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ORIGINAL ARTICLE

Biosynthesis of D-aspartate in mammals: the rat and human homologs of mouse aspartate racemase are not responsible for the biosynthesis of D-aspartate

Satsuki Matsuda · Masumi Katane · Kazuhiro Maeda · Yuusuke Kaneko · Yasuaki Saitoh · Tetsuya Miyamoto · Masae Sekine · Hiroshi Homma

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Abstract D-Aspartate (D-Asp) has important physiological functions, and recent studies have shown that substantial amounts of free D-Asp are present in a wide variety of mammalian tissues and cells. Biosynthesis of D-Asp has been observed in several cultured rat cell lines, and a murine gene (*glutamate-oxaloacetate transaminase 1-like 1*, *Got1l1*) that encodes Asp racemase, a synthetic enzyme that produces D-Asp from L-Asp, was proposed recently. The product of this gene is homologous to mammalian glutamate-oxaloacetate transaminase (GOT). Here, we tested the hypothesis that rat and human homologs of mouse GOT1L1 are involved in Asp synthesis. The following two approaches were applied, since the numbers of attempts were unsuccessful to prepare soluble GOT1L1 recombinant proteins. First, the relationship between the D-Asp content and the expression levels of the mRNAs encoding GOT1L1 and D-Asp oxidase, a primary degradative enzyme of D-Asp, was examined in several rat and human cell lines. Second, the effect of knockdown of the *Got1l1* gene on D-Asp biosynthesis during culture of the cells was determined. The results presented here suggest that the rat and human homologs of mouse GOT1L1 are not involved in D-Asp biosynthesis. Therefore, D-Asp biosynthetic pathway in mammals is still an urgent issue to be resolved.

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S. Matsuda · M. Katane · K. Maeda · Y. Kaneko · Y. Saitoh · T. Miyamoto · M. Sekine · H. Homma (✉)
Laboratory of Biomolecular Science, Graduate School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-Ku, Tokyo 108-8641, Japan
e-mail: hommah@pharm.kitasato-u.ac.jp

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Abbreviations

DAO	D-Amino acid oxidase
DDO	D-Aspartate oxidase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOT	Glutamate-oxaloacetate transaminase
GOT1L1	Glutamate-oxaloacetate transaminase 1-like 1
HPLC	High-performance liquid chromatography
NAC	*N*-acetyl-L-cysteine
NMDA	*N*-methyl-D-aspartate
OPA	*O*-phthalaldehyde
PLP	Pyridoxal phosphate

Introduction

Among the free D-amino acids found in mammals, D-serine (D-Ser) and D-aspartate (D-Asp) have been studied most extensively. D-Ser persists at high concentrations throughout the life of an animal and is concentrated predominantly in the mammalian forebrain, where it is synthesized by Ser racemase (EC 5.1.1.16), a synthetic enzyme that produces D-Ser from L-Ser in a pyridoxal phosphate (PLP)-dependent manner (Wolosker et al. 1999a). D-Ser is a neuromodulator that binds to the glycine-binding site of the *N*-methyl-D-aspartate (NMDA) receptor, a subtype of the L-glutamate (L-Glu) receptor family, and potentiates glutamatergic neurotransmission in the central nervous system (Nishikawa 2011; Wolosker 2007).

Unlike the tissue-specific expression of D-Ser, substantial amounts of free D-Asp are present in a wide variety of mammalian tissues and cells, particularly those of

