanced, but exogenous GSH supplementation decreased, activated T-cell apoptosis. Glutamine increases lymphoproliferation as well as the antiapoptotic protein Bcl-2. Simultaneously, glutamine enhances the Fas receptor CD95 expression but suppressed activation-induced T-cell death in both Jurkat T cells and human peripheral T lymphocytes (11). In other models, very recent results show that glutamine also significantly reduces apoptosis by preventing caspase-8 activation (12).

Apoptosis is induced by oxidative stress and cytokines, but regulation by specific nutrients has been infrequently studied under these conditions. In cultured

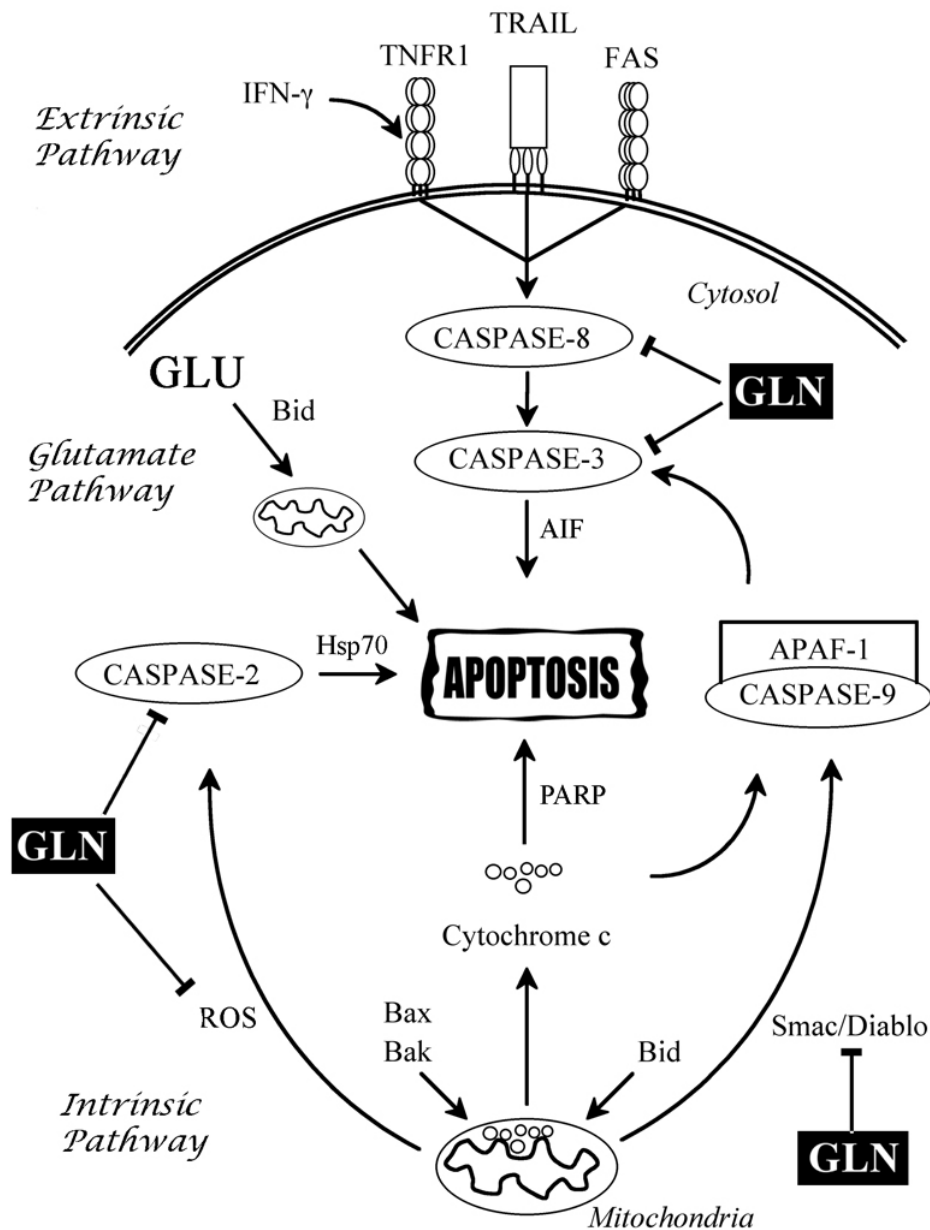
intestinal epithelial cells, glutamine depletion increases oxidant-induced apoptosis. Glutamine -but not other amino acids, including the GSH constituents, glutamate, cysteine and glycine- protects against apoptosis induced by the cytokine tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL) in human colon carcinoma cells. Similar antiapoptotic effects of glutamine occurred when apoptosis was induced by a combination of TNF-alpha and interferon-gamma (Figure 1). Cellular GSH was oxidized during TRAIL-induced apoptosis. This effect was completely blocked by glutamine, however, inhibition of GSH synthesis with buthionine sulfoximine did not alter glutamine antiapoptotic effects. Furthermore, glutamate prevented GSH oxidation in response to TRAIL but did not protect against TRAIL-induced apoptosis. These results show that glutamine specifically protects intestinal epithelial cells against cytokine-induced apoptosis, and that this occurs by a mechanism that is distinct from the protection against oxidative stress mediated by cellular GSH (13). Recent findings show that the antiapoptotic effects of glutamine are independent of DNA/RNA synthesis, pointing that glutamine prevents TRAIL-induced apoptosis in HT-29 cells through a mechanism involving the pyrimidine pathway (14).

Under glutamine-free conditions, which can be obtained when cells are cultivated *in vitro*, tissue cells cannot grow. Therefore, when classifying glutamine as a "non-essential" amino acid, one must consider that in the human body glutamine is synthesized from essential amino acids and is continuously delivered from skeletal muscle to other organs. Further, a glutamine deficiency leads to a cell cycle arrest in G(0) to G(1) and reduces apoptosis. Interestingly, many of these biological activities also are associated with the cellular reduced oxygen potential, which depends mainly on the ratio of reduced to oxidized glutathione. Experimental animal studies have shown that the administration of glutamine increases tissue concentrations of reduced glutathione. Alteration of reduced glutathione metabolism is related to a variety of clinical conditions such as reperfusion injury, myocardial infarction, respiratory insufficiency, cancer, diabetes, liver disease, and protein catabolism diseases (15).

When medications are used to treat life-threatening illnesses, balancing the toxic effects with the therapeutic benefits of the drug can be difficult. Apoptosis is associated with neuropathy after treatment with medications to treat cancer, and HIV, and to prevent transplant rejection. Cisplatin and suramin both result in apoptosis in ganglion neurons that may partially explain the neuropathy that develops with treatment. In contrast, nerve growth factor prevents initiation of the programmed cell death associated with cisplatin neurotoxicity. Glutamine also reduces some drug-induced toxicity (16).

