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Walker 256 tumor-bearing rats demonstrate altered interstitial cells of Cajal Effects on ICC in the Walker 256 tumor model

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Abstract

Background—Cachexia is a significant problem in patients with cancer. The effect of cancer on interstitial cells of Cajal (ICC) and neurons of the gastrointestinal tract have not been studied previously. While supplementation with L-glutamine 2% may have beneficial effects in cancer-related cachexia, and be protective of ICC in models of oxidative stress such as diabetes, its effects on ICC in cancer have also not been studied.

Methods—Twenty-eight male Wistar rats were divided into four groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (WT), and Walker 256 tumor supplemented with L-glutamine (WTG). Rats were implanted with tumor cells or injected with saline in the right flank. After 14 days, the jejunum were collected and processed for immunohistochemical techniques including whole mounts and cryosections and Western blot analysis.

Key Results—Tumor-bearing rats demonstrate reduced numbers of ICC-MY and ICC-DMP and yet increased Anol1 protein expression and enhanced ICC networks. In addition, there is more nNOS protein expressed in tumor-bearing rats compared to controls. L-glutamine treatment had a variety of effects on ICC that may be related to disease state and the interaction of ICC and nNOS neurons. Regardless, L-glutamine reduced the size of tumors and also tumor-induced cachexia that was not due to altered food intake.

Conclusions & Inferences—There are significant effects on ICC in the Walker 256 tumor model. While, supplementation with L-glutamine has differential and complex effects of ICC, it

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DISCLOSURE

The authors have no competing interests.

AUTHOR CONTRIBUTION

LF performed the experiments and research, and JNZ wrote the article. GEV, SRGS, DRL and BCS provided intellectual input. FAG provided the tumor cell. FCVF and JNZ designed the research study. HAM analyzed statistics. JNZ and DRL edited the written article.

reduces tumor size and tumor-associated cachexia, which supports its beneficial therapeutic role in cancer.

Graphical abstract

Keywords

antioxidants; cancer; enteric nervous system; immunohistochemistry; intestinal motility

INTRODUCTION

The interstitial cells of Cajal (ICC) have been established as the cells that generate and propagate the electrical slow wave,^{1,2} the gradient of resting membrane potential,^{3–5} enteric neuromuscular transmission,^{6–8} and they also appear to be involved in mechanotransduction.^{9,10} ICC are classified into several subtypes depending on their location in the tissue layers of the gastrointestinal tract (GIT) and their morphological and functional characteristics.^{11,12} Myenteric ICC (ICC-MY) are located in the external muscle layers, adjacent to the myenteric plexus forming dense, interconnected networks.^{13,14} The function of these cells is to generate slow waves, or rhythmic transient depolarizations that are transferred to the smooth muscle, and thus they serve as pacemaker cells in the small intestine.^{7,15} Deep muscular plexus ICC (ICC-DMP) are located near the inner boundary of the circular muscle layer. They are multipolar cells with thin processes along the nerve fibers in the deep muscular plexus, capable of interacting with the smooth muscle and neurons. They may participate in neurotransmission.^{16–18} The protein anoctamin 1, Ano1 (*Tmem16A*) is a calcium-activated chloride channel^{19,20} highly expressed in ICC.²¹ The channel contributes to the generation and coordination of slow waves.^{22–24} Loss of or defects in ICC can contribute to motility disorders, and dysfunctions are related to inflammatory processes, oxidative stress, and apoptosis.²⁵ Enteric neurons that express Type

Nitric oxide synthase (nNOS) are the major contributor to inhibitory neuromuscular transmission in the gastrointestinal tract. Furthermore, these neurons are particularly susceptible to oxidative stress and may contribute to numerous digestive diseases. There exists an interdependence of nNOS neurons and ICC as they appear to co-dependently promote survival and function of both cell types.^{26–29} Chronic non-transmittable diseases, such as cancer, alter metabolic homeostasis, leading to a syndrome known as cachexia.³⁰ Tumors promote increased oxidative stress and reduced cellular responsiveness by increasing levels of oxidative substances and decreasing the activities of antioxidant enzymes in non-tumor tissues.^{31–33} It seems possible that tumors may directly affect the enteric system by altering cells like ICC, neurons and glia and thus changes in gastrointestinal function that these cells contribute to may also contribute to cancer-induced cachexia. Studies suggest that antioxidant therapy may be effective as adjuvant therapies for cancer.^{34,35} During metabolic stress, L-glutamine increases the synthesis of proteins nucleotides and glucose. L-glutamine is a precursor of glutathione (GSH), which is an important endogenous antioxidant. By enhancing oxidative defenses, L-glutamine attenuates inflammatory processes and preserves immune function.^{36,37,38} The effects of systemic tumors on the enteric system including neurons and ICC have not been directly studied to date. Given its beneficial role on other aspects of systemic issues during cancer, it seems plausible that L-glutamine may reduce or reverse any effects of tumors on ICC. Thus, the objectives this study was 1) to determine the effect of tumors on ICC in the rat jejunum and 2) to investigate the effect of 2% L-glutamine on ICC and tumor-induced changes in ICC. Furthermore, the study aims to determine the effect of tumor load and L-glutamine on myenteric and submucosal nNOS neurons in the rat jejunum.

