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L-Leucine and L-isoleucine enhance growth of BBN-induced urothelial tumors in the rat bladder by modulating expression of amino acid transporters and tumorigenesis-associated genes

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ABSTRACT

We investigated the underlying mechanisms of L-leucine and L-isoleucine mediated promotion of bladder carcinogenesis using an initiation-promotion model. Rats were administered *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine for 4 weeks and then fed AIN-93G basal diet or diet supplemented with L-leucine or L-isoleucine for 8 weeks followed by the basal diet for another 8 weeks. At the end of the experiment, week 20, there was a significant elevation of papillary and nodular (PN) hyperplasia multiplicity in the amino acid groups. L-Leucine and L-isoleucine transporters were up-regulated in PN hyperplasias and/or bladder tumors compared with concomitant normal-appearing bladder urothelium at weeks 12 and/or 20 in all groups. In addition, in normal-appearing bladder urothelium, significantly increased mRNA levels of *y + LAT1, LAT2*, *LAT4*, and *4F2hc* were observed in the amino acid groups compared with the BBN control group at both weeks 12 and 20, and increased mRNA levels of *LAT1* were observed at week 20. Furthermore, up-regulation of *TNF-α*, *c-fos*, *β-catenin*, *p53*, $p21^{Cip1/WAF1}$, *cdk4*, *cyclin D1* and *caspase 3* in the amino acid groups was detected in normal-appearing bladder urothelium. Overall, our results indicate that supplementation with L-leucine or L-isoleucine enhanced growth of bladder urothelial tumors by triggering expression of amino acid transporters and tumorigenesis-associated genes.

1. Introduction

Branched chain amino acids (BCAAs) are essential amino acids which must be obtained from dietary sources (Baracos and Mackenzie, 2006). Retail BCAAs are widely used as dietary supplements (Blomstrand et al., 2006; Shimomura et al., 2010). BCAAs are also used to medicate diseases such as cancer (Baracos and Mackenzie, 2006); however, their effects on the efficacy of cancer treatments are controversial. BCAAs are simultaneously essential for both tumor growth and the physiological well-being of the tumor-bearing host (Baracos and Mackenzie, 2006), and long-term treatment with two BCAAs, L-leucine and L-isoleucine, exerts a promoting effect on rat bladder carcinogenesis (Kakizoe et al., 1983; Nishio et al., 1986; Xie et al., 2012b).

Tumors have various defects that circumvent cell-cycle control (Bartek et al., 1999; Gebhardt and Williams, 1995). In transformed cells, amino acid transporters are up-regulated to support the high-level of protein synthesis required for continuous growth and proliferation (Christensen, 1990). Amino acid transport activity by several amino acid transporters requires the formation of heteromeric complexes with the heavy chain of 4F2 antigen (4F2hc, solute carrier [slc] 3A2, *slc3a2*) (Campbell and Thompson, 2001; Lahoutte et al., 2004), for example, L-type amino acid transporter (LAT) 1 (*slc7a5*), LAT2 (*slc7a8*) and y + LAT1 (*slc7a7*) (Torrents et al., 1998, 1999; Uchino et al., 2002; Verrey, 2003). LAT1 is highly expressed in different proliferating tissues, numerous tumor cell lines and human primary tumors (Yanagida et al., 2001; Kobayashi et al., 2005); the LAT2 gene is a target of the progesterone receptor (Luo et al., 2009), which plays a key role in uterine leiomyoma growth; and y + LAT1-4F2hc complexes are involved in the transport of L-leucine (Torrents et al., 1999). 4F2hc independent LAT3 (*slc43a1*) and LAT4 (*slc43a2*) are also associated with tumorigenesis. Elevated LAT3 expression is associated with prostate cancer outgrowth (Wang et al., 2011), and elevated LAT4 expression is associated with poorly differentiated squamous cell head and neck carcinoma (Haase et al., 2007).

Using a two-stage initiation-promotion rat model, we have previously shown that dietary L-leucine and L-isoleucine promote BBN-initiated rat bladder carcinogenesis. In the present study, we evaluated the effects of short-term supplementation of L-leucine

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and L-isoleucine on the expression of L-leucine and L-isoleucine related transporters and tumorigenesis-associated genes during the early responses of BBN-initiated bladder urothelium to L-leucine or L-isoleucine.