### 4. PATHWAYS TO APOPTOSIS

Following mitochondrial outer membrane permeabilization proteins sequestered in the mitochondrial intermembrane space gain access to other proteins in the cytosol, and this results in apoptosis (17). Cells of higher



**Figure 1.** Schematic representation of glutamine role in extrinsic, intrinsic and glutamate-dependent pathways leading to apoptosis. The extrinsic cell death pathway is mediated by a subgroup of the TNF receptor superfamily called the death receptors (TNFR1, FAS, and TRAIL). Receptor-mediated cell death is initiated by the recruitment of adaptor proteins that leads to activation of caspase-8. Caspase-8 directly cleaves and activates caspase-3, the executioner enzyme of apoptosis. Caspase-2 is only involved in heat shock-induced apoptosis. In enterocytes, immune-derived cells and cancer cells, deprivation of glutamine is able to depresses HSP70 protein expression. In the mitochondrial or intrinsic pathway, proapoptotic Bcl-2 family members Bax and Bak translocate to the mitochondria. The protein Bid activates Bax and Bak to mediate the release of cytochrome c in the cytosol. This triggers the assembly of the apoptosome (APAF1 and caspase-9) and subsequent activation of caspase-3 and cell death. Protein Bid is able to activate Bax and Bak, resulting in cytochrome c release from mitochondria. The inhibition of apoptosis is avoided by the second mitochondria-derived activator of caspases (SMAC/Diablo), that can be down-regulated by glutamine. Glutamine also down-regulates intrinsic pathway to apoptosis acting as a ROS scavenger. In this schema is also included the effect of glutamate inducing the excitotoxic apoptosis. A translocation of a full length Bid to mitochondria produces a collapse of the mitochondrial membrane potential leading to apoptosis. Abbreviations: AIF, apoptosis-inducing factor; APAF-1, apoptotic peptidase activating factor 1; GLN, glutamine; HSP70, heat shock protein of 70 kDa; IF- $\gamma$ , interferon gamma; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; TNFR1, tumor necrosis factor receptor 1; TRAIL, TNF- $\alpha$ -related apoptosis-inducing ligand.

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eukaryotes contain extrinsic receptor pathways and intrinsic pathways that activate effector proteases and induce apoptosis. Most of these proteases are belonging to the cysteine-dependent aspartate-specific proteases or caspases (18). The extrinsic cell death pathway is mediated by a subgroup of the TNF receptor superfamily called the death receptors (TNFR1, FAS, and TRAIL). Receptor-mediated cell death is initiated by the recruitment of adaptor proteins, like FADD, which then bind to procaspases to generate a death-inducing signalling complex (DISC) that leads to activation of caspase 8. Caspase 8 directly cleaves and activates caspase-3, the executioner enzyme of apoptosis (19). The protein Bid amplifies the caspase cascade by connecting the death receptor pathway with the mitochondrial apoptotic pathway (12). After being cleaved by caspase-8 from its inactive form localized in the cytoplasm, the truncated form of Bid activates Bax and Bak to mediate the release of cytochrome c in the cytosol (Figure 1). Cytochrome c triggers the assembly of the apoptosome (APAF-1 and caspase-9) and subsequent activation of caspase-3 and death (20). While a truncated protein Bid acts in both intrinsic and extrinsic pathways to apoptosis, a full length protein Bid is translocated to the mitochondria in the caspase-independent glutamate-induced excitotoxic apoptosis. Following, a collapse of the mitochondrial membrane potential leading to apoptosis will occur (21).

Caspase 2 was exclusively activated in heat shock-induced apoptosis. This activation of caspase 2 was also observed in cells protected from heat-shock-induced apoptosis by Bcl-2 or Bcl-xL (22). As expected, only caspase-8 was present in response to ligation of death receptors, whereas only caspase-9 was present in response to a variety of other apoptosis-inducing agents (Figure 1).

### 5. DEPRIVATION OF GLUTAMINE ENHANCES APOPTOSIS

Deprivation of several amino acids resulted in decreased cell numbers. Nevertheless, in intestinal epithelial cells, only the glutamine-deprived group showed significant induction of apoptosis (23). Other experiments have shown that starvation on rats was associated with lower muscle glutamine levels, a higher level of oxidized glutathione, and greater numbers of apoptotic cells (24).

In human hepatoma cells, glutamine deprivation causes apoptotic cell death with stimulated caspases-2 and -3 but not caspases-8 or -9 activities, and leading to considerable poly (ADP-ribose) polymerase (PARP) cleavage (25). This enzyme is thought to have important function during apoptosis (Figure 1). There is evidence that the enzyme is involved in the stacking of polymers during DNA backbone formation, suggesting the enzyme has a role in DNA damage repair and whether apoptosis is induced cell to die. Recently, it was demonstrated that PARP induces translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus, causing DNA condensation and fragmentation, and subsequent cell death (26).

Both the major energy-source nutrients in the medium, glucose and glutamine, became rapidly exhausted

during the cell incubation of human cells. Glutamine deprivation is associated with cell death by apoptosis independent of energetic failure, whereas glucose deprivation is followed by rapid loss of mitochondrial function with sharp drop of intracellular ATP and cell death by necrosis. A 12-24 h incubation in glutamine-depleted medium was required to direct leukaemia cultured cells toward the apoptotic pathway. Growth arrest followed by apoptotic death was detected in cells when medium glutamine concentration remained below 0.3-0.4 mM for at least 24 h, but a reinstatement of medium glutamine to 2 mM within this period rescued the cells from growth arrest and death (27).

During batch culture, decreases in glutamine correlated with an increase in apoptotic cells. The contribution of apoptosis to overall cell death was smaller in conditions of glucose deprivation than in glutamine deprivation (28). Apoptotic or programmed cell death occurs spontaneously in late exponential phase of batch cultures. Glutamine deprivation induced apoptosis in both hybridoma and myeloma cell lines whereas accumulation of toxic metabolites induced necrotic cell death in these cells. Other triggering factors such as oxygen deprivation might also be responsible for induction of apoptosis. In terms of cellular metabolism, anoxia resulted in an increase in the utilization rates of glucose and arginine, and in a decrease in the utilization rate of glutamine (29).

The phenomenon of starvation-induced apoptosis has been also studied in cultures of a mouse B lymphocyte hybridoma (30) and in human T-lymphoblastic leukemia cell line (31). Suppression of the death rate, and increase of steady-state viable cell concentration, could be achieved by additions glutamine. It is concluded that the apoptosis-preventing glutamine acts as a signal molecule, besides their nutritive function, and that the signal has a character of a survival factor (32). Deprivation of glutamine on human androgen-independent prostate cancer cells, and on nontumorigenic human fibroblasts and human prostate epithelial cells inhibited growth, arresting the cell cycle at G0/G1. However, glutamine deprivation only decreased invasion of prostate cancer cells (33).