MATERIALS AND METHODS

All procedures described in this present study are consistent with the ethical principles adopted by the Brazilian Society for Laboratory Animal Science (SBCAL) and were approved by the Universidade Estadual de Maringá (UEM) Ethics Committee on Animal Experimentation in protocol 099/2012.

Experiment design

Twenty-eight male 57-day-old Wistar rats (*Rattus norvegicus*) were used; these weighed 199 ± 11 g, and were obtained from the Central Animal Facility of the Universidade Estadual de Maringá. The animals were distributed randomly into four groups ($n = 7$ per group): control (C), control supplemented with L-glutamine 2% (CG), Walker tumor-256 (WT), and Walker tumor-256 supplemented with L-glutamine 2% (WTG). Rats were kept in polypropylene boxes ($40 \times 33 \times 17$ cm) for a period of 14 days with a 12h/12h dark/light cycle and a controlled temperature ($24 \pm 2^\circ\text{C}$). Food and water were available *ad libitum*. In the WTG and WT groups, rats were injected subcutaneously in the right rear flank, a 0.5 ml suspension containing 8.0×10^7 viable tumor cells (Walker 256) in phosphate buffered saline (PBS; 16.5 mM, pH 7.5). Walker 256 tumor cells are maintained in live rat cultures in the Laboratory of Pathophysiology at the Universidade Estadual de Londrina (UEL). Tumor and ascites tissue were explanted, transported to UEM where cells were isolated, counted and injected into experimental animals. The control animals (C and CG) were injected with

PBS (16.5 mM, pH 7.5) in the same location.³⁹ L-glutamine treatments (supplementation) were initiated on the same day after injection of tumor cells. Control groups (C and WT groups) received standard balanced Nuvital feed (Nuvilab, Colombo, PR, Brazil) while L-glutamine-treated rats (CG and WTG groups) received the standard feed with L-glutamine (Deg, São Paulo, SP, Brazil) incorporated at a proportion of 2 g/100 g of feed. This feed was ground, L-glutamine added and the mixture was then made into pellets and dried in ovens.⁴⁰ Food intake was assessed every other day (g/day).

Collection and processing of the material

At the end of the experimental period of 14 days, the animals were weighed and received an injection of vincristine sulfate (0.5 mg.kg⁻¹ body weight; Tecnocris™, Eurofarma/Zodiac Laboratories, São Paulo, SP, Brazil) two hours before euthanasia. The administration of this microtubule assembly inhibitor improves immunohistochemistry. Rats were anesthetized with thiopental (40 mg.kg⁻¹ body weight i.p.; Abbott Laboratories, Chicago, IL, USA), tissues were harvested and the animal was killed via thoracotomy. The tumor mass was removed from the animals and then weighed to calculate the percent (%) loss of body mass index (cachexia index) according to the equation:³⁹

$$\text{Cachexia index (\%)} = \frac{[\text{ibm} - \text{fbm} + (\text{mt}) + \text{gmc}]}{(\text{ibm} + \text{gmc})} \times 100$$

Where: ibm = initial body mass of the tumor-bearing animal; fbm = final body mass of the tumor-bearing animal; mt = mass of the tumor; gmc = the mean mass gain of the control group (C). Jejunal tissues were collected, washed in PBS (0.1M, pH 7.4).

