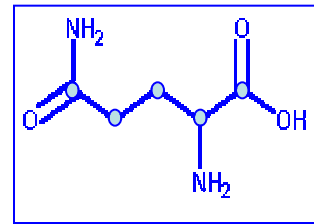


## Q and A: Glutamine, Liver Cancer and Apoptosis

### Background

As you should have learned in BL A302 and 304, amino acids serve several roles in cellular metabolism. They act as precursors in protein synthesis, nitrogen donors in some biosynthetic pathways (nucleotides, amino sugars, amino acids, urea) and carbon donors in other biosynthetic pathways (gluconeogenesis, nucleotides, lipids). Their carbon skeletons can be used as oxidizable substrates (TCA cycle (ATP synthesis)) and they can be utilized in the biosynthesis of polyamines and glutathione. This semester you will learn that they are important for hormone synthesis (thyroid hormones ( $T_4$  and  $T_3$ ), epinephrine) and neurotransmitter biosynthesis (GABA, acetylcholine, serotonin, catecholamines, dopamine, norepinephrine, epinephrine)), and can even act as neurotransmitters (glutamate, glycine).

Amino acid metabolism generates large amounts of ammonia, which can build up to toxic levels in our bodies unless removed by various mechanisms. Glutamine (Gln, Q) serves as the primary nontoxic shuttle for ammonia from extrahepatic tissues to the liver where it is taken up by the amino acid transporter System N [Kilberg, 1980 #39]. Once inside the liver cell (hepatocyte), glutamine is hydrolyzed to glutamate and ammonia (glutamine  $\rightarrow$  glutamate +  $NH_3$ ) by the mitochondrial enzyme glutaminase. The resulting glutamate carbon skeleton is used for gluconeogenesis and other metabolic pathways while the majority of the ammonia is detoxified and converted to urea by the ornithine cycle [Meijer, 1990 #30]. Normally, the liver plays a key role in whole-body glutamine homeostasis, but it can rapidly switch to a center for net glutamine production or consumption [Haussinger, 1990 #27] as discussed later.



Glutamine exhibits the most rapid intracellular turnover (metabolic rate) of all 20 amino acids [Darmaun, 1986 #24] and its importance is further illustrated by the fact that it is the most abundant amino acid in the plasma at levels around 0.6 mmol/L. Glutamine has classically been defined as ‘nonessential’ in biochemistry texts because cells possess the enzyme (glutamine synthetase) required to synthesize this amino acid (glutamate +  $NH_3$  + ATP  $\rightarrow$  glutamine). However, recently glutamine has been termed “conditionally essential” in several medical texts because it is often depleted during times of catabolic stress like cancer, severe infection (sepsis), and burn injury [Souba, 1993 #31]. This characteristic depletion of glutamine is attributed to its role as a metabolic intermediate contributing carbon and nitrogen for the synthesis of other amino acids, fatty acids, nucleic acids and proteins and also supplying a source of fuel for dividing cells [Labow, 2000 #28]. These aspects of glutamine make it essential for cell proliferation, which is demonstrated by the 10-fold increase in glutamine utilization by proliferating cells versus non-proliferating cells [Brand, 1985 #23]. Its use in nutritional therapy in postoperative and severely catabolic patients has been tested experimentally and clinically.

Of all 20 amino acids, glutamine is particularly important in the growth, survival and physiological ‘well-being’ of actively dividing cells such as enterocytes (intestinal epithelial cells), activated lymphocytes, fibroblasts and cancer cells. The experiments we will be doing in lab this year will involve tissue culture. As cells in culture are propagated by growth and serial passage, they are by definition proliferating cells. Similar to actively

dividing cells in the body, cell lines maintained in culture require a lot of glutamine for growth and survival – more than any other amino acid, as shown by the pioneering work of Harry Eagle in the 1950's [Eagle, 1955 #122]. There are several theories as to why actively proliferating cells, especially cancerous ones, consume so much glutamine, but this topic is still highly debated by cellular physiologists.

