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Influence of Progressive Tumor Growth on Glutamine Metabolism in Skeletal Muscle and Kidney

Mike K. Chen, M.D., N. Joseph Espat, M.D., Kirby I. Bland, M.D., F.A.C.S., Edward M. Copeland, III, M.D., F.A.C.S., and Wiley W. Souba, M.D., Sc.D., F.A.C.S.

From the Department of Surgery, Division of Surgical Research, Laboratories for Surgical Metabolism and Nutrition, University of Florida College of Medicine, Gainesville, Florida

Objective
The effects of progressive malignant growth on glutamine metabolism in skeletal muscle and in kidney were investigated.

Summary Background Data
Fast-growing tumors consume considerable quantities of glutamine and lead to a decrease in circulating glutamine concentrations.

Methods
Experiments were performed at various stages of tumor growth in rats implanted subcutaneously with the non-metastasizing methylcholanthrene-induced (MCA) fibrosarcoma and in pair-fed non tumor-bearing controls.

Results
Tumor growth stimulated a twofold increase in hindquarter (muscle) glutamine release, which was not due to an increase in blood flow, but rather to a doubling in the fractional release rate. Consequently, a progressive decrease in skeletal muscle glutamine concentrations was observed over time. Simultaneously, the activity of glutamine synthetase (GS), the principal enzyme of *de novo* glutamine biosynthesis, increased more than twofold. This increase in muscle GS activity was accompanied by an increase in GS mRNA but the augmentation in GS expression apparently could not match the increased rate of efflux since muscle depletion developed. In rats with large tumors and severe glutamine depletion, GS activity was not elevated. Glutamine feeding increased muscle glutamine concentrations and glutamine synthetase specific activity. Although tumor growth led to the development of mild systemic acidemia, the classic renal adaptations normally observed, i.e., elevated glutaminase activity and accelerated renal glutamine utilization, were not present in acidotic tumor-bearing rats. Instead, renal GS activity was increased in tumor-bearing animals and ammoniagenesis was enhanced, in spite of a reduction in net renal glutamine uptake.

Conclusions
These data suggest that marked alterations in muscle and renal glutamine handling occur in the host with cancer; the enhanced muscle glutamine release in conjunction with no increase in renal consumption is consistent with increased glutamine uptake in other organs, most likely the tumor itself and the liver.

Studies in the host with cancer have demonstrated a progressive decrease in blood glutamine levels, indicating a mismatch between the net rates of glutamine uptake and release expressed by the various organs of the body.[1-4] Since glutamine is a key fuel for many cancers and because it is required for cell division, the growing cancer may behave as a glutamine trap and thereby contribute to glutamine depletion. This hypothesis is supported by a substantial body of experimental evidence that indicates that glutamine is the major respiratory fuel for tumor cells.[5,6] Glutamine has been shown to be an unusually good substrate for oxidation by tumor cell mitochondria. Tumor glutaminase activity is high and correlates well with tumor glutamine consumption and growth rates.[7,8]

Although the effects of progressive tumor growth on glutamine utilization in the gut[1,4,9] and liver[2,10,11] have been described, little information is available on how glutamine metabolism in skeletal muscle and in kidney is regulated in the tumor-bearing host. Therefore, in the present work we have used a tumor-bearing rat model to study how glutamine utilization in these two tissues is impacted by progressive tumor growth. Our interest in studying the kidneys in conjunction with skeletal muscle was based on previous studies demonstrating that acidosis is a characteristic feature of the tumor-bearing host.[2] Under normal circumstances, the kidney becomes the major organ of glutamine consumption during metabolic acidosis, a response designed to augment renal ammoniagenesis to help maintain acid/base homeostasis.[12,13] Our results indicate that the tumor-bearing host exhibits alterations in glutamine metabolism that contrast markedly with those normally seen in metabolic acidosis, suggesting the presence of other mediators, rather than changes in regional pH, that control interorgan glutamine flow during advanced malignant disease.

MATERIALS AND METHODS

Tumor Cell Implantation and Animal Maintenance

Male Fischer 344 rats (240–260 g), obtained from the National Cancer Institute, were used in these studies. The animals were allowed at least 1 week to acclimate to the Animal Care Facilities and were fed standard rat chow (Purina Rodent Chow 5001, Purina, Inc., St. Louis, Missouri), given water *ad libitum,* and exposed to alternate 12-hr periods of dark and light cycle. Subsequently, rats underwent bilateral flank implantation with a $2 \times 2 \times 2$ mm cube of viable methylcholanthrene-induced fibrosarcoma cells. This model has been used by other investigators to study host-tumor metabolic interactions. This tumor cell line is an aggressive and rapidly growing malignancy that rarely metastasizes, almost never spontaneously regresses, and is an avid glutamine consumer.[4]

Control rats were subjected to sham implantation and were pair-fed during the remainder of the study period to match carcass weights of the tumor-bearing rats (TBR). A second group of control non tumor-bearing rats were allowed to eat *ad libitum* where indicated. Each animal was weighed on the day implantation and every 4 days thereafter. TBR and their respective controls (10 animals/group) were studied when the tumors were small (2–6% of body weight [BW]), medium (8–13% BW), or large (18–23% BW). A subgroup of rats were fed control and glutamine-enriched diets to study the impact of glutamine feeding on muscle glutamine synthetase activity and on muscle glutamine concentrations (see below). All flank incisions healed without dehiscence or infection. All studies were approved by the Institutional Animal Care and Use Committee at the University of Florida and the Veterans Administration Hospital in Gainesville, Florida.

Study Procedure

Control and TBR were divided into two groups and were studied in the postabsorptive state as either a hindquarter or renal preparation. In the renal studies, a separate group of non-tumor-bearing control animals (n = 9) were given 1.2% NH_4Cl in 5% glucose as a drinking solution for 4 days as previously described[12] to induce a state of chronic metabolic acidosis similar to that observed in rats with large tumor burdens. Each animal was anesthetized with ketamine (7.5 mg/100 g BW IP) and acepromazine (0.1 mg/100 g BW IP) and placed on a heated body board with a heating lamp to maintain euthermia (37.5–38 C). Body temperature was monitored by a rectal thermometer during the procedure.

