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Lactate calcium salt affects the viability of colorectal cancer cells via betaine homeostasis

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ABSTRACT

Aims: Betaine plays an important role in cellular homeostasis. However, the physiological roles of betaine-γ-aminobutyric acid (GABA) transporter (BGT-1) are still being disputed in cancer. In this study, we tried to find the possibility of the antitumor effect on colorectal cancer (CRC) cell via lactate calcium salt (CaLa)-induced BGT-1 downregulation.

Main methods: The CRC cell viability and clonogenic assay was performed using different doses of BGT-1 inhibitor. The expression level of BGT-1 was measured following the treatment of 2.5 mM CaLa. Betaine was treated to confirm the resistance of the antitumor activity by CaLa. Tumor growth was also measured using a xenograft animal model.

Key findings: Long-term exposure of 2.5 mM CaLa clearly decreased the expression of BGT-1 in the CRC cells. As a result of the downregulation of BGT-1 expression, the clonogenic ability of CRC cells was also decreased in the 2.5 mM CaLa-treated group. Reversely, the number of colonies and cell viability was increased by combination treatment with betaine and 2.5 mM CaLa, as compared with a single treatment of 2.5 mM CaLa. Tumor growth was significantly inhibited in the xenograft model depending on BGT-1 downregulation by 2.5 mM CaLa treatment.

Significance: These results support the idea that long-lasting calcium supplementation via CaLa contributes to disruption of betaine homeostasis in the CRC cells and is hypothesized to reduce the risk of CRC. In addition, it indicates the possibility of CaLa being a potential incorporating agent with existing therapeutics against CRC.

1. Introduction

Chemically, betaine is the trimethyl derivative of glycine. Various functions have been defined for the betaine molecule due to its chemical structure, and in upper vertebrates there is evidence for its use as an osmolyte in numerous tissues [8]. Furthermore, betaine provides methyl groups that can be used in transmethylation reactions for the synthesis of various substances [20]. Moreover, betaine is involved in protein and energy metabolism, being widely discussed in animal nutrition due to its lipotropic and growth-promoting effects [7].

The betaine-γ-aminobutyric acid (GABA) transporter (BGT-1) is a member of the solute carrier family 6, and mediates the cellular uptake of betaine [3,11]. The mammalian BGT-1 is predominantly expressed in the kidney, liver, and brain [11]. The majority of studies on BGT-1 concern its function and regulation in the kidney medulla, where its role is best understood. Therefore, the physiological roles of the BGT-1 are still being disputed in other cellular pathogenesis including cancer [11].

In cell physiology, betaine plays central roles in one-carbon metabolism such as homocysteine status, structural integrity, and signaling functions of cell membranes. It also regulates cell volume and protects cells and proteins from environmental stresses including ionic stress [1,26]. Therefore, betaine serves as an osmolyte and plays a major role in protecting cells from external and/or internal stress [1,2].

For this reason, previous reports have also proposed that betaine status in plasma possibly relates to cancer risk [14,26,28], indicating that individuals with different betaine concentrations may be at risk of cancer [14,26]. However, there are few reports regarding the role of betaine in the risk of colorectal cancer (CRC), and as a result, the mechanisms of action pertaining to its activity remain poorly understood.

Lactate calcium salt (CaLa) is a crystalline salt, which is formed by the action of lactic acid on calcium carbonate. CaLa is most commonly used in medicine to treat calcium deficiencies such as hypocalcemia syndromes. In our previous study, we investigated the motility mechanism of metastatic CRC cells based on a single treatment of CaLa [25]. The level of intracellular calcium concentration easily plateaued in

Abbreviations: GABA, betaine-gamma-aminobutyric acid; BGT-1, betaine-gamma-aminobutyric acid transporter-1; CRC, colorectal cancer; CaLa, lactate calcium salt; SEM, standard error of the mean; SD, standard deviation.

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480 s, however, we did not investigate the CRC viability with long-term supplementation of CaLa.

In the present study, CRC cells are subjected to long-term supplementation with CaLa, and the possibility of an antitumor effect via CaLa, with a focus on BGT-1-related betaine homeostasis, is investigated.

2. Materials and methods

2.1. Cell culture and reagents

The human colon cancer cell lines, HCT-116 and HT-29, were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA), 100 IU/ml penicillin (Welgene, Daegu, South Korea), and 100 μg/ml streptomycin (Welgene, Daegu, South Korea). CaLa and betaine were purchased from Sigma-Aldrich (St Louis, MO, USA). The BGT-1 inhibitor, NNC 05-2090 hydrochloride, was obtained from Tocris (Ellisville, MO, USA). Antibodies against BGT-1 and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and EMD Millipore (Billerica, MA, USA), respectively.

2.2. Cell viability assay

HCT-116 and HT-29 cells were seeded at a density of 5000 cells/well in 96-well plates and treated with different concentrations of the BGT-1 inhibitor, betaine, or 2.5 mM CaLa for 48 h. The cell viability assay was performed using 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT, 5 mg/ml). MTT reduction was assessed using an Epoch Micro-Volume Spectrophotometer System (Bio-Tek, Winooski, VT, USA).