the central nervous, neuroendocrine, and endocrine systems. Several lines of evidence suggest that D-Asp plays an important role in regulating developmental processes, hormone secretion, and steroidogenesis (Di Fiore et al. 2014; Katane and Homma 2011; Ota et al. 2012). The amount of D-Asp in human seminal plasma and spermatozoa is significantly lower in oligoasthenoteratospermic and azoospermic donors than normospermic donors (D'Aniello et al. 2005). Furthermore, in female patients undergoing in vitro fertilization, the D-Asp content of pre-ovulatory follicular fluid is lower in older patients than younger patients (D'Aniello et al. 2007); this decrease in D-Asp content appears to reflect a reduction in oocyte quality and fertilization competence. Overall, current evidence suggests that D-Asp may be involved in the pathophysiology of infertility. Meanwhile, D-Asp is also known to stimulate the NMDA receptor by acting as an agonist that binds to the L-Glu-binding site of the receptor (Fagg and Matus 1984; Olverman et al. 1988). Recent studies suggest that D-Asp acts as a signaling molecule in nervous and neuroendocrine systems, at least in part, through the binding to the NMDA receptor, and plays an important role in the regulation of brain functions (Errico et al. 2012; Katane and Homma 2011; Ota et al. 2012). Supporting this, it was recently reported that D-Asp levels in the prefrontal cortex and striatum from postmortem brains of schizophrenic patients are significantly lower than those of non-psychiatrically ill individuals (Errico et al. 2013).

Since the discovery of free D-Ser and D-Asp in mammals, much attention has been paid to the origins and synthetic pathways of these D-amino acids. Wolosker et al. (1999a) were the first to identify and purify mammalian Ser racemase from rat brain. Since then, the cDNAs of mammalian Ser racemases have been cloned from mouse (Wolosker et al. 1999b), human (De Miranda et al. 2000) and rat (Konno 2003), and the recombinant forms of the mouse and human enzymes have been purified and functionally characterized (Hoffman et al. 2009; Stříšovský et al. 2005). In contrast to that of D-Ser, the D-Asp biosynthetic pathway has not yet been characterized in detail. Biosynthesis of D-Asp was first established in cultured rat pheochromocytoma PC-12 cells (Long et al. 1998) and was thereafter demonstrated in rat pituitary tumor GH_3 cells (Long et al. 2000), primary cultured rat embryonic neurons (Wolosker et al. 2000), and MPT1 cells (a subclone of the PC-12 cell line) (Long et al. 2002). In the primary cultured rat embryonic neurons, biosynthesis of [^{14}C]-D-Asp occurs when the cultured neurons are treated with [^{14}C]-L-Asp as the precursor molecule (Wolosker et al. 2000). Moreover, the production of [^{14}C]-D-Asp is markedly inhibited by treatment with aminooxyacetic acid, which is a potent inhibitor of PLP-dependent enzymes. Thus, it appears that the biosynthesis of D-Asp in mammalian cells is mainly mediated by Asp-specific amino acid racemase, which converts L-Asp to D-Asp in a PLP-dependent manner. Indeed, the gene that encodes murine Asp racemase (EC 5.1.1.13) was proposed recently and its cDNA was cloned after long-term exploration (Kim et al. 2010). The recombinant form of this enzyme was reported to exhibit PLP-dependent Asp racemase activity. PLP-dependent Asp racemases of animal origin have also been identified in bivalve *Scapharca broughtonii* (Shibata et al. 2003) and sea slug *Aplysia californica* (Wang et al. 2011). However, while the *S. broughtonii* and *A. californica* Asp racemases belong to the same subfamily as mammalian Ser racemases, the deduced amino acid sequence of mouse Asp racemase is more homologous to that of mammalian glutamate-oxaloacetate transaminase (GOT; EC 2.6.1.1) than those of the mammalian Ser racemases, or the *S. broughtonii* and *A. californica* Asp racemases.