Hindquarter (Skeletal Muscle) Prep

The model used has been previously described.[14] A midline neck incision was made and a tracheostomy was done using a 3 cm segment of PE-240 polyethylene tubing (Intramedic, Clay Adams, Parsipanny, NJ). Next, the right carotid artery was dissected free of surrounding tissue and cannulated with a 10-cm segment of PE-50 tubing attached to a 1-cc syringe containing heparinized saline; 4-0 silk sutures were used to secure the carotid

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Address reprint requests to Wiley W. Souba, M.D., Sc.D., Division of Surgical Oncology, Department of Surgery, Massachusetts General Hospital, Boston, MA 02114.

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cannula in place. A midline abdominal incision was then made and the distal inferior vena cava (IVC) and aorta were identified. A 26-gauge needle attached to a segment of silastic tubing connected to a 1-cc syringe was placed into the distal IVC immediately proximal to the iliac bifurcation. Likewise a 30-gauge needle attached to a segment of PE-10 tubing was introduced into the distal aorta proximal to the iliac bifurcation. Both aortic and IVC catheters were secured in place with one drop of cyanoacrylate adhesive. The aortic catheter was connected to a syringe containing the nonmetabolizable dye C-14 para-aminohippurate (C-14 PAH, Dupont, New England Nuclear, Wilmington, DE) at a concentration of 1–2 μCi in 3 cc of 0.9% saline. This indicator dye was used to determine the hindlimb blood flow by the indicator dilution technique. The C-14 PAH solution was infused at 0.039 cc/minute using a constant infusion pump (Braintree Scientific BS 99, Braintree, MA). During the infusion period, the wounds were covered with warm saline-soaked gauze. After a 30-minute infusion required to reach steady state, 0.6 cc of blood samples were obtained from the distal IVC and carotid artery. The blood samples were processed for C-14 radioactivity and whole blood glutamine. A skeletal muscle biopsy of the anterior quadriceps was obtained and processed for determination of glutamine synthetase activity and glutamine concentration. The animals were killed by cervical dislocation and the tumors dissected free and weighed.

Feeding Studies

To evaluate the effects of glutamine-supplemented nutrition on skeletal muscle glutamine synthetase activity and tissue glutamine concentrations, a separate group of tumor-bearing rats had their drinking water supplemented with glutamine (2% solution). A second group of rats with similar-sized tumors were given an isonitrogenous drinking solution containing a mixture of three other non-essential amino acids (serine, alanine, and glycine). Chow intake was allowed in addition to access to the drinking solution. After 4 days of feeding, skeletal muscle was biopsied for determination of glutamine and GS activity. Studies were done in groups of rats with small tumors (~5% BW) and with large tumors (~20% BW).

Renal Prep

The animals were placed in metabolic cages and urine collected overnight for determination of creatinine and ammonia concentrations. The *in vivo* flux model has been described.[15] After tracheostomy and carotid artery catheterization as described, the left external jugular vein catheter was cannulated and used for a constant infusion of C-14 PAH for determination of renal blood flow. C-14 PAH was infused into the jugular catheter at 0.039 cc/min using a concentration of 1–2 μCi in 3 cc of 0.9% saline. A midline abdominal incision was then made, and the intestines were gently displaced to the right using a cotton swab. The left renal vein was catheterized with a 25-gauge needle attached to a silastic catheter connected to a 1-cc syringe filled with heparinized saline. The renal vein needle was secured with cyanoacrylate adhesive, and warm saline-soaked sponges were placed over both the neck and the abdominal incisions. After a 25-minute infusion period of C-14 PAH (required to reach steady state), 0.8 cc of blood was withdrawn (over 1 minute) from both the renal vein and carotid artery for determination of glutamine, ammonia, blood gases, and C-14 PAH radioactivity. Both kidneys were then removed, weighed, and immediately processed for glutaminase and glutamine synthetase activity as well as for tissue glutamine concentration. The tumors were removed from each flank and weighed.

Processing of Blood, Tissue, and Urine Samples

An aliquot of the heparinized whole blood was mixed with an equal volume of 10% ice-cold perchloric acid then vortexed and centrifuged at 5 C at 1860 g for 10 minutes (Beckman GPR Tabletop Centrifuge, Beckman Instruments, Palo Alto, CA). One hundred microliters of the supernatant was withdrawn and placed in 10 cc of Aquasol liquid scintillation solution (Dupont, Boston, MA). Fifty micoliters of C-14 PAH was placed in 10 cc of Aquasol (100 μl of supernatant was equivalent to 50 μl of infusate since the blood samples are deproteinated with an equal volume of perchloric acid). The C-14 radioactivity was measured using a Beckman LS9800 Series scintillation counter (Beckman Industries, Fullerton, CA). This allowed for determination of whole blood C-14 PAH radioactivity for subsequent calculation of whole blood rather than plasma flow. The remaining supernatant was removed and neutralized with an equal volume of cold 0.48 M K_3PO_4. This was again vortexed and centrifuged at 5 C at 1860 g for 10 minutes. The supernatant was removed and kept frozen at −20 C for later determination of glutamine concentration by a microanalytic method.[16] Using an instrumentation laboratory 1304 pH/blood gas analyzer (Allied, Lexington, MA), pH, PCO_2, and HCO_3- levels were determined on the heparinized blood samples from the renal studies. Whole blood and urinary ammonia concentrations were determined immediately using a microdiffusion assay.[17] Urinary and serum creatinine were determined using Chemetrics II Analyzer (Burlingame, CA).

After removing muscle, a 0.5 gm portion of tissue was immediately minced and homogenized with 50 mM

Na_3PO_4 for 1 minute on a Polytron (Kinematica, Switz. Brinkman Instruments, Westbury, NY) homogenizer at setting #6. An aliquot of this solution was taken for protein determination and the remaining mixture was deproteinated with 10% trichloroacetic acid. The solution was then centrifuged at 5 C at 12,196 g for 15 minutes (Model B-20A Centrifuge, International Equipment Company, Chamblee, GA), and the supernatant was neutralized with 0.48 M K_3PO_4. The concentration of glutamine was measured fluorometrically by the method described by Bergmeyer.[16] Protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) using gamma globulin as the protein standard.