Whole mount immunohistochemistry

To identify Anol and nNOS, the jejunal lumen was filled with acetone p.a. (1A1017.01.B, Prolab; São Paulo, SP, Brazil) for 15 minutes at 4°C. After fixation, the jejunal were cut along the mesenteric border and rinsed in PBS (0.1M, pH 7.4) for 30 minutes. They were stored at 4°C in PBS (0.1M, pH 7.4) with the addition of 0.08% sodium azide (s2002, Sigma-Aldrich, Inc., St. Louis, MO, USA). For some tissues that were used to only identify nNOS neurons, the jejunal were filled with Zamboni's fixative, and then maintained for 18 h at 4 °C. After fixation by Zamboni's, the jejunal were cut along the mesenteric border, rinsed in 80% alcohol to remove picric acid, dehydrated in increasing ethanol series (95 and 100%), and the tissue was cleared in xylene. Following clearing, tissues were rehydrated in decreasing ethanol series (100, 90, 80, and 50%) and stored at 4 °C in PBS (0.1M, pH 7.4) containing 0.08% sodium azide (s2002, Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the segments of jejunum were dissected under a Stemi DV4 stereomicroscope (Zeiss, Jena, Germany) to remove obtain the whole mounts for immunohistochemistry. Whole mounts were either the submucosal layer or the external muscle layers with the most inner layers of circular muscle removed. All whole mounts were washed twice in PBS (0.1M, pH 7.4) solution containing Triton X-100 0.3% (T8532; Sigma-Aldrich, St. Louis, MO, USA) for 10 min. They were then incubated for 1 h in blocking solution containing PBS (0.1M, pH 7.4) + 0.5% Triton X-100 (T8532; Sigma-Aldrich, St. Louis, MO, USA) +

2% bovine serum albumin (A9418 - BSA; Sigma-Aldrich, St. Louis, MO, USA). For dual labeling of Ano1 and nNOS, whole mounts were incubated in a solution containing both primary antibodies: rabbit polyclonal anti-ANO1 antibody (ab53212, 1:200 dilution; Abcam, USA) and mouse monoclonal anti-NOS1 antibody (sc5302, 1:500 dilution; Santa Cruz Biotechnology, CA, USA). Single-label immunohistochemistry used only one antibody. After 48h incubation at 4°C, the jejunal were washed twice in PBS (0.1M, pH 7.4) containing 0.3% Triton X-100 (T8532; Sigma-Aldrich, St. Louis, MO, USA) for 10 min and incubated for 2 h at room temperature with the following secondary antibodies: goat anti-rabbit IgG (Alexa Fluor® 488, A11008, 1:200 dilution; Invitrogen-Molecular Probes, Eugene, OR, USA) and donkey anti-mouse IgG (Alexa Fluor® 568, A10037, 1:500 dilution; Invitrogen-Molecular Probes, Eugene, OR, USA). Whole mounts were washed twice in PBS (0.1M pH 7.4), mounted on slides with buffered glycerol (9:1) and stored at 4°C. For the negative control, the primary antibody was omitted. To avoid potential procedural variation, tissues from all groups of animals were stained at the same time using the same solutions.

Analysis of immunoreactivity intensity

The brightness intensity emitted in immunostaining for Ano1 was quantified using 30 random images of the jejunal whole mounts. The true color images were captured with a 1/600s exposure time through a 40x objective lens by a high resolution 5.0 Mega Pixel Moticam® 2500 camera (Motic China Group Co., Shanghai, China) coupled to an Olympus BX40 optical fluorescence microscope (Olympus Co., Japan), and transferred to the PC using Motic Images Plus® 2.0ML software (Motic China Group Co., Shanghai, China). ImageJ® version 1.43o image analysis software (National Institutes of Health [NIH], Bethesda, MD, USA) was used to quantify the brightness intensity emitted in immunostaining for Ano1 in stored images to determine the brightness (RGB). The analyses were evaluated blindly. All of the images were recorded before the evaluation, and the analysis was conducted by one researcher.

3D Models representing the immunoreactivity intensity

The Interactive 3D surface plot v2.33 plugin for ImageJ® software version 1.43o (National Institutes of Health (NIH), Bethesda, MD, USA) was used to view 30 images of Ano1 immunoreactivity in the jejunal whole mounts for ICC-MY and ICC-DMP, captured with a 1/600s exposure time and a 40x objective lens. The peak height and color represent the magnitude of immunoreactivity intensity. The same parameters, including grid size, surface, perspective, and lighting were used for all the 3D assemblies.⁴¹

Fluorescence area analysis

Semi-quantitative analysis was used to estimate the area fluorescence of Ano1 and nNOS immunoreactivity in whole mount tissues of all animal groups. The mean proportion of the area containing immunoreactivity was obtained using 30 images per preparation captured with a 1/63.2s exposure time and a 20x objective lens. These images, were captured in a similar manner, but were obtained independently of those analyzed above. ImageJ® software version 1.43o (National Institutes of Health (NIH), Bethesda, MD, USA) was used to convert images into 16 bits and subjected to a binarization function to remove background

intensity as described previously.⁴¹ Analyses were conducted blindly. All of the images were recorded before the evaluation, and the analysis was conducted by one researcher.