In the rat and human genomes, the genes annotated as homologous to the mouse Asp racemase are designated *Got 1-like 1* (*Got1l1*) (NCBI Gene ID: 306540) and *GOT1L1* (NCBI Gene ID: 137362), respectively. It is therefore presumed that the proteins encoded by these genes are responsible for the biosynthesis of D-Asp in rat and human cells. However, the lengths of the predicted amino acid sequences of the mouse, rat, and human GOT1L1s (404, 315, and 421 amino acids, respectively) are not conserved and it has not been confirmed experimentally that rat and human GOT1L1s function as Asp racemases. We made a number of attempts using different vectors to prepare soluble recombinant forms of GOT1L1 in *Escherichia coli* cells; however, they were all unsuccessful. Therefore, two approaches were applied to address the issue that *Got1l1* and/or *GOT1L1* are involved in Asp synthesis. First, the correlation between the cellular D-Asp content and the expression level of the mRNA encoding GOT1L1 was examined in several cultured rat and human cells. In addition, the expression levels of the mRNA encoding D-Asp oxidase (DDO, also known as DASPO; EC 1.4.3.1), the sole catabolic enzyme that acts on free D-Asp in mammals (Katane and Homma 2010; Ohide et al. 2011), were also measured. Second, the effect of knockdown of the *Got1l1* gene on D-Asp biosynthesis by the cells was examined. The results presented here suggest that GOT1L1 contributes little to the biosynthesis of D-Asp in rat and human cells. Thus, a whole picture of the biosynthetic pathway of D-Asp in the mammalian body remains to be disclosed.

Materials and methods

Chemicals

D-Asp and L-Asp, catalase from *Aspergillus niger*, and G418 were purchased from Sigma-Aldrich (St Louis, MO,

USA). Dulbecco's modified Eagle's medium, RPMI-1640 medium, penicillin, and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum and horse serum were purchased from Gibco-BRL (Gaithersburg, MD, USA). *N*-acetyl-L-cysteine (NAC), *o*-phthaldehyde (OPA), and flavin adenine dinucleotide were purchased from Wako Pure Chemical Industries (Osaka, Japan). Boc-L-cysteine was purchased from Novabiochem (Läufelfingen, Switzerland). All other chemicals were of the highest grade available and were purchased from commercial sources.

Cell lines

Rat pheochromocytoma PC-12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5 % (v/v) fetal bovine serum and 10 % (v/v) horse serum. Rat pituitary tumor GH_3 cells, rat kidney NRK-52E cells, human cervix adenocarcinoma HeLa cells, human hepatocellular carcinoma Hep G2 cells, human epithelioid carcinoma A431 cells, and human neuroectodermal tumor TASK1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum. Human testicular germ cell tumor NEC8 cells and human umbilical cord endothelial ECV304 cells were cultured in RPMI-1640 medium supplemented with 10 % (v/v) fetal bovine serum. All media contained 100 units/ml penicillin and 100 μg/ml streptomycin. PC-12 cells were cultured on collagen type I-coated polystyrene plates (Iwaki/Asahi Glass, Tokyo, Japan), while the other cell lines were cultured on standard polystyrene plates (Iwaki/Asahi Glass). All cells were maintained at 37 °C in 5 % CO_2/95 % air.

Determination of the amino acid content of cells and culture media

Unless otherwise noted, 1×10^6 cells were seeded into six-well plates and cultured in 2 ml of medium. The following day, the medium was replaced with 1.2 ml of fresh medium and the cells were cultured for a further 24 h before extraction of amino acids. For examination of time-dependent accumulation of D-Asp during culture, 1×10^6 GH_3 cells or 2×10^5 HeLa cells were seeded into six-well plates and cultured in 2 ml of medium. The following day, the medium was replaced with 1.2 ml of fresh medium and the cells were cultured for a further 0, 1 or 2 days before extraction of amino acids from the cells and culture media. The amino acids in the cells and culture media were extracted using methanol, as described previously (Katane et al. 2013; Long et al. 1998) with some modifications. Specifically, the culture media were recovered and centrifuged at 300×*g* for 5 min at 4 °C to remove the cell debris. The supernatant (120 μl) was then mixed with 480 μl of 100 % (v/v) methanol in a 1.5 ml microtube and the mixture was incubated at −80 °C for 1 h to extract the amino acids. The cells on the plates were washed twice with ice-cold 10 mM phosphate-buffered saline (pH 7.4), collected with a cell scraper, and then transferred into a 1.5 ml microtube. Subsequently, the cells were mixed with 600 μl of 100 % (v/v) methanol and the mixture was sonicated in a water bath for 10 min, followed by incubation at −80 °C for 1 h to extract the amino acids. The methanol extracts from the cells and culture media were centrifuged at 10,000×*g* for 10 min at 4 °C to remove the precipitated proteins. The supernatant (500 μl) was then evaporated to dryness, and the residue was dissolved in 50 μl of 200 mM borate buffer (pH 10.2) or 400 mM borate buffer (pH 9.0) and filtered through a 0.45 μm Millex-LH filter (Millipore, Bedford, MA, USA). The filtered solution was diluted with the same buffer and stored at −80 °C until use.