2. Materials and methods

2.1. Chemicals and diets

N-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) was purchased from Tokyo Chemical Industry Co. Ltd., Tokyo, Japan. L-Leucine and L-isoleucine (99.9% pure) were provided by Ajinomoto Co., Inc. (Kanagawa, Japan). Basal diets (powdered AIN-93G; Oriental Yeast Co., Tokyo, Japan) and the diets containing 2% L-leucine or 2% L-isoleucine were prepared once a month by Oriental Yeast Co., Tokyo, Japan.

2.2. Animals

A total of 90 5-week-old male Fisher 344 rats were supplied by Charles River Japan, Inc. (Hino, Shiga, Japan). Animals were housed in polycarbonate cages (three per cage) in experimental animal rooms with a targeted temperature of 22 ± 3 °C, relative humidity of 55 ± 5% and a 12-h light/dark cycle. Diet and tap water were available *ad libitum* throughout the study. Animals were acclimatized for one week prior to beginning the experiment. The experiment was conducted following approval of the Animal Care and Use Committee of the Osaka City University Graduate School of Medicine.

2.3. Experimental design

90 rats were randomly divided into six groups (15 animals in each group) and administered 0.05% BBN in the drinking water for the first 4 weeks. Thereafter, they were fed powdered AIN-93G basal diet (groups 1(G1) and 4 (G4)), AIN-93G supplemented with 2% L-leucine (groups 2 (G2) and 5 (G5)) or AIN-93G supplemented with 2% L-isoleucine (groups 3 (G3) and 6 (G6)) for 8 weeks. Rats in G1, G2, and G3 were sacrificed under diethyl ether anesthesia at the end of the amino acid administration period, week 12. G4, G5, and G6, rats were fed AIN-93G basal diet without amino acid supplementation for an additional 8 weeks (Fig. 1). Rats in groups G4, G5, and G6 were killed under diethyl ether anesthesia at week 20 after the commencement of the experiment. The urinary bladders of the sacrificed rats were inflated by intra-luminal injection of 4% phosphate-buffered paraformaldehyde (PFA) solution and fixed at 4 °C for 4 h, as shown in Fig. 2, for histopathological and molecular biological examination.

2.4. Macroscopic quantitative analysis

PFA-fixed bladders were carefully opened and the lumen inspected for grossly visible lesions. The number of tumors per rat and the volume of each tumor were recorded. Tumor volume was determined using the formula for ellipsoid volumetry $V = diameter1 \times diameter2 \times diameter3 \times \pi/6$. A tumor was defined as a lesion > 0.5 mm in diameter.

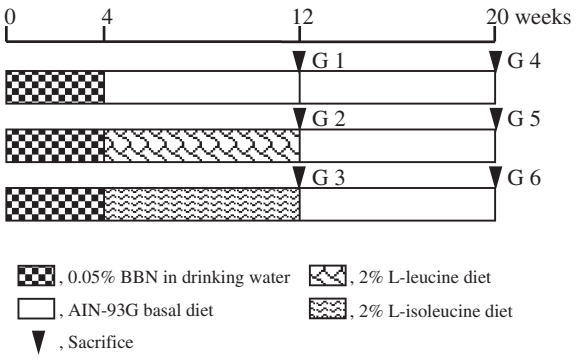

Fig. 1. Experiment design. 90 Fisher 344 rats were randomly divided into six groups (15 animals in each group) and administered 0.05% BBN in the drinking water for the first 4 weeks. They were then fed powdered AIN-93G basal diet (G1 and G4), 2% L-leucine (G2 and G5) in the basal diet, or 2% L-isoleucine (G3 and G6) in the basal diet for 8 weeks. Rats in G1, G2, and G3 were sacrificed at the end of the amino acid supplementation period, and rats in G4, G5, and G6 were fed basal diet without amino acid supplementation for a further 8 weeks. Rats in G4, G5, and G6 were sacrificed at week 20.