Cross section immunohistochemistry

The jejunal segments were cut open along the mesenteric border and stretched with the mucosa facing up onto cork with thorns. The tissues were washed repeatedly with PBS (0.1 M pH 7.4) and immediately fixed in acetone p.a. (1A1017.01.B, Prolab; São Paulo, SP, Brazil) for 15 minutes at 4°C. Next, tissues were washed in PBS (0.1 M pH 7.4) for 30 minutes and submerged in PBS (0.1 M pH 7.4) containing 18% sucrose p.a. (107651, Merck) for 24 h. The tissues were cut longitudinally into 5mm strips, embedded into OCT 4583 (Tissue-Tek) and frozen in liquid nitrogen. Subsequently, semi-serial 10µm cryostat sections were made, arranged on slides, and stored at -20°C. For the tissue of each rat, a slide was made with seven sections to conduct immunofluorescence for Ano1. Accordingly, the sections were initially blocked in PBS (0.1M pH 7.4) containing 0.3% Triton X-100 (T8532; Sigma-Aldrich, St. Louis, MO, USA) and 1% BSA (A9418 - BSA; Sigma-Aldrich, St. Louis, MO, USA) for two hours at room temperature. After blocking, the tissues were incubated in PBS (0.1M pH 7.4) containing 1% BSA (A9418 - BSA; Sigma-Aldrich, St. Louis, MO, USA) and rabbit polyclonal anti-ANO1 antibody (ab53212, 1:200 dilution; Abcam, USA) for 48 hours at 4° C in a humid chamber. The tissues were washed twice in PBS (0.1M pH 7.4) and incubated with goat anti-rabbit IgG (Alexa Fluor® 488, A11008, 1:200 dilution; Invitrogen-Molecular Probes, Eugene, OR, USA) in PBS (0.1M pH 7.4) containing 1% BSA (A9418 - BSA; Sigma-Aldrich, St. Louis, MO, USA) for two h at room temperature. Three successive washes were performed with PBS (0.1M pH 7.4), and tissues were mounted on slides using Prolong Gold antifade with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) and stored in the refrigerator. The negative control was performed by omitting the primary antibody.

Cross section image analysis

Images were captured by a high resolution AxioCam camera (Zeiss, Jena, Germany) coupled to a Axioskop Plus light microscope (Zeiss, Jena, Germany), digitized on a computer using AxioVision version 4.1. software, and recorded on a CD. Image-Pro Plus version 4.5.0.29 image analysis software (Media Cybernetics, Silver Spring, MD, USA) was used to perform the quantification. Ano1-immunoreactive structures that colocalized with DAPI were identified manually in 30 images captured with a 40X objective lens with 6 images from each of five 10µm sections that were separated by at least 50µm. For each animal, data were recorded as the total number of immunoreactive structures in 2.55 mm² 40x fields of view. Data are reported as the mean ± standard error for all eight animals in a group. The analyses were evaluated blindly. All of the images were recorded before the evaluation, and the analysis was conducted by one researcher.

Western blot

The jejunal were removed immediately after the animals were euthanized, and were washed with Krebs-Ringer buffer solution pH 7.4 until complete removal of the contents. Tissues were homogenized in lysis buffer containing protease inhibitor (Tris HCl 50 mM pH 7.2, NaCl 600 mM, EDTA 1mM; Sigma-Aldrich, St. Louis, MO, USA) and then centrifuged for

10 minutes at 3000 cfm. The supernatant was collected and stored at -80°C until use. The total protein concentration was determined by the Bradford method⁴³ (Bio-Rad, Hercules, CA, USA). Western blots were made following standard procedures of the Bio-Rad Mini Protean System. Total protein (40 μg) was separated using SDS polyacrylamide gel (12%) electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After checking the transfer efficiency by Ponceau S staining, the membranes were blocked with 5% nonfat dried milk in TBS buffer (Tris base 2.24g/L and NaCl 8 g/L; pH 7.6) containing 0.1% Tween-20 for 1 h at room temperature. The membranes were incubated with primary rabbit polyclonal anti-ANO1 antibody (ab53212, 1:500 dilution; Abcam, USA), rabbit polyclonal anti-NOS1 antibody (sc8309, 1:500 dilution; Santa Cruz Biotechnology, CA, USA) or mouse monoclonal anti-beta Actin antibody (ab6276, 1:5000 dilution; Abcam, USA) overnight at 4°C , washed with TBS buffer (Tris base 2.24g/L and NaCl 8 g/L; pH 7.6), then incubated with goat anti-rabbit IgG secondary antibody conjugated with peroxidase (31460, 1:2000 dilution; Pierce, Rockford, IL, USA) for 1 h. Immunodetection was performed using the chemiluminescence method with a ChemiDoc MP imager according to the manufacturer's instructions (V3 Western WorkflowTM, Bio-Rad, Hercules, CA, USA). Immunostaining was analyzed through images obtained from Image LabTM software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

The data were statistically analyzed using Statistica 7.1 or GraphPad Prism 5.1 software, and were expressed as mean $\pm$ standard error. Two-way analysis of variance (ANOVA) was used, followed by the Tukey Multiple Comparisons Test. Tumor weight and cachexia data were analyzed by nonparametric t test. P values <0.05 were considered statistically significant.