One gram of minced muscle or kidney was suspended in 5 cc of ice-cold homogenate (300 mM sucrose, 50 mM Trizma HCL, 5 mM MgCl) and homogenized for 1 minute at setting #6 on the Polytron homogenizer. The solution was then centrifuged at 12,196 g for 20 minutes at 4 C and an aliquot taken for protein determination. The remainder of the supernatant was used for measurement of glutamine synthetase activity by the spectrophotometric method described by Lemieux.[18] Protein determination was performed using the Bio Rad protein assay (Bio-Rad Laboratories, Richmond, VA). Renal glutaminase activity was determined using the method of Windmueller.[19]

Quantitation of Glutamine Synthetase mRNA Concentrations in Skeletal Muscle

A rat cDNA (complimentary DNA) probe (a gift from Dr. John Mill and Dr. Steven Max, Bethesda, MD) was used to measure the concentration of glutamine synthetase (GS) mRNA in skeletal muscle by Northern blot analysis.[20,21] Immediately after muscle was biopsied, the tissue was homogenized in guanidinium thiocyanate denaturing solution. Total RNA was extracted[22] after homogenization in a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1% mercaptoethanol, and sequential addition of 2 M sodium acetate (pH 4.0), water saturated phenol (pH 4.0), and chloroform: isoamyl alcohol solution (49:1). RNA was precipitated with isopropanol, centrifuged, and then resuspended in the homogenizing solution. RNA was reprecipitated using isopropanol, washed with 70% ethanol, and resuspended in diethylpyrocarbonate (DEPC)-treated water. Resuspended total RNA was precipitated again using a solution containing 0.1 volume 3 M sodium acetate (pH 5.0) and 2.2 volume 100% ethanol, washed in 70% ethanol, and resuspended in DEPC-treated water. Equal amounts of RNA (40 μg), as determined both spectrophotometrically and through ethidium-bromide staining, were fractionated by electrophoresis through denaturing agarose gels containing formaldehyde.[23] The RNA was transferred to nylon membranes and baked at 80 C for 2 hours. An 800 base pair long rat GS cDNA containing primarily the coding sequence was radiolabeled using an α-^{32}P-CTP (cytidine triphoshate) and a random primer labeling kit (Bethesda Research Laboratories) according to the manufacturers' protocol.[24] Hybridization of the labeled probe was done overnight at 68 C.[25] Autoradiographic detection of the hybridization was done by exposing Kodak XAR film for 48 hours at −70 C. Quantitation of the GS mRNA bands was done by laser densitometry. After quantitation, the GS cDNA probe was stripped off the membrane by boiling in water containing 0.1% SDS. Rehybridization of the blots with a cDNA probe for β-actin was then performed. The β-actin gene was a gift from Dr. Laurence Kedes (Professor and Chairman, Dept. of Biochemistry, USC, Los Angeles, CA). β-actin bands were also quantitated densitometrically.

Calculation of Hindquarter Blood Flow

The concentration and infusion rate of C-14 PAH used in this study is similar to that used by others.[12,14] Previous studies in our laboratory have demonstrated that an infusion period of 30 minutes produces a steady state, as evidenced by a nearly constant concentration (±5%) of C-14 PAH in multiple blood samples obtained before and after the end of the 30-minute infusion period.

Hindquarter blood flow was calculated using the formula:

$$\text{Blood Flow (cc/100 gm BW/min)} = \frac{(\text{CPM infusate})(\text{infusion rate})}{(\text{CPM}_{\text{IVC}}) - (\text{CPM}_{\text{art}})} \times \frac{100}{\text{BW}}$$

where CPM infusate is the number of radioactive counts per minute in 50 μl of infusate, the infusion rate is 0.039 cc/min, CPM_{IVC} is the number of radioactive counts per minute in 100 μl of IVC/perchloric acid supernatant, CPM_{art} is the number of radioactive counts per minute in 100 μl of arterial/perchloric acid supernatant, and BW is the body weight of the rat expressed in grams.

Calculation of Renal Blood Flow

After a 25-minute period of C-14 PAH infusion, a steady state was reached. Under these circumstances, the rate of C-14 PAH excretion in the urine equals the rate of infusion and the fractional excretion of the indicator dye by the kidneys is constant. Renal blood flow was calculated using the following formula:

Blood Flow (cc/100 gm BW/min)

$$= \frac{(CPM_i)(\text{infusion rate})}{(CPM_{art}) - (CPM_{\text{renal vein}})} \times \frac{100}{BW}$$

where CPM_i is the number of radioactive counts per 10 minutes in 50 μl of infusate, the infusion rate is 0.039 cc/min, CPM_{art} is the number of radioactive counts in 100 μl of arterial/perchloric acid supernatant per 10 minutes, $CPM_{\text{renal vein}}$ is the number of radioactive counts in 100 μl of renal vein/perchloric acid supernatant per 10 minutes, and BW is the body weight of the rat expressed in grams.

Calculation of Creatinine Clearance

Creatinine clearance (CrCl) was determined by the following equation:

$$CrCl = \frac{\text{Urine[Cr]} \cdot \text{Urine Volume}}{\text{Plasma[Cr]}} \times \frac{100}{BW}$$

where urine [Cr] is urinary creatinine concentration in mg/dl, urine volume is in cc/min, and plasma[Cr] is plasma creatinine in mg/dl, and BW is the body weight of the rat expressed in grams. Creatinine clearance is expressed in cc/100 g BW/min.

Statistical Analysis and Calculations

Glutamine flux was calculated by multiplying the arterial-venous concentration difference by regional blood flow. All arteriovenous concentration differences were tested for significance with zero. A negative arterial-IVC difference is consistent with net release while a positive difference is considered uptake. Flux was expressed in nmol/100 g BW/min. The Student's unpaired *t*-test and analysis of variance (ANOVA) were used for data analysis. A MacIntosh Plus Computer and Statview 512 software (Apple Computers, Inc.) performed the calculations. All data are expressed as mean ± standard error. Differences between means are considered significant at the $p < 0.05$ level using the unpaired two-tailed Student's *t*-test (MacIntosh Plus Computer, Statview 512 Statistical Program, Apple Computers, Inc).