Cell shrinkage and loss of cell viability by apoptosis have been examined in cultured CD95-expressing leukemia-derived CEM and HL-60 cells subjected to acute deprivation of glutamine. Glutamine deprivation-mediated cell shrinkage promoted a ligand-independent activation of the CD95-mediated apoptotic pathway. The cell-size shrinkage-dependent apoptosis induced by glutamine restriction in CD95-expressing leukemic cells may therefore be of clinical relevance in glutaminase enzyme therapies (34). On the other hand, nutrient starvation and hyperosmolality was associated with increased apoptosis, enhancing the glutamine uptake (35).

Loss of cell viability, through engagement of apoptotic cell death, represents a limitation to maintenance of high levels of productivity of recombinant animal cells in culture. Growth arrest and DNA damage gene 153 (GADD153) is a member of the CCAAT/enhancer-binding

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protein (C/EBP) family of transcription factors and has been associated with apoptosis. Of noteworthiness, depletion of glutamine causes activation of GADD153 expression and susceptibility to apoptosis (36).

Glutamine starvation of epithelial cells resulted in the time-dependent activation of caspases 3 and 2, and the induction of DNA fragmentation. In this model, caspases 1 and 8 remained inactive. ZVAD-fluoromethyl ketone, a general caspase inhibitor, completely blocked glutamine starvation-induced caspase activation, DNA fragmentation, and nuclear condensation. These results indicate that glutamine starvation selectively activates specific caspases, which leads to the induction of apoptosis (37). In some cells types (RIE-1, rat neutrophils, human CEM and HL-60), glutamine deprivation alone is sufficient to trigger apoptosis. In Sp2/0-Ag14 hybridoma cells, lower GSH levels per se are not sufficient to trigger cell death (38). Caspase-9 is also involved in this process, suggesting that glutamine deprivation initiates an intrinsic apoptotic pathway. Supporting this idea, the cytosolic release of the mitochondrial proteins Smac (also known as DIABLO) and cytochrome c was observed during glutamine deprivation (Figure 1). The latter occurred simultaneously with the translocation of the pro-apoptotic protein Bax to the mitochondria (39). In some situations, the use of caspase inhibitors has enabled maintenance of hybridoma cell line viability during a significant period of time, when glutamine depletion was maintained in the culture. Nonetheless, when the culture was exposed to non-apoptotic conditions under apoptosis protection conditions, a normal growth pattern was not recovered. Interestingly, the simultaneous use of inhibitors made the recovery of the cell culture possible even after a period of 36 h under glutamine depletion, indicating that the inhibition of the effector caspases occurs upstream of the point in which hybridoma cells enter into the commitment step of apoptosis (40).

In addition, acivicin, a glutamine antagonist, causes a dose- and time-dependent apoptosis in a gamma-glutamyltranspeptidase (GGT)-negative cell line as well as in its GGT-positive counterpart line. This is the evidence that acivicin induces apoptosis in these cells working as a glutamine antagonist, independently of their GGT activity level (41).

### 6. SUPPLEMENTATION OF GLUTAMINE DIMINISH APOPTOSIS BUT REDUCES TUMOR PROLIFERATION

Intestinal epithelial cell turnover (proliferation, migration, differentiation, and apoptosis) and gut barrier functions are dynamic processes that are markedly affected by nutritional status, the route of feeding, and the adequacy of specific nutrients in the diet. Emerging studies are defining potential therapeutic roles for specific nutrients and diet-derived compounds (including glutamine, glutamate, and glutathione) in turnover, repair and adaptation (42).

Retention and accumulation of toxic bile salts in hepatocytes may cause toxicity by inducing apoptosis. Oral

glutamine administration can attenuate or abolish hepatocyte apoptosis. In fact, after administration of glutamine the increased hepatocyte apoptosis was significantly diminished (43).

In cell cultures, apoptosis can also be prevented by glutamine feeding (44). Elevated concentrations (mM) of glutamine have been shown to protect cell lines exposed to two very different environmental stresses: nutrient starvation and hyperosmolality or elevated pCO<sub>2</sub> (45). Supplementation of hypertonic storage solutions with glutamine might exert a partial osmoprotective effect and prevent endothelial damage (46).

Glutamine has a protective effect on neutrophil apoptosis induced by acute exercise. When rats received oral glutamine supplementation, the phagocytosis capacity was significantly increased, the decrease in nitric oxide production induced by exercise was abolished and production of reactive oxygen species was raised (47).

On the other hand, it has been established that oral glutamine supplementation significantly reduced tumor development and restored the depressed GSH production. Glutamine supplementation resulted in a significant decrease in the levels of insulin-like growth factor-1 (IGF-1), type I insulin-like growth factor receptor (IGF-IR), Akt, and Bcl-2 in nontumorous samples. At the same time, the levels of pro-apoptotic protein Bad were significantly elevated. The samples collected from tumor tissues showed lower levels of IGF-1, IGF-IR, Akt, Bcl-2, and Bad in comparison with nontumorous tissues. Glutamine supplementation inhibited the PI-3K/Akt pathway that is thought to be important in increasing cell survival during tumorigenesis. On the contrary, 7,12-dimethylbenz [a] anthracene (DMBA) administration to pubertal rats causes breast tumors and inhibits glutathione production. These results are in agreement with glutamine counteracting the effects of DMBA and blocking carcinogenesis *in vivo* (48). Glutamine transport was decreased in a human neuroblastoma cell line in the presence of IGF-IR. In accordance, activation of IGF-IR have been shown to partially mediate neuroblastoma cell proliferation by regulating membrane glutamine transport (49).

### 7. NEURONAL APOPTOSIS AND GLUTAMATE

Elevated levels of glutamate, a glutamine-related excitatory amino acid, contribute to the development of neuronal injury in various cerebral diseases. Decrease of glutamine levels during methionine sulfoximine (specific inhibitor of glutamine synthetase) treatment, was accompanied by a decrease of glutamate but it did not caused neuronal apoptosis. A higher functional capacity of the cortical glutamine synthetase (GS) may be possible explanations for the delayed decline of extracellular glutamate concentrations. (50).

Of interest, glufosinate (that structurally resembles glutamate and blocks glutamine synthetase) was recently found to be dysmorphogenic in mammals *in vitro*, specifically inducing apoptosis in the neuroepithelium of embryos (51).

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The treatment of some cells types with L-asparaginase induces an apoptotic process that is forerun by a marked intracellular depletion of glutamate and glutamine, and a complete suppression of cell proliferation (52). Under these conditions, the activity of glutamine synthetase is very low. Recent findings indicate that, in the presence of L-asparaginase, the inhibition of GS triggers apoptosis (53).