The concentrations of D-Asp and L-Asp in the cells were determined by high-performance liquid chromatography (HPLC) using the OPA precolumn derivatization technique, as described previously (Nimura and Kinoshita 1986). An aliquot (10 μl) of the sample was prepared as described above, and then 30 μl of 200 mM borate buffer (pH 10.2) and 20 μl of OPA/NAC reagent (prepared by mixing 8 mg of OPA with 10 mg of NAC in 1 ml of 100 % [v/v] methanol) were added to fluorescently derivatize the amino acids in the sample. After incubation at room temperature for 2 min, a 10 μl aliquot of the sample was injected into a Jasco chromatographic system, which consisted of a model PU-2089 pump, a model FP-2025 fluorescence detector, and a model 807-IT integrator (Jasco Corp., Tokyo, Japan). The amino acids were separated on an octadecylsilyl silica gel column (TSK gel ODS-100Z, 250 × 4.6 mm internal diameter; Tosoh, Tokyo, Japan) at a flow rate of 1 ml/min, with a mobile phase of 15 mM phosphate buffer (pH 6.0):methanol (93:7 v/v). The fluorescence was detected at an excitation wavelength of 350 nm and an emission wavelength of 445 nm. The levels of D-Asp and L-Asp were determined based on the peak areas in the chromatograms. Under these conditions, D-Asp was eluted with a retention time of approximately 7 min (see Fig. 1). To confirm that this peak corresponded to D-Asp, recombinant human DDO was used to digest the product. Human DDO was expressed in *E. coli* and purified to near homogeneity as described previously (Katane et al. 2010). An aliquot (20 μl) of the sample was prepared as described above and then added to a solution comprising 40 mM sodium pyrophosphate buffer (pH 8.3), 60 μM flavin adenine dinucleotide, 0.033 μg/μl *A. niger* catalase, and 0.067 μg/μl purified recombinant human DDO in a final volume of 150 μl. After incubation at 37 °C for 6 h, 600 μl of 100 % (v/v) methanol was added to the mixture, which was incubated at −80 °C for 1 h and then subjected to centrifugation at 10,000×*g* for 10 min at

