Pharmacological Research 76 (2013) 119–131

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Functional expression of choline transporter-like protein 1 (CTL1) in small cell lung carcinoma cells: A target molecule for lung cancer therapy☆

Masato Inazu[a,b,*], Tomoko Yamada[c], Nobuo Kubota[d], Tsuyoshi Yamanaka[b]

[a] Institute of Medical Science, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
[b] Department of Molecular Preventive Medicine, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
[c] Department of Pharmacology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
[d] POLA Pharma, Inc., R&D Laboratories, 560 Kashio-cho, Totsuka-ku, Yokohama 244-0812, Japan

ARTICLE INFO

Article history:
Received 12 June 2013
Received in revised form 22 July 2013
Accepted 29 July 2013

Keyword:
Choline
Acetylcholine
Transporter
Small cell lung carcinoma
Cell proliferation
Apoptosis

ABSTRACT

Choline is essential for the synthesis of the major membrane phospholipid phosphatidylcholine and the neurotransmitter acetylcholine (ACh). Elevated levels of choline and up-regulated choline kinase activity have been detected in cancer cells. Thus, the intracellular accumulation of choline through choline transporters is the rate-limiting step in phospholipid metabolism and a prerequisite for cancer cell proliferation. However, the uptake system for choline and the functional expression of choline transporters in lung cancer cells are poorly understood. We examined the molecular and functional characterization of choline uptake in the small cell lung carcinoma cell line NCI-H69. Choline uptake was saturable and mediated by a single transport system. Interestingly, removal of Na^+ from the uptake buffer strongly enhanced choline uptake. This increase in choline uptake under the Na^+-free conditions was inhibited by dimethylamiloride (DMA), a Na^+/H^+ exchanger (NHE) inhibitor. Various organic cations and the choline analog hemicholinium-3 (HC-3) inhibited the choline uptake and cell viability. A correlation analysis of the potencies of organic cations for the inhibition of choline uptake and cell viability showed a strong correlation ($R = 0.8077$). RT-PCR revealed that choline transporter-like protein 1 (CTL1) mRNA and NHE1 are mainly expressed. HC-3 and CTL1 siRNA inhibited choline uptake and cell viability, and increased caspase-3/7 activity. The conversion of choline to ACh was confirmed, and this conversion was enhanced under Na^+-free conditions, which in turn was sensitive to HC-3. These results indicate that choline uptake through CTL1 is used for ACh synthesis. Both an acetylcholinesterase inhibitor (eserine) and a butyrylcholinesterase inhibitor (ethopropazine) increased cell proliferation, and these effects were inhibited by 4-DAMP, a mAChR3 antagonist. We conclude that NCI-H69 cells express the choline transporter CTL1 which uses a directed H^+ gradient as a driving force, and its transport functions in co-operation with NHE1. This system primarily supplies choline for the synthesis of ACh and secretes ACh to act as an autocrine/paracrine growth factor, and the functional inhibition of CTL1 could promote apoptotic cell death. Identification of this new CTL1-mediated choline transport system provides a potential new target for therapeutic intervention.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; ChAT, choline acetyltransferase; CHT1, high-affinity choline transporter 1; CTL1, choline transporter-like protein 1; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; D-PBS, Dulbecco's phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; DFP, diisopropyl fluorophosphates; DMA, dimethylamiloride; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HC-3, hemicholinium-3; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAChR3, muscarinic cholinergic receptor 3; MES, 2-(N-morpholino)ethanesulfonic acid; NHE, Na^+/H^+ exchanger; NMDG, N-methyl-D-glucamine; OCT, organic cation transporter; PAH, *p*-aminohippuric acid; PCho, phosphocholine; PET, positron emission tomography; SCLC, small cell lung carcinoma; siRNA, small interfering RNA; SNRI, serotonin and norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; TEA, tetraethylammonium chloride; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; VAChT, vesicular acetylcholine transporter.

* Corresponding author at: Institute of Medical Science, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan. Tel.: +81 3 3351 6141.
E-mail address: inazu@tokyo-med.ac.jp (M. Inazu).

1043-6618/$ – see front matter © 2013 The Authors. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.phrs.2013.07.011

1. Introduction

Lung cancer is the most frequently occurring cancer in the world, and is classified as either small cell lung carcinoma (SCLC) or non-SCLC, the former of which accounts for 20–25% of primary lung cancer [1]. SCLC are strongly associated with smoking and, beyond the well-established causal connection with carcinogens in cigarette smoke, nicotine also appears to affect lung cancer growth. SCLC is a chemo-sensitive disease with a response rate ranging from 70 to 90% for first-line treatment; however, relapse is very common and, as a result, long-term survival is poor. Current therapies for SCLC rarely extend survival beyond 5 years. Recent studies have suggested that postoperative radiation therapy is not associated with improved survival for elderly patients with N2 disease [2]. Thus, novel approaches for research on and medical treatment of SCLC are required.

Acetylcholine (ACh) can stimulate cell growth through either nicotinic or muscarinic cholinergic pathways. It has been well established that SCLC express receptors for ACh and that stimulation of these receptors by nicotine or other cholinergic agonists stimulates cell growth [3]. Furthermore, SCLC can synthesize, secrete, and degrade ACh, and this released ACh stimulates SCLC cell growth. Spindel's group has proposed that the interruption of autocrine muscarinic cholinergic signaling has the potential to inhibit SCLC cell growth [4,5]. Another group also found that muscarinic cholinergic receptor 3 (mAChR3) modulated SCLC cell proliferation, adhesion and migration [6]. These reports suggest that a mAChR3 antagonist may be a beneficial therapeutic modality for SCLC patients. However, the uptake system of choline, which is a precursor of ACh, in SCLC is poorly understood.