RESULTS

Physiological parameters

There were no significant differences in final weight when comparing groups C and CG ($p>0.05$). The final weight for groups WT and WTG were lower by 16.5% and 11.0%, respectively, when compared to C ($p<0.05$; Table 1). Food intake for the four groups over the 14 days of treatment was similar ($p>0.05$, Table 1). Tumor mass contributed to overall weight in the WT and WTG groups. The tumor mass was significantly smaller in the WTG group compared to the WT group ($p<0.05$; Table 1). Cachexia was evidenced by loss of body mass, using to calculate: $80.7\pm3\text{g}$ mass gain of the control group and $193\pm2\text{g}$ and $191\pm2\text{g}$ initial weight for WT and WTG group, respectively. In the WT group a reduction of $19\pm3\%$ was seen, and in the WTG group a reduction of $10\pm3\%$. Thus, L-glutamine treatment reduced cachexia ($p<0.05$; Table 1).

Ano1 Immunoreactivity Intensity

When the intensity of Ano1 immunoreactivity in the myenteric region was compared, no changes were seen between groups C and GC ($p>0.05$); on the other hand, in the deep muscular plexus region, an increase of 14% in CG compared to C was observed ($p<0.01$; Figure 1). The Walker 256 tumor (WT) provoked an increase in Ano1 immunoreactivity

intensity in both the myenteric and deep muscular plexus regions compared to relative control groups ($p < 0.05$). L-glutamine increased the Ano1 immunoreactivity intensity in rats with tumors in the myenteric region ($p < 0.01$), but did not change the intensity any further in the deep muscular plexus region. The differences between the image intensities are represented graphically in 3D intensity plots demonstrated in Figure 1C.

Cross Sectional Density of Ano1 Immunoreactive Structures

The number of structures containing both Ano1 immunoreactivity and DAPI staining were quantified in 30 images from five non-adjacent cross sections from each of eight animals within the four treatment groups. This measurement is used to determine the number of ICC in these tissues. Within the myenteric region, the number of ICC decreased by 38% and 36% in the WTG and WT animals compared to C, respectively ($p < 0.05$; Figure 2). There were no differences between the WT and WTG groups. Within the deep muscular plexus region, the number of ICC decreased 18% in the WT group compared to C ($p < 0.05$; Figure 2), whereas there was no significant difference in the number of ICC between the WTG and C groups ($p > 0.05$; Figure 2).

Ano1 Fluorescence Area Analysis

To determine the density of Ano1 immunoreactivity as a measure of the ICC network and connectivity, the immunoreactivity intensity was subjected to thresholding and binarization. Within the myenteric region, an increase of 9% was observed in the fluorescence area analyzed by Ano1 immunoreactivity between groups C and CG ($p < 0.01$; Figure 3). The Walker 256 tumor (WT) provoked an increase in the fluorescence area analyzed by Ano1 immunoreactivity compared to group C ($p < 0.05$; Figure 3) to similar levels as observed in group CG ($p < 0.05$; Figure 3). L-glutamine treatment in animals with tumors (WTG) further increased the fluorescence area analyzed by Ano1 immunoreactivity compared to groups C, CG and WT ($p < 0.01$; Figure 3). Within the deep muscular plexus region, the fluorescence area analyzed by Ano1 immunoreactivity was increased in animals with tumors (WT and WTG groups) compared to group C ($p < 0.05$; Figure 3). Within this region, there was no effect of L-glutamine treatment in either control or tumor groups ($p > 0.05$; Figure 3).

The fluorescence area analyzed by nNOS immunoreactive myenteric neurons was not different between groups C, CG and WT but was reduced by 11% in group WTG compared to group C ($p < 0.05$; Figure 4). In the submucosal plexus, the fluorescence area analyzed by nNOS immunoreactivity was not different between groups C, WT and WTG groups but was increased by 8% in group CG compared to C ($p < 0.05$; Figure 4). Some tissues were double labeled for Ano1 and nNOS to determine the relative spatial distribution between these cells. There are few observations of close apposition of immunoreactive structures in the myenteric region (Figure 5). Conversely, there is a relatively high degree of apposition between Ano1 immunoreactivity and nNOS immunoreactivity within the submucosal/deep muscular plexus region (Figure 6).