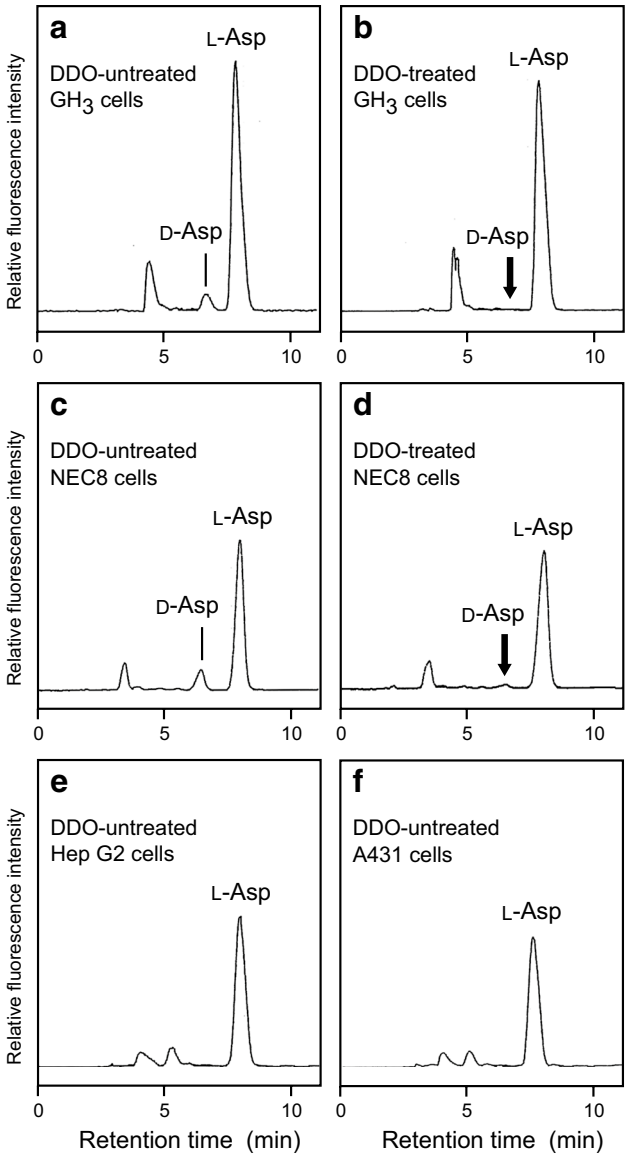


Fig. 1 Typical examples of the measurement of the D-Asp and L-Asp content of cultured rat and human cells by HPLC using the OPA/NAC precolumn derivatization technique. Chromatograms of cell samples prepared from untreated and DDO-treated GH_3 cells (**a**, **b**), untreated and DDO-treated NEC8 cells (**c**, **d**), untreated Hep G2 cells (**e**), and untreated A431 cells (**f**) are shown. Details of the analytical conditions are provided in the "Materials and methods". Peaks corresponding to D-Asp were detected in the samples prepared from GH_3 and NEC8 cells (**a**, **c**). When the samples were treated with DDO, these peaks disappeared almost completely (**b**, **d**), confirming the identity of D-Asp. D-Asp was not detected in Hep G2 (**e**) and A431 (**f**) cells

4 °C to remove the precipitated proteins. The supernatant (500 µl) was evaporated to dryness, and the residue was dissolved in 50 µl of 200 mM borate buffer (pH 10.2) and filtered through a 0.45 µm Millex-LH filter (Millipore). The filtered solution was used for HPLC analysis following the OPA/NAC derivatization method described above.

The concentrations of D-Asp and L-Asp in the cells and culture media were also determined by HPLC using another OPA precolumn derivatization technique, based on the method described by Hashimoto et al. (1992). Specifically, a 10 µl aliquot of the sample was prepared as described above, and then 30 µl of 400 mM borate buffer (pH 9.0) and 20 µl of OPA/Boc-L-cysteine reagent (prepared by mixing 10 mg of OPA with 10 mg of Boc-L-cysteine in 1 ml of 100 % [v/v] methanol) were added to fluorescently derivatize the amino acids in the sample. After incubation at room temperature for 2 min, a 10–20 µl aliquot of the sample was injected into the Jasco chromatographic system. The amino acids were separated on an octadecylsilyl silica gel column (Mightysil RP-18GP, 150 × 4.6 mm internal diameter; Kanto Chemical, Tokyo, Japan) at a flow rate of 1 ml/min. The column was first equilibrated with a 9:1 ratio of solvent A (200 mM sodium acetate buffer [pH 5.8 or 6.2]) to solvent B (100 % [v/v] acetonitrile). After injection, the sample was eluted for 20 min with a linear gradient (10–14 %) of solvent B. The fluorescence was detected at an excitation wavelength of 344 nm and an emission wavelength of 443 nm. The levels of D-Asp and L-Asp were determined based on the peak areas in the chromatograms. Under these conditions, D-Asp was eluted with a retention time of approximately 18 min (see Fig. 3). This peak was confirmed to be derived from D-Asp by specific degradation of D-Asp in the sample with DDO, as described above.