RESULTS

Body Weights and Food Intakes

Body weights were the same between the two groups on the day of tumor or sham-implantation (Table 1). Likewise, pair-feeding resulted in no significant difference in carcass weights between control and tumor-bearing rats (Table 1).

Hindquarter Studies

Small Tumor Burden

In this group of animals, the tumor weighed 8 ± 3 g and represented 2–6% of the animal's body weight. The only significant difference between TBR and pair-fed controls was the diminished arterial glutamine concentration in the TBR (Table 2). Hindquarter blood flow, glutamine flux, skeletal muscle glutamine concentration, and glutamine synthetase activity between the control and TBR were not different at a time when the tumor burden was small. Feeding glutamine to these tumor-bearing rats did not affect muscle glutamine synthetase activity or glutamine concentrations (data not shown).

Medium Tumor Burden

When the tumor comprised 8–12% of the animal's body weight, skeletal muscle glutamine metabolism was markedly altered in tumor-bearing rats compared with both controls fed *ad lib* and to control rats that had been pair-fed to carcass weight (Table 3). Hindquarter blood flow was the same for all three groups. The arterial-inferior vena cava glutamine concentration difference indicated that the TBR released nearly twice the amount of glutamine compared with both control groups. This ac-

Table 1. HOST (CARCASS) AND TUMOR WEIGHTS*

	Implantation	Small Tumor	Medium Tumor	Large Tumor
TBR				
Carcass weight	248 ± 3	269 ± 8	240 ± 9	222 ± 8
Tumor weight	—	8 ± 3	25 ± 4	57 ± 5
Tumor (% BW)	—	(2–6%)	(8–12%)	(18–23%)
Controls†				
Body weight	251 ± 2	261 ± 7	244 ± 12	230 ± 11

* Expressed in grams.
† Pair-fed to match carcass weights.

Table 2. SMALL TUMOR BURDEN HINDQUARTER GLUTAMINE METABOLISM

	Art GLN (μmol/l)	Flow (cc/100 g BW/min)	Art-IVC GLN (μmol/l)	HQ Release (nmol/100 g BW/min)	Muscle GLN Conc (μmol/g)	GLN SYN (nmol/mg protein/hr)
CONT†						
(n = 10)	680 ± 10	3.7 ± 0.3	−38 ± 9	−158 ± 36	2.3 ± 0.1	54 ± 12
TBR						
(n = 10)	603 ± 17*	3.6 ± 0.2	−43 ± 8	−145 ± 35	2.1 ± 0.1	68 ± 7

* $p < 0.01$ vs. controls.
† Pair-fed to carcass weight. HQ = hindquarter, GLN = glutamine, IVC = inferior vena cava, Art = arterial, GLN SYN = glutamine synthetase activity.

celerated release was associated with a 2.5-fold increase in glutamine synthetase activity in the TBR muscle as compared with the two control groups. However, this augmented synthetic activity could not match the increased hindquarter glutamine efflux, and the muscle glutamine concentration decreased by 30%. Of note, the intracellular glutamine concentration of the pair-fed controls was also significantly diminished as compared to *ad lib* fed controls, but not to the level of depletion observed in the TBR.

Large Tumor Burden

In animals with large tumors (18–23% BW), there was evidence of profound cachexia and anorexia (Table 1). The average tumor weight was 57 ± 5 g. Again, the arterial glutamine concentration was significantly depressed in the TBR as compared with controls (Table 4). The lack of difference between the two groups studied at 22 days may be due to the inherent increased hindquarter glutamine efflux in these partially starved controls due to pair-feeding. The glutamine synthetase activity was not further augmented in these markedly cachectic animals and the muscle intracellular glutamine concentration became further depleted.

Feeding a glutamine-supplemented diet to rats with large tumors increased the muscle glutamine concentration by 20% (Fig. 1). In addition, glutamine feeding increased the specific activity of glutamine synthetase (Fig. 1). Glutamine feeding did not alter muscle glutamine levels or GS activity in control non-tumor-bearing rats (Fig. 1).

Skeletal Muscle Glutamine Synthetase mRNA Concentrations

A steady-state increase in skeletal muscle GS mRNA was present in TBR with medium-sized and large tumors. A representative Northern blot is shown in Figure 2. No change in the constitutively expressed β-action gene was noted (Fig. 3). When normalized to actin, the amount of GS mRNA in muscle of tumor-bearing rats was increased 2–3-fold (Fig. 4).

Table 3. MEDIUM TUMOR BURDEN HINDQUARTER GLUTAMINE METABOLISM

	Art GLN (μmol/l)	FLOW (cc/100 g BW/min)	Art-IVC GLN (μmol/l)	HQ Release (nmol/100 g BW/min)	Muscle GLN Conc (μmol/g)	GLN SYN (nmol/mg protein/hr)
CONT†						
(n = 10)	689 ± 12	3.6 ± 0.3	−57 ± 7	−207 ± 27	2.3 ± 0.13	56 ± 13
TBR						
(n = 10)	606 ± 22*	3.5 ± 0.1	−111 ± 16†	−383 ± 48*	1.6 ± 0.04†	138 ± 34†
CONT§						
(pair-fed)						
(n = 10)	629 ± 17	3.7 ± 0.2	−65 ± 6	−233 ± 22	1.9 ± 0.1	61 ± 9

* $p < 0.05$ vs. controls.
† $p < 0.01$ vs. pair-fed controls.
‡ Non tumor-bearing with ad lib food intake.
§ Pair-fed to carcass weight. HQ = hindquarter, GLN = glutamine, IVC = inferior vena cava, Art = arterial, GLN SYN = glutamine synthetase activity.

Table 4. LARGE TUMOR BURDEN HINDQUARTER GLUTAMINE METABOLISM

	Art GLN (μmol/l)	FLOW (cc/100 g BW/min)	Art-IVC GLN (μmol/l)	HQ Release (nmol/100 g BW/min)	Muscle GLN Conc (μmol/g)	GLN SYN (nmol/mg protein/hr)
CONT† (n = 10)	641 ± 21	3.4 ± 0.2	−68 ± 10	−237 ± 37	2.0 ± 0.1	83 ± 9
TBR (n = 10)	544 ± 35*	3.6 ± 0.1	−74 ± 18	−280 ± 75	1.4 ± 0.1*	88 ± 13

* $p < 0.01$ vs. control.
† Pair-fed to carcass weight. IVC = inferior vena cava, HQ = hindquarter, GLN = glutamine, Art = arterial, GLN SYN = glutamine synthetase activity.