Western Blot

Western blot analyses were conducted as a semi-quantitative measure of Ano1 and nNOS proteins within the jejunal of rats and compared between the four treatment groups. The β -

actin, Ano1, and nNOS proteins migrated with molecular weights of approximately 42 kDa, 114 kDa, and 160 kDa, respectively (Figure 7). The relative intensity of Ano1 immunoreactive bands were increased by 80% and 53% in the WT and WTG groups compared to C, respectively ($p < 0.05$; Figure 7A). L-glutamine treatment did not alter Ano1 levels in either control or tumor groups. Likewise, the relative intensity of nNOS immunoreactive bands were increased by 54% and 48% in the WT and WTG groups compared to C, respectively ($p < 0.05$; Figure 7B). L-glutamine treatment did not alter nNOS levels in either control or tumor groups.

DISCUSSION

The experimental model of the Walker 256 tumor in rats used in this study demonstrated tumor growth at the site of injection in the flank, and yet overall weight loss which confirmed the model as cachexia similar to results observed previously.^{42,43} Cachexia associated with cancer is characterized by a loss of at least 10% of body mass and complex metabolic changes.^{43–44} In this study, the cachexia was likely caused by increased metabolism and not anorexia, because there were no changes in food intake between the control groups and the tumor groups. This observation is also similar to previous reports.⁴⁴

In the present study we made several observations regarding ICC-MY and ICC-DMP, immunostained by Ano1 protein. First, there was an overall increase in the intensity of immunoreactivity for Ano1 in both the myenteric plexus and deep muscular plexus in the tumor-bearing groups compared to the control groups. Conversely, there was a decrease in the number of ICC, confirmed by structures containing both Ano1 and DAPI, in both of these regions in tumor-bearing rats compared to controls. Finally, there was an increase in the fluorescence area analyzed by ICC in both the myenteric plexus and deep muscular plexus in tumor-bearing rats compared to controls. These are the first observations of the effect of tumors on intestinal ICC. Together, they suggest that while the tumors may decrease the number of ICC, there is an increased expression of Ano1, observed as staining intensity, and also increased density of ICC processes in these tissues. Western-blot analyses support the concept that Ano1 protein levels are increased in tumor-bearing rats. The reduced number of ICC may be related to oxidative stress, which is high in this disease.³² In other models of oxidative stress such as diabetes mellitus, there are previous observations of reduced numbers of ICC.^{45,46} Gibbons *et al.*⁴, in experiments with healthy human colon, noted that ICC die by apoptotic mechanisms, yet continuously regenerate and maintain intact networks via cell proliferation. In support of this concept, several studies have attributed the maintenance of ICC function after damage to: a) repair and proliferation by adult ICC;^{47,48} b) replacement of ICC precursor cells;⁴⁹ c) protection through the heme oxygenase-1 (HO-1);^{50,51} and d) signaling pathways contribute to the survival and proliferation of ICC, including neuron-derived NO.²⁹ In the present study, there appears to be a tumor-induced plasticity that may maintain and enhance the ICC-networks for both ICC-MY and ICC-DMP. Ano1 contributes to ICC proliferation⁵² and the observed increase in Ano1 protein in the present study may contribute to enhanced proliferation, though this was not tested directly. However, proliferation does not likely explain enhanced networks, because we also observed a decrease in the number of ICC. Therefore, the most likely explanation of these data is that the oxidative stress of the tumors causes a loss of ICC, and

yet ICC networks are maintained and enhanced by increased protein synthesis including Ano1. Support for this form of ICC plasticity comes from a previous study that found that recovery of ICC networks and intestinal motor function following acute damage was not due to restored numbers of ICC.⁵³

Nitric oxide contributes to maintaining ICC networks.^{29,50} Therefore, we sought to test the effect of Walker 256 tumor on nNOS in the jejunum of tumor-bearing rats. As measured by Western blot, the nNOS enzyme was increased in tumor bearing animals. The fluorescence area analysis of nNOS immunoreactive structures in either the myenteric region, or the submucosal region was not different between control and tumor-bearing animals. Therefore, it seems plausible that while the structure of neural networks, as evidenced by nNOS immunoreactivity, are not altered by tumors, the neurons may be capable of producing more nitric oxide. A direct link between increased nNOS and increased ICC networks requires further study.