It is now widely accepted that neuronal damage in HIV infection involves apoptosis, oxidative stress and glutamate-mediated neurotoxicity. Glutamate toxicity acts via 2 distinct pathways: an excitotoxic one in which glutamate receptors are hyperactivated, and an oxidative one in which cystine uptake is inhibited, resulting in glutathione depletion and oxidative stress. A number of studies show that astrocytes normally take up glutamate, keeping extracellular glutamate concentration low in the brain and preventing excitotoxicity. This hypothesis might explain the discrepancy between microglial activation which occurs early in the disease, and neuronal apoptosis and neuronal loss which is a late event. There are possible neuroprotective and neurotrophic roles of activated microglia and macrophages that may be generated by the expression of high affinity glutamate transporters and glutamine synthetase, two major effectors of glial glutamate metabolism (54).

Astrocytes protect neurons against glutamate excitotoxicity through exchange of glutamine for glutamate, and reactive oxygen and nitrogen (peroxynitrite) species. Astrocytes and microglia also protect neurons by storing excess iron and by brushing away from the brain tissue damaged cell constituents such as aged mitochondria, peroxidized cytomembranes and nitrated proteins (55). Astrocyte uptake and recycling of synaptic glutamate as glutamine is a major metabolic pathway dependent on energy metabolism, which inter-relationships are not fully understood and remain controversial. The glutamate/glutamine cycle is a generally accepted picture of the interaction between astrocytes and neurons to deal with glutamate toxicity; however, the process that shuttles the nitrogen produced in neurons and needed by astrocytes is poorly understood (56). Experiments altering the glutamate-glutamine cycle and glucose metabolism have been made. A reduction of glutaminase (GA), the glutamate synthetic enzyme, was evidenced combined with an increase in glutamine synthetase, the inactivating glutamate enzyme. Increased lactate dehydrogenase (LDH) activity was only present after a more severe injury. These results indicate an *in vivo* adaptation of the glutamate-glutamine cycle in order to increase the net glutamine output, reduce glutamate excitotoxicity, and avoid neuronal death. It is concluded that the graded modification of the glutamate-glutamine correlation and neuronal lactate availability may be key factors in the apoptotic and necrotic neuronal demise, whose control may prove highly useful to potentiate neuronal survival (57).

Additionally, glutamine synthetase and neuronal apoptotic cell death were evaluated in a rabbit model of pneumococcal meningitis. GS activity is higher in the

neocortex but not in the hippocampal formation compared to the respective brain region of uninfected control animals. This may represent a protective mechanism for cortical neurons. The inability of hippocampal GS to counteract the detrimental effects of glutamate may be the cause of neuronal apoptosis observed during meningitis (58). Apoptosis of granular cells in the hippocampal formation during bacterial meningitis may be mediated by glutamate toxicity. After intravenous administration of methionine sulfoximine in treated rabbits with meningitis, hippocampal glutamine synthetase is unable to metabolize excess amounts of glutamate, contributing to neuronal apoptosis in the hippocampal formation during meningitis (59). It is suggested that GS may execute a neuroprotective feature of rabbit brain during meningitis since neuronal apoptosis occurs only in brain regions showing diminished GS activity (60).

## 8. HIGH GLUCOSAMINE AND LOW GLUCOSE LEVELS INDUCE APOPTOSIS

A common characteristic of tumor cells is the constant overexpression of glycolytic and glutaminolytic enzymes. So, UDP-N-acetyl glucosamine is enhanced during cell proliferation (61). An important link has recently been shown *in vivo* between beta-cell O-linked protein glycosylation, providing substrate for the glucosamine pathway, and beta-cell apoptosis. Ability to stimulate O-glycosylation was only consistently observed when pancreatic islets were isolated in the presence of glucose and glutamine. These data suggest that during islet isolation, beta-cell enzymes responsible for regulating O-glycosylation may be adversely affected by the absence of glucose and glutamine, which together are necessary for O-linked N-acetylglucosamine synthesis (62).

In a different context, neuronal apoptosis occurs early in diabetic retinopathy. Insulin may act as a neurotrophic factor in the retina via the phosphoinositide 3-kinase/Akt pathway. Glucosamine not only impaired the neuroprotective effect of insulin but also induced apoptosis in a dose-dependent fashion. Glucosamine also damaged insulin receptor processing in a dose-dependent manner. By contrast, in muscle cells, glucosamine impaired insulin receptor processing but did not induce apoptosis. These results suggest that the excessive glucose flux may direct retinal neurons to undergo apoptosis in a bimodal fashion; i.e. via perturbation of the neuroprotective effect of insulin mediated by Akt and via induction of apoptosis possibly by altered glycosylation of proteins (63).

Conversely, inhibition of glucose uptake markedly increased the rate of apoptosis in several cell lines, an effect that could be reversed by the provision of alternative energy sources such as glutamine (64). Decrease of pancreatic beta-cell viability (by increasing apoptosis), are associated with significant increases in glucose and glutamine consumption as well as nitric oxide and ammonia production. This regulatory effect may be compromised by high circulating levels of glucose, which is elevated in type II diabetes and may impact upon dysfunctional and apoptotic intracellular events in the beta-

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cell (65). Rat RPE cells require glucose as their primary metabolic substrate in culture, but can metabolize glutamine in its absence, by altering their pathways of energy production (66). On the other hand, nutritional support with glutamine promotes mucosal cell proliferation significantly, and prevents mucosal cell from undergoing apoptosis (67).

Prolonged culture in low-glucose concentrations induces apoptosis in beta-cells. Culture in the presence of 3 or 5mM instead of 10mM glucose induces a large increase in c-myc expression before onset of a caspase-dependent apoptosis. These effects were prevented by addition of glutamine. In rat beta-cells, adenovirus-mediated c-myc overexpression increased their rate of apoptosis, whereas antisense-c-myc expression reduced low-glucose-induced apoptosis by approximately 50%. In the insulin producing MIN6 cell line, apoptosis induction by either low glucose or an activator of AMP-activated protein kinase (AMPK) was associated with c-myc mRNA and protein upregulation (68).

Insulin-dependent diabetes mellitus (IDDM) serum induced apoptosis in islet cells. Glutamine and the potent antioxidant 1-pyrrolidinedithioic acid partially reversed cell death induced by IDDM patient serum in a concentration-dependent manner. Pathway of IDDM patient serum-induced islet cell apoptosis involve free radical generation (69).

### 9. GLUTAMINE FIGHTING AGAINST OXIDATIVE STRESS

Oxidative stress results in the release of free radicals and aggressive reactive oxygen species (ROS) that alter the lipids, proteins and nucleic acids. ROS function as intracellular second messengers activating, among others, apoptosis, whereas glutamine is an apoptosis suppressor (70).

Recent evidence indicates that in addition to their antioxidant function, several redox species and systems are involved in regulation of biological processes, including cellular signaling, transcription factor activity, and apoptosis in normal and cancer cells. The survival and overall well-being of the cell is dependent upon the balance between the activity and the intracellular levels of these antioxidants as well as their interaction with various regulatory factors (71).