Renal Studies

Arterial and Renal Vein pH, PCO2, and Bicarbonate

In rats with small tumors, no disturbance in acid/base balance occurred. Rats with large tumors had reduced arterial bicarbonate concentrations compared with pair-fed controls and were acidotic (Table 5). Net renal bicarbonate release was increased by approximately 40%. There was no statistically significant difference in the pCO_2 between the two groups although there appeared to be a trend toward respiratory compensation for the metabolic acidosis in the TBR. The NH_4Cl fed acidotic controls had a blood pH value similar to that present in the TBR.

Renal Glutamine Flux

The arterial glutamine concentration was diminished significantly in rats with small tumors (628 ± 18 μM in control vs. 538 ± 26 in TBR, $p < 0.05$) and became further depressed when the disease was advanced. The arterial glutamine concentration of the acidotic controls was similar to that of the TBR (Fig. 5). There was no difference in renal blood flow between the TBR and the control (pair-fed and acidotic) groups (mean = 4.5 ml/100 g BW/min). Renal glutamine flux was unchanged in rats with small tumors although there was a trend toward a decrease in uptake (121 ± 74 nmol/100 g BW/minute in control vs. 65 ± 66 in TBR). In rats with large tumors, the kidney switched from an organ of glutamine uptake in the controls to balance or slight release in the TBR (Fig. 6). This occurred at a time when the TBR were acidotic, which under normal circumstances would result in a marked increase in renal glutamine uptake. This marked elevation in renal glutamine consumption dur-

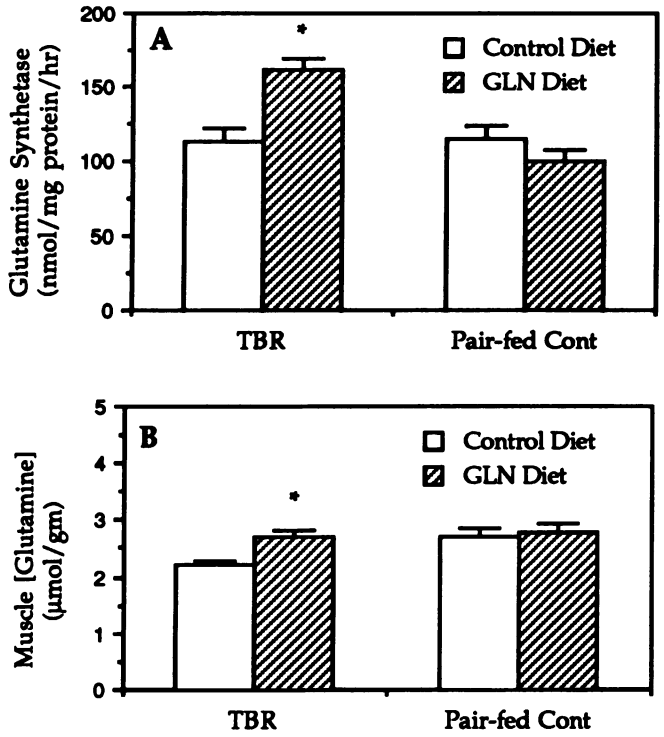

Figure 1. Effects of glutamine feeding on muscle GS and glutamine concentration in pair-fed controls (non tumor-bearing) and in rats with large tumors (TBR). *$p < 0.05$, **$p < 0.01$ versus controls.

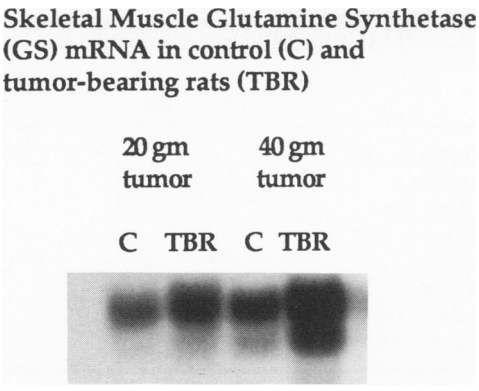

Figure 2. Photograph of a representative Northern autoradiogram showing the effect of tumor growth on skeletal muscle (anterior quadriceps) glutamine synthetase mRNA.

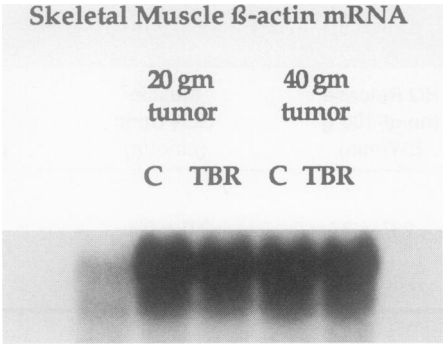

Figure 3. Autoradiogram of rehybridization of glutamine synthetase Northern blot shown in Figure 1 for β-actin.

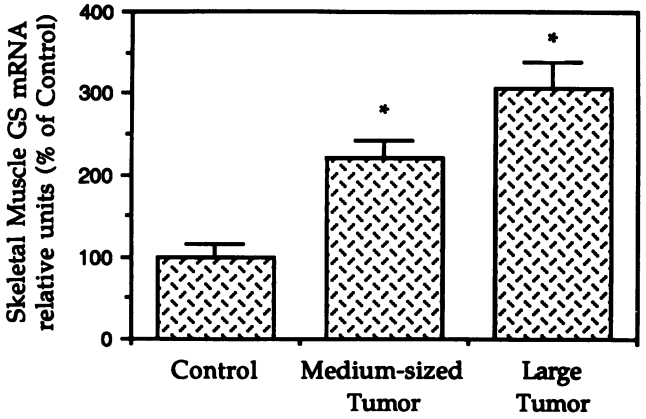

Figure 4. Concentrations of muscle glutamine synthetase mRNA in control and tumor-bearing rats at various stages of tumor growth. Data represent the mean ± SEM of three separate autoradiograms normalized to actin. *p < 0.05 versus control.

ing acidosis was observed in the acidotic controls as demonstrated by a fivefold increase in uptake despite a diminished arterial glutamine concentration (Fig. 6).

