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Full paper

Identification and functional analysis of choline transporter in tongue cancer: A novel molecular target for tongue cancer therapy

Ryohta Nishiyama [a], Fumiaki Nagashima [a], Beniko Iwao [b], Yuiko Kawai [c], Kana Inoue [d], Arisa Midori [d], Tsuyoshi Yamanaka [c], Hiroyuki Uchino [a], Masato Inazu [c, e, *]

[a] Department of Anesthesiology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
[b] Department of Psychiatry, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
[c] Institute of Medical Science, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
[d] School of Nursing, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
[e] Department of Molecular Preventive Medicine, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan

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ABSTRACT

We examined the functional characteristics of choline uptake in human tongue carcinoma using the cell line HSC-3. Furthermore, we explored the possible correlation between the inhibition of choline uptake and apoptotic cell death. Both choline transporter-like protein 1 (CTL1) and CTL2 mRNAs and proteins were expressed, and were located in plasma membrane and mitochondria, respectively. Choline uptake was saturable and mediated by a single transport system, which is pH-dependent. Several cationic drugs inhibited cell viability and [^{3}H]choline uptake. Choline uptake inhibitors and choline deficiency inhibited cell viability and increased caspase-3/7 activity. We conclude that extracellular choline is mainly transported via a CTL1 that relies on a directed H^+ gradient as a driving force. The functional inhibition of CTL1 by cationic drugs could promote apoptotic cell death. Furthermore, CTL2 may be the major site for the control of choline oxidation in mitochondria and hence for the supply of endogenous betaine and *S*-adenosyl methionine, which serves as a major methyl donor. Identification of this CTL1- and CTL2-mediated choline transport system provides a potential new target for tongue cancer therapy.

1. Introduction

Choline is an essential nutrient for all cells, and is needed for the synthesis of the major membrane phospholipids phosphatidylcholine (PC) and sphingomyelin. Large degrees of choline uptake and PC biosynthesis are necessary for new membrane synthesis. Choline is also used as a precursor for the neurotransmitter acetylcholine (ACh) and betaine, and participates in several vital biological functions. Betaine, an oxidized metabolite of choline, is a source of methyl groups for the production of *S*-adenosyl methionine (SAM), which serves as a substrate for DNA and histone methyltransferases, and is thus required for the establishment and maintenance of the epigenome (1). Epigenetic mechanisms play important roles in carcinogenesis. Aberrant DNA methylation is observed in tongue squamous cell carcinoma (TSCC) tissue and plays an important role in the tumorigenesis, development and progression of TSCC (2). Previous studies have demonstrated abnormalities in choline uptake and choline phospholipid metabolism in tongue cancer based on imaging with magnetic resonance spectroscopy (MRS) (3). Furthermore, positron emission tomography (PET) and PET computed tomography (PET-CT) studies with ^{11}C-labeled choline are used in tongue cancer patients (4). The aberrant choline metabolism in cancer cells is strongly correlated with the progression of malignancy (5–7). Thus, the intracellular accumulation of choline through choline transporters is the rate-limiting step in choline phospholipid metabolism, and a prerequisite for cancer cell proliferation. However, the uptake system for choline and the functional expression of choline transporters in tongue cancer are not totally understood.

The choline transport system has been categorized into three transporter families: high-affinity choline transporter 1 (CHT1/SLC5A7), intermediate-affinity choline transporter-like proteins (CTL1-5/SLC44A1-5), and polyspecific organic cation transporters (OCT1-2/SLC22A1-2) that exhibit low affinity for choline (8,9). CHT1

* Corresponding author. Institute of Medical Science, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan. Tel.: +81 3 3351 6141; fax: +81 3 3351 6166.
E-mail address: inazu@tokyo-med.ac.jp (M. Inazu).
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is thought to play an important role in cholinergic neurons, and may be part of the rate-limiting step in ACh synthesis. In addition, CHT1 is a Na^+-dependent co-transporter that is highly sensitive to the choline analogue hemicholinium-3 (HC-3) in the nM range. Choline is known to be a substrate for carriers of OCTs, and these transporters accept choline as a substrate with comparatively low affinity. Recently, distinct choline transporters called CTLs have been shown to be present in various human tissues (10,11). CTL1 is a Na^+-independent, intermediate-affinity transporter of choline that can be completely inhibited by a high concentration of HC-3 in the μM range. CTL2 is expressed as two isoforms (CTL2-P1 and CTL2-P2) in the cochlea, tongue, heart, colon, lung, kidney, liver and spleen, which suggests that tissue-specific differences may influence its function in each tissue. While human CTL2-P1 does not transport choline, human CTL2-P2 exhibits detectable choline transport activity (12). However, the functions of other transporters in this family (CTL3-5) are not fully understood. They are expressed in different organisms and cell types, and this implies that they are important for choline transport for a broader purpose, such as phospholipid synthesis and epigenetics.

In this study, we examined the functional characteristics of choline uptake and sought to identify the transporters that mediate choline uptake in the tongue cancer cell line HSC-3. We also examined the effects of existing drugs on choline uptake and cell viability, to investigate the possible benefits of drug repositioning.