Oxidative stress during islet isolation induces a cascade of events injuring islets. In an up to date study it was concluded that administration of glutamine reduces oxidative injury and apoptosis and improves islet yield and function after transplantation. The amount of apoptotic cells per islet was smaller in an enriched-glutamine group than at the control. Glutamine administration increased GSH levels and reduced lipid-peroxidation (72).

TNF-alpha induces a caspase-independent but mitochondria-dependent cell death process in a mouse fibrosarcoma cell line. Mitochondria actively participate in

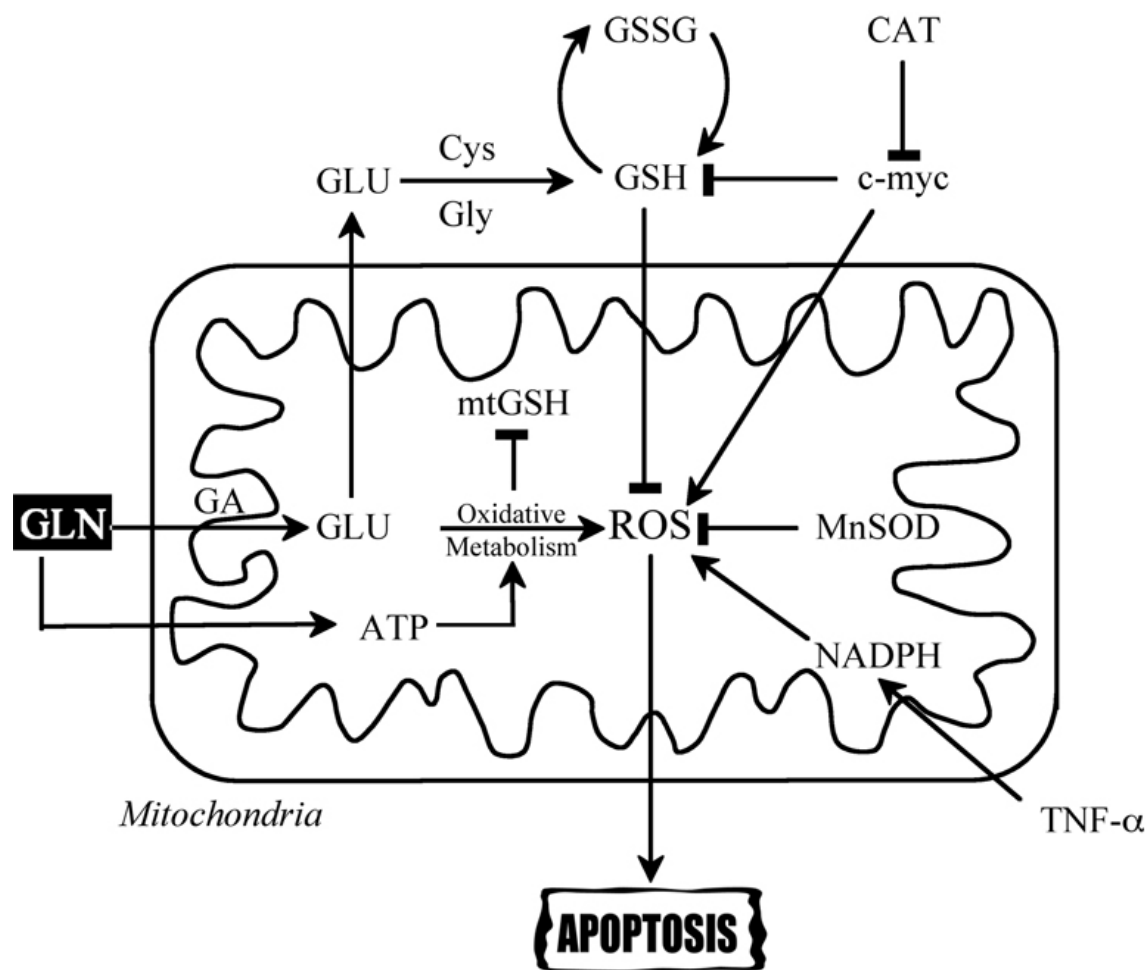
this TNF-induced necrotic cell death by the generation of mitochondrial ROS. TNF-alpha increases the levels of NADPH (Figure 2). Also, the sensitizing effect of glutamine metabolism correlates with an enhanced contribution of complex I to the overall electron flux. TNF-induced ROS generation and cell death are strongly regulated by bioenergetic pathways that define electron flux through complex I of the electron transport chain (73).

The role of antioxidants in preventing apoptosis and viral activation is well documented. Glutathione has been shown to interrupt the process of viral activation and CD4 cell death; and glutamine has been shown to improve glutathione levels and significantly increase lean body mass in HIV infection (74).

Expression of c-myc regulates apoptotic cell death in a human hepatoma cell line during culture in serum-free medium. Cell death was prevented by serum-free medium supplementation with the amino acid glutamine but not serine or asparagine. Improved cell survival with glutamine was associated with increased levels of glutathione. In that hepatoma cell line, c-myc expression led to decreased levels of GSH, and elevated intracellular levels of hydrogen peroxide (Figure 2). Cell death induced by c-myc expression was inhibited by the addition of catalase or dimethyl sulfoxide, a hydroxyl radical scavenger, or by increased intracellular expression of catalase. In contrast to findings in fibroblasts, c-myc-dependent apoptosis during serum deprivation in the hepatoma cells was unrelated to a loss of growth factors. Therefore, apoptosis resulted from H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress with associated glutamine dependent intracellular GSH depletion (75).

Glutamine sensitizes tumor cells to TNF-alpha-induced cytotoxicity. A high rate of glutamine oxidation promotes a selective depletion of mitochondrial glutathione. The mechanism of mtGSH depletion involves an inhibition of GSH transport from the cytosol into mitochondria. The increase in reactive oxygen intermediates production induced by TNF-alpha further depletes mtGSH, and elicits mitochondrial membrane permeabilization and release of cytochrome c (Figure 2). Mitochondrial membrane permeabilization is lethal because it results in the release of caspase-activating molecules and caspase-independent death effectors, metabolic failure in the mitochondria, or both (76). It was also found in intact tumor cells cultured with a glutamine-enriched medium under conditions of GSH synthesis inhibition. Enforced expression of the bcl-2 gene in tumor cells could not avoid the glutamine- and TNF-alpha-induced cell death under conditions of mtGSH depletion. However, addition of GSH ester, which delivers free intracellular GSH and increases mtGSH levels, preserved cell viability. These findings show that glutamine oxidation and TNF-alpha, by causing a change in the glutathione redox status within tumor mitochondria, activates the molecular mechanism of apoptotic cell death (77).

Other authors have equally shown that glutamine may be protective to cells during periods of stress.