Renal Glutaminase and Glutamine Synthetase Activity

Renal glutaminase and glutamine synthetase activity were unchanged in rats with small tumors. In rats with large tumors (20 ± 4% BW), glutaminase activity was not elevated despite the presence of systemic acidosis (Fig. 7). As expected, NH_4Cl-fed rats exhibited a marked increase in renal glutaminase activity (Fig. 7). The activity of glutamine synthetase was 41% higher in the TBR when compared to pair-fed controls (Fig. 8). This is consistent with the switch towards net glutamine release and/or an increased utilization of endogenously synthesized glutamine.

Renal Ammonia Metabolism

In rats with small tumors, arterial ammonia concentrations demonstrated no significant difference between TBR and pair-fed controls. In rats with large tumors, the arterial ammonia concentration in the TBR was significantly higher than in pair-fed controls and was associated with significantly increased renal ammonia release (Table 6). There was also a 2.5-fold increase in urinary ammonia excretion, consistent with the kidneys' attempt to maintain acid-base homeostasis by eliminating excess acid load.

Renal Function

No difference was noted in the creatinine clearance between the two groups (0.85 ± 0.06 cc/100 g BW/min in pair-fed controls vs. 0.88 ± 0.06 in TBR). In addition, blood creatinine was not different between the two study groups (0.32 ± 0.25 mg/dl in controls vs. 0.34 ± 0.22 in TBR). This indicates no defect in glomerular filtration as the cause for the altered glutamine metabolism seen the acidotic TBR.

Table 5. ACID-BASE STATUS* LARGE TUMOR BURDEN

	pH	PCO_2 (mm Hg)	HCO_3^- (mg/dl)	Renal HCO_3^- Release (μg/100 g BW/min)
CONT (n = 12)				
Arterial	7.36 ± 0.01	43 ± 1	23.7 ± 0.4	
Renal vein	7.34 ± 0.01	48 ± 1	25.9 ± 0.4	99 ± 9
TBR‡ (n = 12)				
Arterial	7.30 ± 0.01†	40 ± 1	18.5 ± 0.5†	
Renal vein	7.27 ± 0.01†	46 ± 1	21.4 ± 0.4†	139 ± 13§
Acidotic (n = 9)				
Arterial	7.31 ± 0.02	37 ± 2	17.0 ± 1.0	

* Derived from renal studies.
† p < 0.01 compared to control.
‡ Pair-fed to carcass weight.
Data = mean ± SEM.

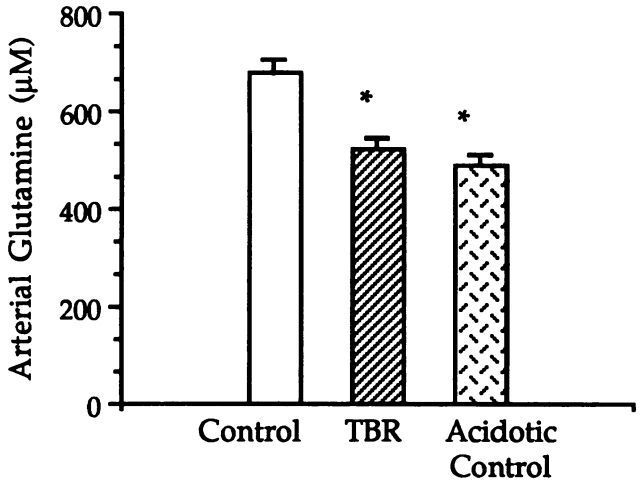

Figure 5. Arterial glutamine concentrations in control rats (pair-fed to carcass weights of TBR), tumor-bearing rats (TBR, large), and acidotic controls. Metabolic acidosis results in a decrease in blood glutamine levels similar to that observed in TBR. *p < 0.01 versus controls.

DISCUSSION

The effects of progressive malignant growth on glutamine metabolism in skeletal muscle and in kidney were studied to gain further insight into the alterations and adaptive changes in glutamine handling that occur in the tumor-bearing rat. The methylcholanthrene-induced sarcoma (MCA tumor) model has been extensively used to investigate the effects of malignant disease on interorgan glutamine metabolism.[1,2,9–11,26] This tumor is locally aggressive but rarely metastasizes, causing death (from inanition) 5–6 weeks after tumor implantation, at which

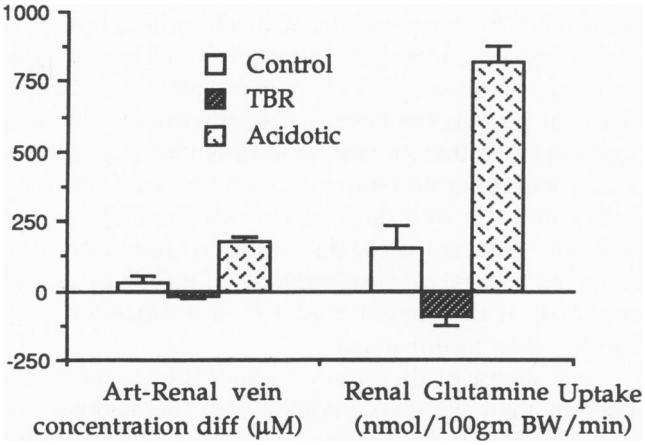

Figure 6. Renal glutamine arteriovenous differences and uptake in pair-fed controls, tumor-bearing (large tumor burden), and acidotic (NH_4Cl-fed) rats. Renal blood flow was similar in each group such that extraction reflected consumption. The classic 5–10-fold increase in renal glutamine consumption that is characteristic of acidosis was not observed in tumor-bearing rats even though their acid/base parameters were similar to those of the acidotic controls.