Hepatocellular Carcinoma (HCC) or liver cancer is the leading cause of cancer death in the developing world and is on the rise in the United States (~75% increase in cases between 1979 and 1995) due to higher incidence of viral hepatitis. HCC had been typically seen as a late complication of cirrhosis in the United States [Di\_Bisceglie, 1998 #17]. Hepatitis (HBV, HCV and HDV), aflatoxin and cirrhosis are the major etiologic causes of HCC [Ogunbiyi, 2001 #15]. Other risk factors for HCC include inherited metabolic diseases like hemochromatosis,  $\alpha$ -1-antitrypsin deficiency, tyrosinemia, porphyria cutanea tarda and glycogen storage disease [Di\_Bisceglie, 1998 #17]. Treatment of HCC is usually unsuccessful, with a 5-year survival rate of less than 5%. Resection and transplant are the two most common treatment modalities (if possible), and effective chemo- or molecular therapeutic interventions are currently lacking.

Hepatocellular transformation to a cancerous phenotype is characterized by increased transport of amino acids, especially glutamine, to be used as metabolic fuel for cell division [Medina, 1992 #29] [Bode, 1999 #45]. In fact, tumor cells may consume more nutrients than required for their own metabolic demands. The liver tumor becomes a net glutamine consumer which is why it is often defined as a “nitrogen trap”. Because tumors are often poorly vascularized, they must have efficient mechanisms for extracting plasma amino acids in order to compete with their host tissues [Medina, 1992 #29]. One such mechanism results in part by differential gene expression between normal human hepatocytes (liver cells) and hepatomas (liver cancer cells) allowing the cancer cells to transport and metabolize glutamine at such high rates. This increase in glutamine transport is due to the expression of the ASCT2 or ATB<sup>0</sup> amino acid transporter in the liver cancer cells. This transporter is not expressed in primary human hepatocytes or growing fetal liver tissue which both express the System N transporter [Bode, 1995 #50], and may be responsible for the observed decrease in plasma concentrations of glutamine in patients with hepatocellular carcinoma [Hirayama, 1987 #38]. Our hypothesis is that increased glutamine transport by ASCT2 is necessary to drive the growth of human hepatoma cells and we are currently exploring the feasibility of using ASCT2 as a selective target in the fight against HCC.

In this lab, we will be studying the effects of glutamine on the survival of six human hepatoma cell lines: HepG2, Hep3B, HuH-7, Focus, P/5, and SK-Hep. Extensive gene expression analysis through microarrays have divided these six hepatomas into two groups. The former three (**Group I**) are more differentiated than the latter three (**Group II**), and our lab has shown that the Group II hepatomas are more sensitive to growth arrest by competitive inhibition of glutamine transport. Using the SK-Hep cells, we have previously shown that glutamine deprivation causes a significant 80% decrease in cell number after 48 h as a result of **apoptosis** characterized by increases in both **caspase-2** and **caspase-3** activity. The purpose of this laboratory is to determine if glutamine deprivation elicits similar apoptotic mechanisms in both “classes” of human hepatomas.

**Motivation for Study:** Our approach to therapy for hepatocellular carcinoma involves choking off the glutamine supply to cancerous liver cells by selectively silencing the expression of ASCT2 – a transporter that mediates most glutamine uptake in all human liver cancers examined, but is not expressed in normal hepatocytes. We have demonstrated that this approach works in one human liver cancer cell line, but the question is: will it work in all hepatocellular cancers? To test this approach, we will starve individual human liver cancer cell lines representing a spectrum of cancerous phenotypes for glutamine and determine if an apoptotic cascade results, as indexed by the activities of an initiator caspase (2) and effector caspase (3).

**Description:** All six of the hepatoma cell lines will be grown in the absence and presence of glutamine ( $\pm$ GLN) for 48 h after which time cellular lysates will be prepared and [caspase-2](#) (morning lab) and [caspase-3](#) (afternoon lab) activities will be determined. Each group will perform the assays on one of the six cell lines. The data will be analyzed by each of the groups in lab the following week (2/5) and a group lab report will be generated and turned in two weeks after the experiment (2/12) – including a list of which team members performed which task on the report. A quiz on the entire cellular physiology sequence will be given on February 12<sup>th</sup> based on the learning objectives listed below. The results will be combined into a final report by the TA's.