2. Materials and methods

2.1. Materials

The human tongue cancer cell line HSC-3 was provided by the JCRB Cell Bank (Osaka, Japan). [^{3}H]Choline chloride (specific activity: 3182 GBq/mmol) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). A QIA shredder and RNeasy Mini Kit were obtained from Qiagen Inc. (Valencia, CA, USA). A TaqMan® RNA-to-CT™ 1-Step Kit and TaqMan® Gene Expression Assays were obtained from Applied Biosystems (Foster City, CA, USA). Detector Blocking Solution, Wash Solution, and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD, USA). VECTASHIELD mounting medium with 4′, 6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Alexa Fluor 488 goat anti-rabbit and anti-mouse IgG, and Alexa Fluor 568 goat anti-rabbit and anti-mouse IgG were purchased from Molecular Probes Inc. (Eugene, OR, USA). RIPA Lysis Buffer System (sc-24948) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Tris-SDS β-ME sample solution was purchased from Cosmo Bio Corporation (Tokyo, Japan). Protein Multicolor III was purchased from BioDynamics Laboratory Inc. (Tokyo, Japan). A DC Protein Assay kit, Mini-PROTEAN® TGX™ Gel and Trans-Blot® Turbo™ Transfer Pack were purchased from Bio-Rad Laboratories (Tokyo, Japan). Anti-CTL1 polyclonal antibody (ab110767), anti-pan Cadherin antibody (CH-19, ab6528) and anti-COX IV antibody (ab16056) were purchased from Abcam plc (Cambridge, UK). Anti-CTL2 monoclonal antibody (clone 3D11), which recognizes both CTL2-P1 and CTL2-P2, was purchased from Abnova Corporation (Taipei, Taiwan). Anti-Calnexin antibody (PM060MS) and anti-MG130 antibody (PM061) were purchased from Medical Biological Laboratories Co., LTD. (Nagoya, Japan). ATPLite™ was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Caspase-Glo® 3/7 Assay was purchased from Promega Corporation (Madison, WI, USA). Alimemazine tartrate, hydroxyzine dihydrochloride, ketotifen fumarate, azelastine hydrochloride and ebastine were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Choline chloride and RPMI 1640 medium, clemastine fumarate, mequitazine, oxatomide and D-MEM medium were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Homochlorcyclizine hydrochloride, cyproheptadine hydrochloride, paroxetine hydrochloride, quinidine hydrochloride, mebendazole, bepridil hydrochloride, lomerizine dihydrochloride, HC-3, Triton X-100, *N*-methyl-D-glucamine (NMDG), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2-(*N*-morpholino) ethanesulfonic acid (MES) were purchased from Sigma–Aldrich Co. LLC. (St. Louis, MO, USA). Promethazine hydrochloride, loratadine, levocetirizine dihydrochloride, and tranilast were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Rupatadine fumarate was purchased from COSMO BIO Co., Ltd. (Tokyo, Japan). Citalopram hydrobromide and fluoxetine hydrochloride were purchased from LKT Laboratories, Inc. (St Paul, MN, USA). Reboxetine mesylate was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Fluvoxamine maleate was a generous gift from Meiji Yakuhin Co., Ltd. (Tokyo, Japan). D-MEM medium without choline chloride was obtained from Cell Science & Technology Institute, Inc. (Miyagi, Japan).

2.2. Cell culture

HSC-3 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 20 mg/L kanamycin (Gibco) on non-coated flasks and in 24-well plates. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, and the medium was changed every 3–4 days.

2.3. [^{3}H]Choline uptake into HSC-3 cells

Cells were cultured in 24-well culture plates. The growth medium was removed from the 24-well culture plates. Cells were washed twice with uptake buffer, consisting of 125 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5.6 mM glucose, 1.2 mM $MgSO_4$ and 25 mM HEPES adjusted to pH 7.4 with Tris. This was followed by the addition of [^{3}H]choline. Choline uptake was terminated by removal of the uptake buffer and three rapid washes with ice-cold uptake buffer. The cultures were dissolved in 0.1 N NaOH and 0.1% Triton X-100, and aliquots were taken for liquid scintillation counting and protein assay. Radioactivity was measured by a liquid scintillation counter (Tri-Carb® 2100 TR, Packard, USA). When Na^+-free buffer was used, the Na^+-free buffer was modified by replacing NaCl with an equimolar concentration of NMDG chloride. Uptake buffers with different pH values (pH 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) were prepared by mixing 25 mM MES (pH 5.5) and 25 mM Tris (pH 8.5). Both buffers contained 125 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5.6 mM glucose and 1.2 mM $MgSO_4$. In experiments on saturation kinetics, the concentration of [^{3}H]choline was kept constant at 10 nM and unlabeled choline was added to give the desired choline concentration. The specific uptake of [^{3}H]choline was calculated as the difference between total [^{3}H]choline uptake in the presence and absence of 30 mM unlabeled choline. Protein concentrations were determined using a DC Protein Assay Kit.

2.4. RNA extraction and real-time polymerase chain reaction (PCR) assay

Cells were washed with sterile D-PBS and total RNA was extracted from the cells using QIA shredder and an RNeasy Mini Kit according to the manufacturer's instructions. The TaqMan probes for the target mRNAs (CHT1, OCT1-3, CTL1-5 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were