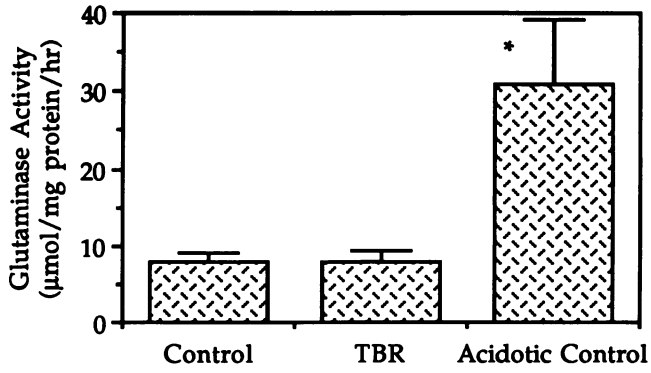

Figure 7. Renal glutaminase activity in control, tumor-bearing (TBR), and NH_4Cl-fed rats (acidotic controls). Despite the systemic acidemia, tumor-bearing animals did not exhibit the classic increase in renal glutaminase expression. *p < 0.01 versus control.

time tumor size may account for approximately half of total body weight. The tumor grows most effectively in the Fisher 344 rat and has several advantages over murine models. First, the larger size of the rat makes the technical skills involved in regional flux measurements easier to complete. Second, the larger organs in the rat often do not require pooling of tissue from several animals to do metabolic studies. Furthermore, the volume of blood that can be sampled for repeated analysis is greater. Because a portion of the tumor-induced cachexia in this model is due to a fall in voluntary food intake (tumor-induced anorexia) this must be taken into account when trying to differentiate between derangements in interorgan glutamine metabolism as a consequence of simple starvation as opposed to abnormalities that are secondary to the growing tumor (i.e., elaboration of a circulating factor that alters intermediary metabolism). Therefore, control non–tumor-bearing rats

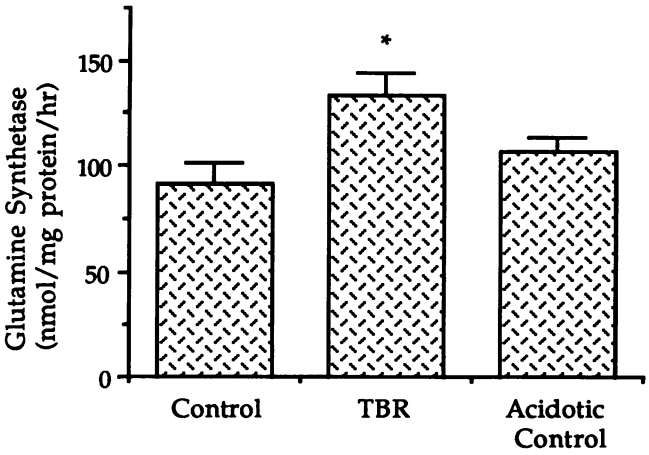

Figure 8. Renal (whole kidney) glutamine synthetase activity in control, tumor-bearing (large tumor burden), and acidotic control rats. p < 0.05 versus controls.

Table 6. RENAL AMMONIA METABOLISM LARGE TUMOR BURDEN

	A NH_3 (μmol/l)	RV NH_3 (μmol/l)	Urinary Volume (cc/12 hr)	Urinary NH_3 (μmol/12 hr)	Renal NH_3 Release (nmol/100 g BW/min)
CONT (n = 12)	27 ± 4	49 ± 6	6.5 ± 0.6	396 ± 49	101 ± 13
TBR (n = 12)	47 ± 3*	79 ± 4*	6.1 ± 0.5	1056 ± 121†	162 ± 14*

* $p < 0.05$ compared to control.
† $p < 0.001$ compared to control. A = arterial, RV = renal vein.

should be pair-fed to carcass weight in order to control for simple starvation effects.

The surgical preparations used in this study are attractive because they allow *in vivo* blood flow measurements as well as sampling of arterial and venous blood. An indicator dye dilution method was chosen to measure blood flow because insertion of intravascular sampling catheters was required to measure substrate concentration and thus the need to implant flow probes was unnecessary. The 0.8-ml sample of venous blood was drawn slowly over a 60-second period, a rate of withdrawal that is less than 10% of the flow of blood through the hindquarter or through the kidney. Such a rate of withdrawal is unlikely to disturb regional hemodynamics. Similar preparations have been used by others for the study of glutamine metabolism and the values we obtained in control rats are similar to those reports.[12,13,27]

Predictably, both the magnitude and direction of glutamine flow in tumor-bearing rats changes during the course of the disease process. The changes that develop appear to be designed, in part, to maintain the blood glutamine concentration as the tumor grows and utilizes more glutamine. In the case of the MCA tumor, the cancer becomes the principal glutamine consumer with time, exceeding glutamine uptake by the intestinal tract.[4] The rate at which this tumor can extract glutamine from plasma is impressive and may be as high as 45%, greater than the rate of glutamine extraction for any organ under conditions of health. The high rates of intracellular glutaminolysis (when the sarcoma is large) are evident by the enormous release of ammonia into the venous effluent.[26]

Glutamine release from the hindquarter is largely secondary to muscle metabolism rather than metabolism in skin or bone.[27] Hindquarter glutamine release was accelerated in rats with tumors; progressive muscle glutamine depletion developed despite an increase in GS mRNA and specific activity, indicating an inability of endogenous biosynthesis to keep up with the rate of efflux. The accelerated release was not due to an increase in muscle blood flow but instead to an increase in the fractional release of glutamine. Induction of glutamine synthetase mRNA in skeletal muscle was not merely secondary to a global response to tumor growth since β-actin mRNA levels were unchanged. Thus, the increase in GS expression may represent an important priority in the muscle response to the growing tumor. This response may be adaptive in nature and may represent an attempt to compensate for the decrease in circulating glutamine levels that develop as the tumor grows. A small tumor burden caused a diminished arterial glutamine level but did not alter skeletal muscle glutamine metabolism using our model. This elevated rate of muscle glutamine release observed in rats with medium-sized tumors was not due solely to starvation or to diminished arterial glutamine level since neither pair-fed controls or controls allowed to eat *ad lib* exhibited such an increase in hindquarter glutamine efflux. In contrast to rats with medium-sized tumors, animals with large sarcomas did not exhibit a further increase in muscle glutamine efflux and and actually released less glutamine than the hindquarter of medium-sized animals (280 nmol/100 g BW/min vs. 383). In addition, these animals with large tumors did not exhibit an increase in GS activity, although GS mRNA levels were increased. It is unclear whether this is consistent with an impairment in synthesis of the GS protein from pre-existing GS transcripts; however, the muscle glutamine concentration (which is markedly depleted with advanced malignant disease) may be a regulator of protein synthesis.[28–31] Furthermore, our data demonstrating that glutamine feeding partially repletes muscle glutamine and simultaneously increase GS activity is consistent with this concept. Muscle may reach a point of "depleted cachexia" where release cannot be sustained because *de novo* synthesis of the GS protein is impaired; in the present model such a timepoint may signify a near terminal state.