Choline is an organic cation that plays a critical role in the structure and function of biological membranes in all cells as an essential component of the membrane phospholipids phosphatidylcholine and sphingomyelin. Large degrees of choline uptake and phosphatidylcholine biosynthesis are necessary for new membrane synthesis. In the brain, choline plays an additional role as a precursor for synthesis of the neurotransmitter ACh [7]. To date, the choline transport system has been categorized into three transporter families according to their affinity for choline. A high-affinity choline transporter, CHT1, has recently been cloned and characterized, and is thought to be unique to cholinergic neurons [8,9]. CHT1 is a Cl^--dependent and Na^+-dependent co-transporter that is highly sensitive to the choline analog hemicholinium-3 (HC-3), and is thought to be part of the rate-limiting step in ACh synthesis in the cholinergic neurons. As an organic cation, choline is known to be a substrate for carriers of organic cation transporters (OCTs). To date, three different OCTs (OCT1-3) have been cloned and their function, which involves a Na^+-independent uptake mechanism, has been characterized [10,11]. These transporters recognize a multitude of endogenous and exogenous organic cations as substrates and exhibit considerable overlap in substrate specificity. Choline also interacts with these transporters with varying affinity. While OCT1 and OCT2 accept choline as a substrate with comparatively low affinity, OCT3 does not recognize choline as a substrate [12–16]. Recently, a distinct choline transporter called choline transporter-like (CTL) protein (human CTL1-5) has been shown to be present in a various human tissues [17]. CTL1 has been cloned from *Torpedo marmorata*, and was first cloned as a suppressor for a yeast choline transport mutation from a Torpedo electric lobe yeast expression library by functional complementation [17,18]. Functional characterization studies have been performed with CTL1 in rats, and have shown that CTL1 is a Na^+-independent, intermediate-affinity transporter of choline that can be completely inhibited by a high concentration of HC-3 [19–22]. CTL2 mRNA and protein are expressed in human inner ear [23] and rat renal epithelial cells [22]. It has recently been demonstrated that CTL1 and CTL2 are functionally expressed as choline transporters [24,25]. However, the function of CTL2 has not been fully characterized. Other transporters in this family are completely unknown.

Uptake studies have shown that enhanced choline transport may play a role in the elevation of phosphocholine (PCho) levels in cancer cells. The elevation of PCho and total choline is one of the most widely established characteristics of cancer cells [26,27]. PCho is a precursor and a breakdown product of phosphatidylcholine, the most abundant phospholipid in biological membranes. The biosynthesis and hydrolysis of phosphatidylcholine are essential processes for mitogenic signal transduction events in cells [28]. Previous studies have demonstrated that abnormalities of choline uptake and choline phospholipid metabolism in cancer cells based on imaging with magnetic resonance spectroscopy [29–31] and positron emission tomography (PET) [32,33]. The aberrant choline metabolism in cancer cells is strongly correlated with their malignant progression [34–36]. The utility of a PET scan with ^{11}C-choline for detecting various tumors including lung cancer has been well established [37–40]. Because tumor cells duplicate very quickly, the biosynthesis of cell membranes is also very fast. Consequently, the uptake of choline in tumors represents the rate of tumor cell duplication. 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 molecular and functional characteristics of choline transporters in lung cancer cells are poorly understood.

In this study, we examined the functional characterization of choline uptake and sought to identify the transporters that mediate choline uptake in the SCLC cell line NCI-H69. We also examined the correlations between choline uptake and both cell viability and ACh synthesis.

2. Materials and methods

2.1. Materials

The human small cell lung carcinoma cell line NCI-H69 was purchased from American Type Culture Collection (Manassas, VA). COS-7 cells were purchased from Japanese Collection of Research Bioresources of the Human Science Research Resources Bank (Osaka, Japan). [Methyl-^{3}H]choline chloride (specific activity: 3182 GBq/mmol) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA). Hemicholinium-3 (HC-3), choline chloride, tetraethylammonium chloride (TEA), clonidine, quinine, quinidine, desipramine, diphenhydramine, *p*-aminohippuric acid (PAH), dimethyl amiloride (DMA), Triton X-100, N-methyl-D-glucamine (NMDG), D-mannitol, choline oxidase, acetylcholinesterase (type V), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(N-morpholino)ethanesulfonic acid (MES), eserine, ethopropazine and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) were obtained from Sigma–Aldrich Inc. (St. Louis, MO). Choline chloride and diisopropyl fluorophosphates (DFP) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A QIA shredder and RNeasy Mini Kit were obtained from Qiagen Inc. (Valencia, CA). A TaqMan® RNA-to-CT™ 1-Step Kit and TaqMan® Gene Expression Assays were obtained from Applied Biosystems (Foster City, CA). A protein detector Western Blotting Kit (TMB system), Milk Diluent/Blocking Solution and Wash Solution were purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). VECTASHIELD mounting medium with 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories, Inc. (Burlingame, CA). Alexa Fluor 568 goat anti-rabbit IgG was purchased from Molecular Probes Inc. (Eugene, OR). Tris-SDS β-ME sample solution was purchased from Cosmo Bio