Real-time PCR analysis

For real-time PCR analyses, cells were cultured in 60 mm dishes or 6-well plates to near confluency. The cells were then collected and total RNAs were extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For first-strand cDNA synthesis, the RNA samples (5 µg) were reverse transcribed for 1 h at 50 °C in a 20 µl mixture containing 200 units of SuperScript™ III Reverse Transcriptase and 0.5 µg of oligo$(dT)_{12–18}$ primer (Invitrogen). Two units (1 µl) of RNaseH (Invitrogen) were then added and the sample was incubated at 37 °C for 20 min. A 5 µl aliquot of the first-strand cDNA was mixed with 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 2 µl (20 pmol) of forward primer, 2 µl (20 pmol) of reverse primer, and 1 µl of H_2O. The mixture was subjected to PCR amplification using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). The primers used were as follows: rat *Got1l1*, 5′-GAC TCA T CT TCC GGG ACA TGG GCT-3′ (forward) and 5′-GCC G GG ATA TGC TGT AGA ACT TCG A-3′ (reverse); rat *Ddo*, 5′-GAG ACG CCA CGG TTT CCC CG-3′ (forward) and 5′-GCC CCC GTC GCG GAT GAA AT-3′ (reverse);

human *GOT1L1*, 5′-GGG CAG AAG CGG AAC ACA G GA-3′ (forward) and 5′-CTT GTG GGC GAG GGG CAC A TC-3′ (reverse); and human *DDO*, 5′-TCC CTC CAC GGA G CC TGC AA-3′ (forward) and 5′-GCC TCT GTC CAT CTC GCG CA-3′ (reverse). The cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 66 °C for 10 s. Raw threshold cycle (C_t) values were determined using CFX Manager software (Bio-Rad Laboratories) and were used to calculate relative expression levels. The expression level of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*/*GAPDH*) mRNA was also determined as an internal control to compensate for variations in the efficiencies of the reverse transcriptions and PCRs. The primers used were as follows: rat *Gapdh*, 5′-TGC TGG GGC TGG CAT TGC TC-3′ (forward) and 5′-GGC CAT GAG GTC CAC CAC CC-3′ (reverse); and human *GAPDH*, 5′-AGC ACC CCT GGC CAA GGT CA-3′ (forward) and 5′-CCG GAG GGG CCA TCC ACA GT-3′ (reverse). The PCR products were confirmed by dye terminator cycle sequencing.

Construction of siRNA expression plasmids

Two sequences in the rat *Got1l1* gene (+587 to +605 and +669 to +687) were selected as targeting sequences for construction of the siRNA expression plasmids, and hairpin-type siRNA expression sequences were chemically synthesized (siGOT1L1-1 and siGOT1L1-2, respectively). Two scrambled siRNAs, which consisted of randomized siGOT1L1-1 or siGOT1L1-2 sequences (siGOT1L1-1-Ctrl and siGOT1L1-2-Ctrl, respectively), were also chemically synthesized and used as negative controls. The sequences of the siRNAs were as follows: siGOT1L1-1, 5′-GAT CCG GAC AAA GTT GAT GTC CAT CTG TGA AGC CAC AGA TGG GAT GGA CAT CAA CTT TGT CCT TTT TTA-3′ (top strand) and 5′-AGC TTA AAA AAG GAC AAA GTT GAT GTC CAT CCC ATC TGT GGC TTC ACA GAT GGA CAT CAA CTT TGT CCG-3′ (bottom strand); siGOT1L1-2, 5′-GAT CCG CCT TGA AGA AGA TAG CAA ACT GTG AAG CCA CAG ATG GGT TTG CTA TCT TCT TCA AGG CTT TTT TA-3′ (top strand) and 5′-AGC TTA AAA AAG CCT TGA AGA AGA TAG CAA ACC CAT CTG TGG CTT CAC AGT TTG CTA TCT TCT TCA AGG CG-3′ (bottom strand); siGOT1L1-1-Ctrl, 5′-GAT CCG GAT GAA TAG TCT GAC CAT CTG TGA AGC CAC AGA TGG GAT GGT CAG ACT ATT CAT CCT TTT TTA-3′ (top strand) and 5′-AGC TTA AAA AAG GAT GAA TAG TCT GAC CAT CCC ATC TGT GGC TTC ACA GAT GGT CAG ACT ATT CAT CCG-3′ (bottom strand); and siGOT1L1-2-Ctrl, 5′-GAT CCG CCT AGA ATA GAC GAG TAA ACT GTG AAG CCA CAG ATG GGT TTA CTC GTC TAT TCA AGG CTT TTT TA-3′ (top strand) and 5′-AGC TTA AAA AAG CCT TGA ATA GAC GAG TAA ACC CAT CTG TGG CTT CAC AGT TTA CTC GTC TAT TCT AGG CG-3′ (bottom strand). These sequences were designed to generate *Bam*HI and *Hin*dIII cohesive ends at the 5′- and 3′ sides of the top strands, respectively, when the top and bottom strands of each pair were annealed. Double-stranded oligonucleotides prepared by annealing the top and bottom strands of each pair were subcloned into pBAsi-mU6 Neo (Takara Bio, Shiga, Japan) to construct the rat GOT1L1-specific siRNA expression plasmids (pBAsi-mU6-GOT1L1-1 and pBAsi-mU6-GOT1L1-2) and the control siRNA expression plasmids (pBAsi-mU6-GOT1L1-1-Ctrl and pBAsi-mU6-GOT1L1-2-Ctrl).