The response by the kidneys in the TBR was markedly different than the classic response to the acidosis observed in the non–tumor-bearing acidotic rat, which has been previously reported by others.[12,13,32] These metabolic changes occurred at a time when the functional capacity of the kidney (as assessed by creatinine clearance) was not different than the control animals. In the tumor-bearing acidotic rat, there was no compensatory increase in glutaminase and the activity of glutamine syn-

thetase actually increased. This is in contrast to the classic compensation observed in acidotic non–tumor-bearing animals. Normally, chronic acidosis leads to a diminished glutamine synthetase activity and an increased glutaminase activity associated with a markedly increased renal glutamine consumption to maintain acid-base homeostasis.[33] This elevated renal uptake occurs despite a diminished arterial glutamine level observed in the chronic acidotic state. Our results suggest that the growing tumor can modulate glutamine metabolism in muscle and in kidney via some, as yet undefined, mechanism. The impaired glutamine utilization by the kidney may provide necessary substrate for the tumor which has been shown to compete with visceral organs for circulating glutamine.[4] In the present study, the rats appeared to be able to respond to the acidemia by generating ammonia and bicarbonate from other pathways and possibly from endogenously synthesized glutamine. The specific mediators responsible for this divergent regulation are unknown.

In the kidney, the major enzymes of glutamine metabolism are confined principally to the renal tubular cells. Glutaminase is located in both the proximal and distal renal tubular cells[34] in contrast to glutamine synthetase which is located in the proximal straight tubule.[35] During metabolic acidosis in the otherwise healthy rat, the marked increase in renal glutamine uptake is associated with a significant increase in glutaminase enzyme activity and a fall in glutamine synthetase activity, in order to support ammoniagenesis at the expense of glutamine nitrogens. In the current study, renal ammoniagenesis in response to acidosis appears to be maintained but the source of the excreted ammonia is not entirely clear. Although glutaminase activity did not increase in the acidotic TBR, glutamine may account for a considerable portion of the ammonia excreted into the urine of the tumor-bearing rats since *de novo* glutamine biosynthesis (secondary to an increase in glutamine synthetase activity) was increased. This endogenous synthesis (as opposed to glutamine extracted from the bloodstream) may provide a source of intracellular glutamine some of which may be subsequently hydrolyzed by glutaminase to generate urinary ammonia. The increase in glutamine synthetase activity also occurred at a time when the net renal glutamine uptake decreased, suggesting that a portion of the glutamine synthesized in the proximal tubular cell may be released into the bloodstream rather than used for renal ammoniagenesis.

Regardless of the specific mediator(s) involved in the abnormal renal response to acidosis in the TBR, the data in this model suggest that the growing tumor can modulate enzymatic regulation of renal tubular glutamine metabolism and alter glutamine metabolism in the kidney. These metabolic derangements occur without a detectable associated functional or morphologic impairment and thus the kidney is able to adapt by augmenting ammoniagenesis via other pathways. As the cachexia worsens secondary to progressive growth, renal failure develops, an event that appears to be preceded by distinct alterations in the classic renal glutamine response to acidosis.

In other models, glutamine nutrition has been shown to be effective at partially replenishing glutamine stores.[36,37] However, until proven otherwise, the use of glutamine-enriched diets in patients with cancer must be approached with caution because of the relationship between glutamine utilization and rates of tumor cell proliferation. Nevertheless, the potential benefits of glutamine-enriched nutrition have been observed in animals treated with chemotherapy[38] or with radiation therapy[39,40] and the best designed clinical study to date has shown clear-cut benefits of glutamine-enriched total parenteral nutrition in patients undergoing bone marrow transplantation.[41,42]

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Discussion

DR. COURTNEY TOWNSEND (Galveston, Texas): The investigators have begun to determine the molecular mechanisms to explain the altered glutamine metabolism in tumor-bearing rats. It is interesting that they find the dislocation or uncoupling of the process by which glutamine synthetase activity is regulated as tumor growth progresses. What is the signal? Does the tumor say, "feed me?" Is the increased efflux of glutamine from the muscle a result of increased synthetic activity both mRNA transcription and translation, or are those molecular events results of some other effect that causes the outpouring, or is it a primary event of molecular regulation? What is the temporal relationship? Further, the measurements of messenger RNA levels is, in fact, a steady-state measurement. Do you know that there is increased transcription early on and that later on when there appears to be uncoupling, is there a translational block or is there some effect on the enzyme itself? Finally, when you see increased levels of messenger RNA as well as enzyme activity, is there an absolute increase in enzyme protein as well?

DR. ACHILLES DEMETRIOU (Los Angeles, California): In an elegant series of experiments the authors have demonstrated that in a rodent tumor model a growing tumor can modulate glutamine metabolism in muscle and kidney. The investigators described changes in glutamine metabolism in these tissues, and they demonstrated an increase in glutamine release from muscle, increased activity of glutamine synthetase, decreased consumption of glutamine in spite of developing acidosis, and in short, demonstrated a series of changes that make physiologic sense. This is a well-thought out and well-executed study that uses a variety of experimental tools including direct glutamine synthetase messenger RNA determinations to study a specific aspect of tumor metabolic response and adaptation. You have described changes in glutamine metabolism in the tumor, muscle, and kidney. What happens in the intestine, liver and lung? Is the rate of tumor growth important in altering glutamine metabolism in addition to the actual tumor mass? How does the adaptation take place with fast- versus slow-growing tumors? Is there a critical tumor mass, i.e., percent body weight beyond which the tumors alter glutamine