**Figure 2.** Mechanisms by which glutamine may regulate oxidative stress. In many cell types, limiting glutamine supply or inhibiting glutamine metabolism via glutaminase inhibits the supply of intracellular glutamate available for GSH biosynthesis. On the contrary, *in vivo* studies have shown that GLN feeding actually reduces mtGSH levels in some cancer cells. This apparent paradox appears to be the additive result of enhanced ROS generation by oxidative GLN metabolism, which reduces mtGSH. Depressed mtGSH in turn induces the cancer cells to be more susceptible to TNF-alpha-induced apoptosis via NADPH action. In addition, glutamine is an oxidative metabolic substrate and a precursor of ATP synthesis that can lead to superoxide production. Conversely, catalase is able to inhibit c-myc-induced apoptosis as well as MnSOD depletes ROS-induced cell death. Catalase influence has been associated to glutamine-dependent intracellular GSH depletion. Abbreviations: CAT, catalase; GA, glutaminase; GLN, glutamine; GLU, glutamate; GSH, reduced glutathione; GSSG, oxidized glutathione; MnSOD, Manganese-dependent superoxide dismutase; mtGSH, mitochondrial GSH; ROS, reactive oxygen species; TNF-alpha, tumor necrosis factor-alpha.

Supplemental glutamine enhances cell growth in cells exposed to moderate concentrations of oxygen, causing higher concentrations of GSH, suggesting that glutamine confers protection to the cell during exposure to hyperoxia through up-regulation of GSH (78).

On the contrary, glutamine increased superoxide generation in the presence of PMA, in rat neutrophils maintained previously in medium deprived of this amino acid. DON (6-diazo-5-oxo-L-norleucine), an inhibitor of phosphate-dependent glutaminase and thus of glutamine metabolism, caused a significant decrease in superoxide production by neutrophils stimulated with PMA both in the absence and in the presence of glutamine. PMA markedly

increased the expression of NADPH oxidase components mRNAs. Glutamine leads to superoxide production in neutrophils, probably via the generation of ATP and regulation of the expression of components of NADPH oxidase (79).

To explore the effects of glutamine on growth and apoptosis of hepatoma cells, mice inoculated with hepatoma cell suspension were orally administered with glutamine. When different concentrations of glutamine solution were added in human hepatoma cell culture, the hepatoma cells' proliferation was inhibited and cells were induced to apoptosis. Cell death was dependent on glutamine concentration; meanwhile the contents of NO



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rose, however malondialdehyde contents were slightly lowered, and the activity of GSH increased. Then, hepatoma cell apoptosis and tumor growth inhibition by glutamine may equally be associated with its antioxidative activity and its intervention in hepatoma cell proliferation, and simultaneous release of NO (80).

ATP is also required for apoptosis to proceed after oxidant stress, otherwise necrosis occurs. Agents like glutamine that enhance ATP levels in oxidant-stressed cells may be potent means of shifting cell death during inflammation to the noninflammatory form of death, as cell death due to necrosis results in acute inflammation, while death by apoptosis generally does not (81). Glutamine is a metabolic substrate and a precursor of ATP synthesis (Figure 2). Its mechanism of action probably involves prevention of mitochondrial damage causing recovery of ATP to baseline levels (82). Alterations in ATP production, intracellular energy levels and mitochondrial function have been shown to trigger cytokinetic events *in vitro*, including inhibition of cell division, abnormal or blocked differentiation and inhibition of apoptosis. Changes in colonic cytokinetics are directly related to colon tumorigenesis and energy metabolism during the tumorigenic process. Cellulose feeding resulted in decreased oxidation of glucose, and glutamine (83).

### 10. GLUTAMINE IN THE ROUTES LEADING TO APOPTOSIS

Glutamine is a fuel for the synthesis of RNA, DNA, phospholipids, UDP sugars and glycogen. Consequently, cooperation between key cell signalling pathways and glutamine, a basic element of cellular metabolism, suggests that this amino acid have the potential to determine distinct cellular fates, including growth, differentiation and death (84).

Glutamine has been known to function as an anti-apoptotic agent, since it blocks apoptosis induced by heat shock, irradiation, and c-myc overexpression. HeLa cells were susceptible to Fas-mediated apoptosis under the condition of glutamine deprivation. Fas ligation activated apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK), in glutamine-deprived cells but not in normal cells, suggesting that glutamine might be involved in the activity control of ASK1 and JNK/SAPK. As one of the possible mechanisms for the suppressive effect of glutamine on ASK1, there exists a molecular interaction between human glutaminyl-tRNA synthetase (QRS) and ASK1 (Figure 3). Besides it has been described the glutamine-dependent association of the two molecules. Finally, it has been shown that QRS inhibited the cell death induced by ASK1, and this antiapoptotic function of QRS was weakened by the deprivation of glutamine. Concentration of Gln-charged tRNA decreases during Gln-depletion leading to an increased need for active QRS and thus inducing a dissociation of QRS-ASK1 complexes. Thus, the antiapoptotic interaction of QRS with ASK1 is controlled positively by the cellular concentration of glutamine. These results provide one possible explanation

for the working mechanism of the antiapoptotic activity of glutamine (85). However, recent studies have found that even a decrease of extracellular Gln to 0.05 mM has no effect on the intracellular concentration of Gln-charged tRNAs in monocytic cells (86).

On the other hand, embryonic astrocytes respond readily to serine/threonine kinase regulation. These responses include apoptosis; and induced apoptosis resulted in the activation of glutamine synthetase activity (87).