2.3. Clonogenic assay

HCT-116 and HT-29 cells were seeded at a density of 200 cells/well in 6-well plates and treated with different concentrations of BGT-1 inhibitor, betaine, or 2.5 mM CaLa in complete medium. After 10 days, the colonies were fixed with methanol and stained using crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Finally, images of the stained colonies were counted (G16, Canon INC., Tokyo, Japan).

2.4. BGT-1 knockdown assay

Small interference RNA (siRNA) of BGT-1, 5′-GCAUUGGUCUGGCA UCUGUTT-3′ (sense) and 5′-ACAGAUGCCAGACCAAUGCTT-3′ (antisense) were purchased from GenePharma (Shanghai, China). The cells were transfected with the siRNA in RPMI 1640 medium using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.5. Western blotting

Whole-cell lysates were prepared using lysis buffer (20 mM Tris–HCl (pH 7.6), 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate). Equivalent amounts of protein (20 μg) were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Nonspecific binding was blocked with 5% nonfat powdered milk. The membrane was incubated with primary antibodies against BGT-1 and GAPDH (1:1000) in 5% bovine serum albumin at 4 °C for 16 h. The membrane was then washed three times with Tris-buffered saline containing 0.1% Tween-20 and incubated with an anti-mouse secondary antibody (1:2000) for 2 h at room temperature. Immunoblots were developed using Western blotting detection reagents (Abclon, Seoul, Korea) and exposed to X-ray film (Agfa, Leverkusen, Germany) according to the manufacturer's recommended protocol.

2.6. Immunocytochemistry

CRC cells were fixed on bio-coated coverslips (BD bioscience, NJ, USA) using 4% paraformaldehyde, and incubated for 15 h with the primary antibody against BGT-1 (1:500). Subsequently, coverslips were incubated with an anti-rabbit secondary biotinylated antibody and visualized with fluorescein-conjugated streptavidin (Vector, Burlingame, CA, USA). Cells were then washed and mounted using Vectashield mounting medium (Vector Laboratories, Burlingham, CA, USA) for observation using confocal microscopy (Nikon, Tokyo, Japan).

2.7. Animals and treatment

The Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved the animal facilities, and all experiments were performed under the institutional guidelines established by the Institutional Animal Care and Use Committee at Gachon University (IACUC-2014-0046). Female balb/c nude mice (5 weeks of age) were purchased from the Charles River Breeding Laboratories (Wilmington, MA, USA). All animals were maintained in a 12-h light/dark cycle (lights on, 08:00) at 22–25 °C, with free access to food and water. In order to establish the heterotopic model, HCT-116 cells (5×10^6) were suspended in 100 μl phosphate-buffered saline and injected subcutaneously into the left hind flank of the mice. When tumor diameters reached 8 mm, 2.5 mM CaLa was perorally administered ad libitum. Tumor growth was monitored every 2 days.

2.8. Immunohistochemisty

Tumor tissues ($n = 5$) were dissected and fixed in 4% paraformaldehyde/PBS solution for 15 h at 4 °C. Fixed tissues were embedded in paraffin and sectioned at 5 mm. Slides were incubated at 55 °C for 2 h, and subsequently deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked with 3% H_2O_2 in distilled water for 30 min. Antigen retrieval was performed by heating the slides in 10 mM citrate buffer (pH 6.0) for 10 min and washing several times in PBS. Blocking was subsequently performed using a blocking agent (Invitrogen, Frederick, MD, USA) and slides were incubated with primary antibody (BGT-1) overnight at 4 °C.

2.9. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM) or standard deviation (SD). Statistical significance was analyzed using the Student's *t*-test or the Mann–Whitney rank sum test, depending on the normality of the data. A difference of $P < 0.05$ was considered to be statistically significant. All statistical analyses were carried out using Sigma Stat (ver. 3.5, Systat Software Inc., Chicago, IL, USA).

3. Results

3.1. Inhibition of betaine transport decreases the viability of CRC cells

To confirm the viable effect of betaine homeostasis, BGT-1 was inhibited using NNC 05-2090 hydrochloride, which is a known selective inhibitor of BGT-1. The inhibitor was treated with increasing concentrations up to 5 μM, and the CRC cell viability was calculated (Fig. 1). Concentrations of the inhibitor between 0.5 and 2 μM had no effect on the viability of HCT-116 cells. The viability was significantly decreased to 29.2 ± 2.3% at a concentration of 5 μM (Fig. 1A). In HT-29 cells, 2 and 5 μM BGT-1 inhibitor decreased the cell viability significantly (2 μM, 65.2 ± 2.6%; 5 μM, 35.1 ± 3.6%). However, there was no effect on the cell viability at concentrations of 0.5 and 1 μM (Fig. 1B).