Antioxidant therapy may be effective as adjuvant therapies for cancer.^{34,35} L-glutamine is a precursor of glutathione (GSH), an important endogenous antioxidant, and attenuates inflammatory processes and preserves immune function.^{36,37} The epithelial cells of the intestinal mucosal have a high concentration of glutaminase compatible with high rates of uptake and use of L-glutamine.⁵⁴ Supplementation of glutamine in the gut reduces the permeability of the intestinal mucous membrane, helping to maintain the integrity of the intestinal barrier.⁵⁵ L-glutamine treatment protects enteric neurons and enteric glia in animal models of oxidative stress including diabetes.³⁸ Walker 256 tumors change L-glutamine metabolism in rats^{56,57} and cancer patients often demonstrate L-glutamine depletion.⁵⁸ Tumors often have high glutaminase activity,⁵⁹ which may contribute to reduced L-glutamine levels. L-glutamine did not stimulate tumor growth, which may have been expected, but rather there was a significant reduction in tumor mass in rats supplemented with L-glutamine. Supplementation with L-glutamine decreases the rate of degradation and increases protein synthesis in the skeletal muscle and intestinal mucosa, and increased levels in plasma to help reduce weight loss.⁸ In the present study, cachexia, or the loss of body mass in tumor-bearing rats, was significantly reduced in the WTG group compared to the WT group, confirming a beneficial role of this supplementation.

We investigated the effect of L-glutamine on ICC in control rats and rats with Walker 256 tumors. L-glutamine had various effects in these studies. L-glutamine did not change the number of ICC-MY in either control or tumor-bearing animals. Nor did L-glutamine change the number of ICC-DMP in control animals. L-glutamine treatment partially restored the number of ICC-DMP in tumor-bearing rats suggesting that the treatment may be protective of ICC-DMP to the stress of tumors. The effect of L-glutamine on the fluorescence area analyzed by Ano1 immunoreactivity was region-dependent. In the deep muscular plexus, L-glutamine had no effect on the network of ICC in either control or tumor-bearing animals. However, in the myenteric region, L-glutamine enhanced ICC-MY networks in both control and tumor-bearing animals, such that the fluorescence area analyzed by Ano1 immunoreactivity was highest in the WTG group compared to the other groups. Ano1 immunoreactivity intensity was increased in control animals within the deep muscular plexus but was not changed in the myenteric region. Conversely, L-glutamine treatment

increased Ano1 immunoreactivity intensity in myenteric region but not the deep muscular plexus of tumor-bearing rats. There was no effect of L-glutamine on the protein levels of Ano1 in the intestine in either control or tumor-bearing animals. Thus, L-glutamine appears to have differential effects on ICC depending on the region and the disease state of the animal. Likewise, L-glutamine alters nNOS immunoreactivity in a region- and disease-dependent manner. Like its effects on Ano1 protein levels, L-glutamine did not alter nNOS protein levels in either control or tumor-bearing animals. However, the fluorescence area analyzed by nNOS immunoreactivity was significantly increased in the submucosal plexus of control animals, and significantly decreased in the myenteric plexus of tumor-bearing animals by treatment with L-glutamine. There is a complex protective relationship between nNOS neurons and ICC.^{29,50} Perhaps the region-specific effects of L-glutamine may depend on the spatial relationship of nNOS neurons to ICC in these different regions. Consistent with a recent review,¹³ we observed a close relationship between nNOS and ICC-DMP with less structural overlap between nNOS and ICC-MY. One could postulate that the differential effects of L-glutamine on ICC-DMP and ICC-MY may be due to alterations in the nNOS/ICC relationship in each region. This hypothesis requires further in depth analysis to fully elucidate what is likely complex molecular and cellular interactions, but must include not only co-localization with NO-producing enzymes but also measures of NO bioavailability that are likely to be altered by both tumors and L-glutamine.⁶⁰

In conclusion, this study demonstrates for the first time that tumors have significant effects on intestinal ICC. Tumors decrease the number of ICC and yet increase the networks of both ICC-MY and ICC-DMP and increase tissue Ano1 protein expression. In addition, tumors increase the levels of nNOS in the jejunum. While L-glutamine treatment may be protective of ICC-DMP cell numbers and improve the network of ICC-MY, it remains unclear whether these effects contribute to the improved health of tumor-bearing rats. Furthermore, L-glutamine appears to be uncovering even more complexity in the already complex nNOS/ICC relationship. Regardless, L-glutamine treatment has beneficial effects on both cachexia and tumor size and thus supports previous reports of the benefits of L-glutamine for cancer.^{36, 37}

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Key messages

The aim of the study was to investigate Ano1 protein present in ICC-MY and ICC-DMP in the jejunum of Walker 256 tumor-bearing rats using immunohistochemical techniques including whole mounts and cryosections and Western blot analysis and to test the effect of L-glutamine treatment on these measurements. In addition, nNOS expression was also assessed.

Tumor-bearing rats demonstrate wasting or cachexia as evidenced by reduced body mass. Supplementation with 2% L-glutamine partially reduces cachexia and reduces the tumor mass.