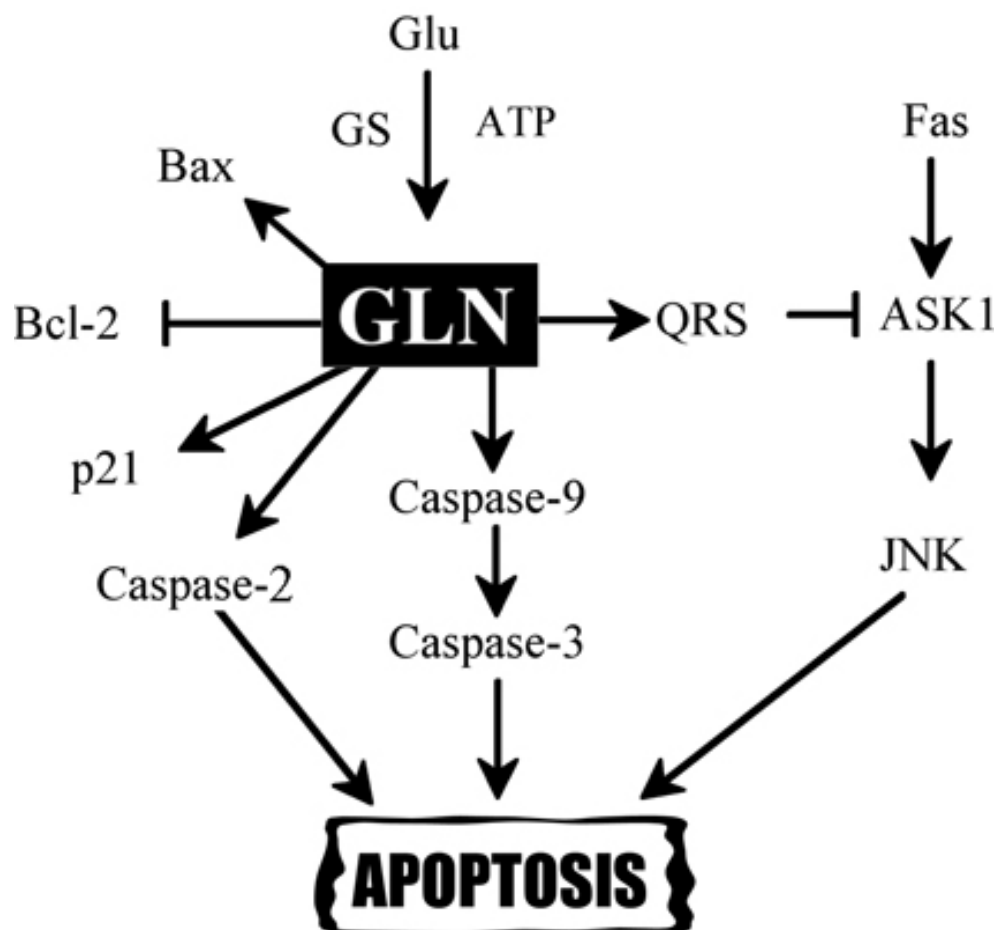
Fas receptor CD95 shows a predominant intracellular localization in normoosmotically exposed rat hepatocytes, whereas hyperosmotic exposure induces CD95 trafficking to the plasma membrane followed by activation of caspase-3 and -8. Hyperosmotic CD95 membrane targeting was sensitive to inhibition of JNK, protein kinase C (PKC), and cyclic adenosine monophosphate. Hyperosmotic CD95 targeting to the plasma membrane was dose-dependently diminished by glutamine, probably caused by an augmentation of volume regulatory increase. Despite CD95 trafficking to the plasma membrane and caspase activation, hyperosmolarity per se did not induce apoptosis (88).

In an interesting study Voehringer *et al.* (89) have found that T lymphocytes undergoing apoptosis are depleted of reduced glutathione coinciding with the onset of chromatin fragmentation. In contrast, augmentation of intracellular GSH is sufficient to reduce the Fas-triggered increase in apoptosis. Overexpression of Bcl-2 causes accumulation of glutathione in the nucleus, thereby altering the nuclear redox state and blocking caspase activity and other nuclear features of apoptosis.

Glutamine supplementation causes an up-regulation of Bax and caspase-3, and down-regulation of Bcl-2 and GSH (Figure 3). These findings suggest that dietary glutamine supplementation suppresses mammary carcinogenesis by activation of apoptosis in tumor cells and this probably is a result of GSH down-regulation (10).

In addition, a glutamine enriched-diet substantially increases lower-expressing Bcl-xL cells viability and total cell density, concomitant with a decrease in the rate of cell death. This effect was not seen when other amino acids or glucose replaced glutamine. The improvement in the culture behavior of cells was attributed to a reduction in the rate of accumulation of apoptotic cells (90).

Surprisingly, in human glioblastoma cell lines, induction of apoptosis has been related to the downregulation of Bcl-2 expression, but not to p53 expression. The remaining non-apoptotic cells presented overexpression of GS (91). Over-expression of the human Bcl-2 protein in retinal glial cells of a transgenic mice leads to early postnatal apoptotic cell death and retinal degeneration. In parallel there was a progressive disappearance of Bcl-2 over-expression, as well as GS. This phenomenon led to photoreceptor apoptosis. Besides its generally accepted anti-apoptotic function, over-



**Figure 3.** Pathways from glutamine to apoptosis. HeLa cells are susceptible to Fas-mediated apoptosis under the condition of glutamine deprivation. Fas ligation activated ASK1 and JNK. The suppressive effect of glutamine on ASK1 has been associated to QRS. In several cell types, expression of p21 and Bax, as well as decreased expression of Bcl-2, have been related to glutamine availability. Note caspase activities countered by glutamine. See text for details. Abbreviations: ASK1, apoptosis signal-regulating kinase 1; Glu, glutamate; GS, glutamine synthetase; JNK, c-Jun N-terminal kinase; QRS, glutaminyl-tRNA synthetase.

expression of Bcl-2 also exerts a pro-apoptotic action, at least in immature glia (92).

Glutamine synthetase is a key enzyme necessary for ammonia detoxification in the brain, but excessive activation of this enzyme can be cytotoxic to neural cells as a consequence of excessive consumption of ATP and glutamate. The stomach also expresses high levels of GS and can be possible pathophysiological roles of GS in ammonia-induced gastric mucosal injury. Increased expression of p21 and Bax, decreased expression of Bcl-2, cytochrome c release from the mitochondria into the cytosol and subsequent activation of caspase-9 and -3 were identified in the cells treated with ammonia. Pretreatment with various concentrations of methionine sulfoximine reduced the GS activity in ammonia-treated cells, and prevented the induction of apoptosis. Thus, energy exhaustion which resulted from an overload of ammonia to GS may have initiated the apoptotic signalling in gastric mucosal cells (93).

Other experiments were performed using cell lines expressing IFN-gamma or cell lines obtained by transfection with the human Bcl-2. Either cell line could grow in media devoid of glutamine with minimal cell death due to endogenous glutamine synthetase activity that allowed cells to synthesize glutamine from glutamic acid in the medium. However, compared to control cultures in glutamine-containing media, the cell growth rate in glutamine-free media was slower with an increased fraction of cells distributed in the G0/G1 phase. The slower rate of cell cycling apparently protected the cells from entering apoptosis when they were stimulated to proliferate in an environment devoid of other protective factors, such as serum or over-expressed Bcl-2. The depletion of both glutamine and glutamic acid did cause cell death, which could be mitigated by Bcl-2 over-expression (94). In this sense, targets for metabolic engineering have been identified in a hybridoma cell line to make it more robust in culture toward potential limitations inducing apoptosis. The cells were genetically modified with plasmids harboring

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endogenous bcl-2 gene and also with viral bcl-2 homologues (95).

Another research have shown livers of TGF-beta1 transgenic mice harbour a reduced number of GS-positive hepatocytes. Starved mice served as controls during TGF-beta1 exposure while expression of GS should be downregulated. Particularly these control mice showed an impressive amplification of GS-positive hepatocytes. It was found that reduction of GS in TGF-beta1 transgenic mice results from apoptosis of GS-positive hepatocytes rather than downregulation of GS expression (96).

### 11. SUMMARY

Apoptosis plays an important role in the homeostasis and development of all tissues within an organism. In contrast to necrosis (cell death by accident), apoptosis is a well-regulated physiological process. Any disturbance of the balance between cell proliferation and cell death maintained by apoptosis can result in serious disease, in particular cancer.