2.5. Quantitative analysis of histological parameters

PFA-fixed rat bladders were cut into eight strips, routinely processed for embedding in paraffin, sectioned at 3 μm thickness and processed for histopathological analysis. After deparaffinization, slides were stained with hematoxylin and eosin (H&E) for histopathological classification and assessment of the incidence of bladder papillary or nodular (PN) hyperplasia, papillomas and carcinomas, which were counted using a light microscope and categorized based on tumor size and presence of mitotic figures according to the diagnostic criteria of Boorman et al. (1990). The average number of PN hyperplasia, papillomas, transitional cell carcinomas (TCCs) and total tumors per rat was calculated and expressed as multiplicity.

2.6. Extraction of total RNA

Tissues from 8 rats in each group were used for RT-PCR analysis. Total RNA was extracted from the PFA-fixed paraffin-embedded rat bladders. After deparaffinization, normal-appearing bladder urothelium, PN hyperplasia and bladder tumors, papillomas and transitional cell carcinomas (TCCs) were collected using sterile toothpicks under a light microscope, and total RNA was extracted using the RNeasy FFPE kit according to the protocol supplied by the manufacturer (QIAGEN, Tokyo, Japan).

2.7. Real-time quantitative RT-PCR analysis of L-leucine and L-isoleucine transporters and tumorigenesis-associated genes in rat bladder lesions and normal-appearing urothelium

cDNA copies of rat bladder total RNA were obtained using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Tokyo, Japan). Primers and probes (Taqman Gene Expression Assay) were purchased from Applied Biosystems, Inc., Carlsbad, CA, USA. The PCR program cycles were set as follows: initial denaturing at 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s, and 60 °C for 30 s. PCR reactions were performed as described previously (Xie et al., 2012a), with primers for rat branched-chain amino acid-related transporters 4F2hc (Rn01759899_g1), LAT1 (Rn00569313_m1), y + LAT1 (Rn00580189_m1), LAT2 (Rn00584909_m1), LAT3 (Rn01513966_m1) and LAT4 (Rn01751916_m1) and tumorigenesis-associated genes TNF-α (Rn01525859_g1), c-fos (Rn02396760_g1), β-catenin (Rn00584431_g1), p53 (Rn00755717_m1), $p21^{Cip1/WAF1}$ (Rn01427989_s1), cdk4 (Rn00585909_m1), cyclin D1 (TaqMan probe, TCAAGCCTG- CGCCAGGCCC, and forward, 5′ GCCTGCCAGGAACAGATTGA, and reverse, 5′ GGCCTTGGGATCGATGT TCT, primers), cyclin E (Rn01457760_m1) and caspase-3 (Rn00563902_m1). β-actin mRNA (TaqMan probe, TGAGACCTTCAACACCCC- AGCCATG, and forward, 5′ CCGTG AAAAGATGACCCAGATC, and reverse, 5′ACCAGAGGCATACAGGGACAAC, primers) was employed as an internal standard; the mRNA levels of the target gene were normalized to the β-actin mRNA level. mRNA expression in each treated group was expressed as a fold change compared to the mean value of the BBN control group, which was given an arbitrary value of 1.

2.8. Statistical analysis

All values were expressed as means ± standard deviations (SDs). Statistical analyses were performed using the Statlight program (Yukms Co., Ltd, Tokyo, Japan). Incidences of pathologic lesions were compared using the Chi-squared test.

Homogeneity of variance was tested by the F test in the basal diet groups and each treatment group. Differences in mean values between the control and each treatment group were evaluated by the two-tailed Student t-test when variance was homogeneous and the two-tailed Aspin-Welch t-test when variance was heterogeneous. P values less than 0.05 were considered significant.

3. Results

3.1. General observations

Final rat body weights, water and food intakes are shown in Table 1. All treatment diets were well tolerated and there were no differences among the groups with regard to food and water consumption or body weight gain. No rat died before the termination of the experiment.

3.2. Macroscopic observation

Incidences and multiplicities of macroscopic tumors at weeks 12 and 20 are shown in Supplemental Table 1. Overall, incidences and multiplicities tended to be increased in the L-leucine and L-isoleucine supplemented groups compared with the BBN alone control; although, there were no significant differences between any