Expression of siRNAs in cultured mammalian cells

To generate mammalian cell lines overexpressing rat GOT1L1-specific siRNA, pBAsi-mU6-GOT1L1-1 and pBAsi-mU6-GOT1L1-2 were transfected into GH_3 cells using the Agilent Mammalian Transfection Kit (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. The cells were selected for resistance to 800 μg/ml G418 and several drug-resistant cell clones were isolated. The efficiency and specificity of knockdown of the rat *Got1l1* gene were determined by real-time PCR as described above, as well as by electrophoretic separation of the PCR products on 2 % (v/w) agarose gels. Clones GH_3.si-1-20, GH_3.si-1-21, GH_3.si-2-10, and GH_3.si-2-11 displayed the most efficient and specific knockdown of the rat *Got1l1* gene and were used for further experiments. To prepare cell lines expressing the control siRNAs, pBAsi-mU6-GOT1L1-1-Ctrl and pBAsi-mU6-GOT1L1-2-Ctrl were transfected into GH_3 cells as described above. The cells were selected for resistance to 800 μg/ml G418, and two drug-resistant cell clones (GH_3.si-1-Ctrl and GH_3.si-2-Ctrl) were isolated and used for further experiments. The GH_3.si-1-20, GH_3.si-1-21, GH_3.si-2-10, GH_3.si-2-11, GH_3.si-1-Ctrl, and GH_3.si-2-Ctrl cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum and 800 μg/ml G418 at 37 °C in 5 % CO_2/95 % air.

Measurement of cell proliferation

To measure cell proliferation, GH_3, GH_3.si-1-20, GH_3.si-1-21, GH_3.si-1-Ctrl, GH_3.si-2-10, GH_3.si-2-11, and GH_3.si-2-Ctrl cells (5×10^3 cells) were seeded in 96-well plates and cultured in 100 μl of medium. The cells were cultured continuously for 1–6 days and cell proliferation was assessed on days 1, 2, 3, 4, and 6 using the Cell Count Reagent SF kit (Nacalai Tesque), according to the manufacturer's instructions.

Table 1 D-Asp and L-Asp levels in cultured rat cells

Cell line	D-Asp (pmol/well)	L-Asp (pmol/well)	*D* %
GH_3	155 ± 26	2,324 ± 408	6.2 ± 0.1
PC-12	111 ± 29	2,359 ± 165	4.5 ± 1.5
NRK-52E	52.5 ± 37.7	788 ± 45	6.2 ± 4.5

Data are represented as the mean ± standard deviation ($n = 3$). *D* % is the proportion of D-Asp to total Asp [D-Asp level/(D-Asp level + L-Asp level) × 100 %]

Statistical analysis

Data are represented as the mean ± standard deviation ($n = 3$). For evaluating the statistical significance of differences among the means, the data were analyzed by one-way analysis of variance followed by a Tukey–Kramer multiple comparison test; $P < 0.05$ was considered to be statistically significant.