**Learning Objectives:**

- To understand the basic principles of cell culture, and some of the unique roles of glutamine in cellular growth and metabolism
- To understand the concept and application of a *standard curve* for the calculation of unknown values (enzyme activities in this case) from experimental samples.
- To learn the principles behind the use of cellular lysates in measuring biological endpoints, and the importance of *normalization* and *replication* of data in cellular physiology research. Also, to appreciate the importance of fastidious/careful pipetting technique in achieving quality data/results.
- To learn the general mechanisms of apoptosis including the distinction between *extrinsic* and *intrinsic* pathways, and the role of *initiator* and *executioner (a.k.a. effector) caspases* in this process. Also, why apoptosis is an important normal physiological process.
- To appreciate the link between cellular growth, proliferation and viability (survival) and specific nutrients.
- To learn the process of hypothesis driven research, and the presentation of results from such studies, including proper *controls*, data analysis, graphical representation of results and the distinction between *dependent* and *independent variables*.

## **Methods**

### **Cell Culture (This will be done by the TA's before lab)**

The human hepatoma cell lines mentioned above are maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air in Dulbecco's Modified Eagle Medium (DMEM, 4.5 mg/ml D-glucose) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G and 100 µg/ml streptomycin (all from Invitrogen Life Technologies, Carlsbad, CA). For glutamine deprivation studies, cells were grown in DMEM ± 2 mM L-glutamine, containing 10% dialyzed FBS (dFBS), 100 U/ml penicillin G and 100 µg/ml streptomycin.

### **Caspase Assays**

Cells will be plated in 100 mm plates (Corning, Acton, MA) at a density of 5x10<sup>4</sup> cells/ml, allowed to grow for 2 days, and subsequently treated with the experimental conditions (±GLN). After 48 h, the cells will be lysed and protein extracts collected. The specific activities of caspases 2 & 3 will be measured in cellular extracts using colorimetric kits according to the manufacturer's instructions (BioSource, Camarillo, CA). In these assays, caspase-2 (VDVAD), and -3 (DEVD) recognition sequence peptides conjugated to the chromophore para-nitroaniline (pNA) are cleaved by the activated enzymes and the liberated pNA quantified by spectrophotometric absorbance at 405 nm. The pNA released is normalized to total protein content in the lysates by the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL), and the results should be expressed as pmoles pNA released per µg protein per hour.

## **Protocol**

1. Aspirate the media and wash the 100 mm plates gently 2X with ice-cold PBS.
2. Place trays on ice-cold metal pan and scrape cells into 1 ml ice-cold PBS.
3. Harvest cells with 2 ml pipet and place in a labeled 1.5 ml tube on ice.
4. Place tubes in pre-cooled centrifuge in a 50 ml tube and centrifuge @ 250 x g for 10 minutes at 4°C.
5. Aspirate supernatant and add ~175 µl of Cell Lysis Buffer and resuspend pellet with large-bore pipet tips.
6. Incubate the cell lysates on ice for 10 minutes, and then centrifuge at 10,000 x g for 1 minute at 4°C. Transfer the supernatant to a new tube and keep on ice.
7. Add 10 µl of fresh DTT stock per 1 ml of 2X Reaction Buffer.
8. Pipet 150 µl of cell lysate (from 3-5 x 10<sup>6</sup> cells or 100-200 µg protein) and 150 µl of 2X Reaction Buffer containing DTT into a labeled 1.5 ml tube on ice and dispense 95 µl into each of three designated wells of a 96 well flat bottom microplate.
9. Add 5 µl of caspase colorimetric substrate to each well.
10. Incubate the plate at 37°C for 4 hours in the dark. Read the plate at 405 nm every hour.
11. Quantitate protein content of lysate by the method of BCA.