Glutamine deprivation elicits apoptosis by intrinsic pathways, depending on cell type. Conversely, an enhanced glutamine supply represses death receptor-mediated apoptosis in certain cell types, but may actually enhance it in some cancer cells. Glutamine limitation promotes adaptative stress response pathways that aid in survival such as cell cycle arrest, and angiogenesis, again depending upon cellular context, but subverts other stress response pathways necessary for cell survival such as heat shock. Glutamine transporters, cellular hydration, glutaminyl-tRNA synthetase, ATP levels, mRNA stability, and glutathione availability have been variably implicated in glutamine-dependent survival signalling (97). In any case glutamine plays a decisive role in the maintenance of the right balance. There are many examples pointing the relevance of glutamine in the control of cell proliferation and apoptosis:

1. During fasting and many systemic diseases tissues and cells suffer important changes in several mRNAs involved in glutamine metabolism (98).

2. Frequent intense exercise and training has been shown to impair the immune response and might increase the susceptibility to infections (6). Some authors explain the increase in susceptibility to infections due to a decrease in plasma glutamine concentration, which impairs some neutrophil functions (99).

3. In human neutrophils undergoing constitutive or tumor necrosis factor alpha stimulated apoptosis, levels of glutamine decreased. Identical changes were also observed in neutrophils stimulated to undergo apoptosis over a shorter time period in the presence of TNF-alpha and a phosphatidylinositol-3-kinase inhibitor. The increased levels of apoptosis obtained, suggest a synergistic effect by these compounds (100). Therefore, glutamine protects from events associated with triggering and executing apoptosis in human neutrophils (101).

4. After major trauma and sepsis, patients frequently show a decreased blood glutamine level. When induced by TNF-alpha, Fas ligand, or heat shock, the apoptosis rate was significantly lower in the presence of glutamine than in the absence of glutamine. Nevertheless, glutamine had no effect on UV irradiation-induced apoptosis. These results suggest that glutamine serves as a selective immunomodulating factor (102).

5. Glutamine is also necessary for intestinal cell proliferation, intestinal fluid/electrolyte absorption, and mitogenic response to growth factors. Glutamine stimulation of quiescent cells produces immediate-early gene expression and MAP kinase activation. Interestingly, EGF-stimulated mitogenesis is ineffective in the absence of glutamine (103).

6. In relation with cancer, glutamine deamination may indirectly cause an enhancement in leukemia blasts apoptosis (104). Among others, human promyelocytic leukemia, human oral squamous carcinoma, human salivary gland tumor and rat neuron cells consume glutamine. Glutamine utilization varied considerably from cell to cell. For example, during apoptosis induction of HL-60 cells by dopamine or ascorbate derivatives the consumption of glutamine was reduced (105).

7. The ether lipid 1-octadecyl-2-methyl-rac-glycero-3-phosphocholine (ET-18-OCH3) is a membrane interactive drug selectively cytotoxic toward neoplastic cells compared to normal cells. It induces apoptosis in human leukemic cell lines. ET-18-OCH3 promotes a moderate induction of HSP70 and the increase of GS activity. Conversely, at high doses, the drug shows toxic effects on astrocytes inducing decrease in GS activity, low molecular weight DNA formation, and release of LDH in the culture medium, thus increasing apoptosis (106).

8. In critically ill patients, glutamine is used as an energy substrate by monocytes. Glutamine deprivation of these cells results in an increased susceptibility to cell stress and apoptosis. Proteomic analysis has revealed that glutamine depletion is associated with specific changes in the protein expression pattern of HSP70, showing the highest reduction in protein synthesis due to a decreased mRNA stability in Gln-depleted cells. Thus, a specific link between glutamine metabolism and the regulation of heat shock proteins is clearly established (86).

### 12. PERSPECTIVE

All these evidences confirm that glutamine is an essential amino acid in healthy and unhealthy cells, and that it is required for the growth and the adequate function of most cell types. Concerning cancer, glutaminase activity is positively correlated with malignancy in tumors and with growth rate in normal cells. In this field, expression of antisense mRNA for glutaminase induces apoptosis and reduces glutathione antioxidant capacity (107), as well as sensitises Ehrlich ascitic tumor cells to methotrexate, widely used against several malignant tumors (108). Very recent results show a L-glutamine-enriched diet promoting

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in the tumor cells an increase in glutaminase activity, accumulation of cytosolic L-glutamate and competitive inhibition of GSH transport into mitochondria. Helping to glutamine diet, Bcl-2 and MnSOD antisense oligodeoxynucleotides also facilitate elimination of cancer cells by TNF-alpha and chemotherapy. Finally, cell death was associated with increased generation of superoxide and H<sub>2</sub>O<sub>2</sub>, opening of the mitochondrial permeability transition pore complex, and releasing of proapoptotic molecular signals (109).

These results all together open interesting perspectives to be exploited as research tools to gain new insights into the underlying biological basis against cancer. In fact, extensive advances have been achieved in the field of apoptosis-based therapies (110). Numerous novel approaches are currently being followed employing gene therapy and antisense strategies, recombinant biologics, or classical organic and combinatorial chemistry to target specific apoptotic regulators (111). In this context, glutaminase inhibition and glutamine supplementation could be useful to increase the comfort and health of patients suffering from many diseases. Eventually, combined with classic molecular signalling investigations, further studies will also yield a comprehensive assessment of potential mechanisms for glutamine-modulated survival signalling *in vivo*.

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**Abbreviations:** AIF: apoptosis-inducing factor; AMPK: AMP-activated protein kinase; ASK1: apoptosis signal-



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regulating kinase 1; DISC: death-inducing signalling complex; DMBA: 7,12-dimethylbenz [a] anthracene; DON: 6-diazo-5-oxo-L-norleucine; ET-18-OCH<sub>3</sub>: ether lipid 1-octadecyl-2-methyl-rac-glycero-3-phosphocholine; GA: phosphate-activated glutaminase; GADD153: growth arrest and DNA damage gene 153; GGT: gamma-glutamyltranspeptidase; GS: glutamine synthetase; GSH: glutathione; HSP: heat shock proteins; IDDM: insulin-dependent diabetes mellitus; IGF: insulin-like growth factor; JNK: c-Jun N-terminal kinase; LDH: lactate dehydrogenase; PARP: poly (ADP-ribose) polymerase; PMA: phorbol myristate acetate; QRS: glutaminyl-tRNA synthetase; ROS: reactive oxygen species; SAPK: stress-activated protein kinase; TGF: transforming growth factor; TNF: tumor necrosis factor; TRAIL: TNF-alpha-related apoptosis-inducing ligand

**Key Words:** Cancer, Glucosamine, Glutamate, Glutaminase, Glutathione, Oxidative stress, Review

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