Tumors in the right flank have significant effects on the jejunum ICC, decreasing the number of both ICC-MY and ICC-DMP, and yet increasing Ano1 protein levels and the fluorescence area analyzed by Ano1 immunoreactivity. nNOS protein levels are also increased in tumor-bearing rats. L-glutamine treatment may be protective of ICC-DMP and promote stronger ICC-MY networks, but do not alter nNOS or Ano1 protein levels.

Figure 1.

Figure 1A. Immunoreactivity (IR) intensity for ICC-MY (A) and ICC-DMP (B). Results were expressed as mean $\pm$ standard error. Different letters above the bars indicate statistical differences ($p < 0.05$). $n = 8$ rats per group. **1B.** Representative photomicrographs of the whole-mounts of ICC-MY (A) and ICC-DMP (B) immunostained with the Anol protein in rat jejunal. **1C.** Representative 3D surface modeling representing immunoreactivity intensity for ICC-MY (A) and ICC-DMP (B). Groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (WT) and Walker 256 tumor supplemented with L-glutamine (WTG). Calibration bar 15 μ m.

Figure 2.

Figure 2A. Density for ICC-MY (A) and ICC-DMP (B). Results were expressed as mean $\pm$ standard error for the total of Ano1⁺/DAPI⁺ structure observed in 2.55 mm² 40x fields of view for n = 8 rats per group. *p < 0.05 when compared to C. **2B.** Representative photomicrographs of the histological section of ICC-MY and ICC-DMP. The arrows indicate co-localization of ICC/DAPI. Calibration bar 50 μm. Groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (WT) and Walker 256 tumor supplemented with L-glutamine (WTG).

Figure 3.

Figure 3A. The fluorescence area analyzed by Anl immunoreactivity in the myenteric (A) and deep muscular plexus (B) regions. Results are expressed as mean $\pm$ standard error. Mean values followed by different letters are statistically different ($p < 0.05$). $n = 8$ rats per group.

3B. Representative binarized photomicrographs of whole-mount preparations of Anl immunoreactivity in the myenteric region (A) and deep muscular plexus region (B). Calibration bar 15 μ m. Groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (WT) and Walker 256 tumor supplemented with L-glutamine (WTG).

Figure 4.

Figure 4A. Fluorescence area analysis by the nNOS-IR neurons myenteric (A) and submucosal (B). Results were expressed as mean $\pm$ standard error. $p < 0,05$ when compared to C. $n = 8$ rats per group. **4B.** Photomicrographs of whole-mount preparations of nNOS-IR myenteric (A) and submucosal (B), 16-bit images. Calibration bar 30 μ m. Groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (WT) and Walker 256 tumor supplemented with L-glutamine (WTG).

Figure 5.

Representative photomicrographs of Anl (green) and nNOS (red) in the myenteric plexus region of the rat jejunum. Calibration bar 30 µm. Groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (WT) and Walker 256 tumor supplemented with L-glutamine (WTG).

Figure 6.

Representative photomicrographs of Ano1 (green) and nNOS (red) in the depp muscular plexus/submucosal plexus region of the rat jejunum. Arrowheads indicate examples of nNOS-IR nerve fibers and networks of ICC-DMP in close apposition. *Calibration bar 30 µm.* Groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (WT) and Walker 256 tumor supplemented with L-glutamine (WTG).

Figure 7.

Figure 7A. Representative immunoblots for Ano1 and nNOS and the corresponding immunoblots for beta actin for the matched protein samples. **7B.** Compiled results of Western blot analyses expressed as the percentage of the control group. * p < 0,05 when compared to C. n = 4 rats per group. Groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (TW) and Walker 256 tumor supplemented with L-glutamine (TWG).

Table 1

Physiological parameters: final weight (FW), cachexia index (CI), variation of the mass gain (VMG)*, tumor mass (TM), and food intake (FI). Groups: control (C), control supplemented with L-glutamine (CG), Walker 256 tumor (WT) and Walker 256 tumor supplemented with L-glutamine (WTG).

	C	CG	WT	WTG
FW (g)	298±6 ^a	286±5 ^a	249±5 ^b	265±6 ^b
CI (%)	-	-	19±3 ^a	10±3 ^b
VMG (g)	-	-	29±7 ^a	47±4 ^b
TM (g)	-	-	27±2 ^a	21±3 ^b
FI (g/dia)	23.3±0.6 ^a	22.9±0.4 ^a	21.4±0.6 ^a	21.4±0.4 ^a

* Variation of the mass gain = (final weight – tumor weight – initial weight). Results were expressed as mean ± standard error. Mean values followed by different letters in the same row are statistically different (p <0.05). n = 7 rats per group.