Results

The relationships between the D-Asp content and the expression levels of *Got1l1* and *Ddo* mRNAs in cultured rat cells

To investigate the relationship between the D-Asp content and the expression of GOT1L1 in rat cells, the concentrations of D-Asp and L-Asp and the expression levels of the *Got1l1* and *Ddo* mRNAs were determined in several rat cell lines (GH_3, PC-12, and NRK-52E cells). The D-Asp and L-Asp content of the cells was determined by HPLC using the OPA/NAC precolumn derivatization technique (see "Materials and methods"). As expected, D-Asp was detected at a relatively high level in GH_3 cells (Fig. 1a; Table 1) and a slightly lower level in PC-12 cells (data not shown and Table 1), both of which are known to synthesize D-Asp (Long et al. 1998, 2000). D-Asp was also detected in NRK-52E cells, albeit at a much lower level than those in GH_3 and PC-12 cells (Table 1). L-Asp was detected in GH_3 and PC-12 cells at similar levels, and the L-Asp contents of these cells were approximately three times higher than that in the NRK-52E cells (Table 1). The proportion of D-Asp to total Asp (D-Asp level/[D-Asp level + L-Asp level] × 100 %) was similar in all three cell lines (4.5 ± 1.5 to 6.2 ± 0.1 %; Table 1).

The expression levels of the *Got1l1* and *Ddo* mRNAs in GH_3, PC-12, and NRK-52E cells were determined by real-time PCR. In contrast to the relative D-Asp contents of the three cell lines, the expression levels of the *Got1l1* and *Ddo* mRNAs were much higher in NRK-52E cells than GH_3 and PC-12 cells (Fig. 2a). The inverse correlation between *Ddo*

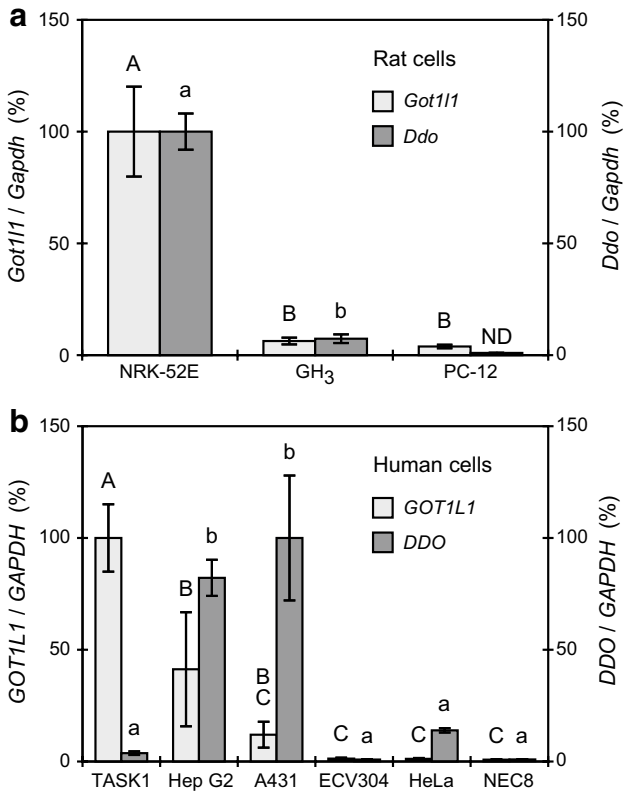


Fig. 2 Real-time PCR analyses of the levels of *Got1l1*/*GOT1L1* and *Ddo*/*DDO* mRNAs in cultured rat (**a**) and human (**b**) cells, normalized to those of the mRNA encoding GAPDH. The *Got1l1*/*GOT1L1* expression levels in each cell line are expressed relative to those in the NRK-52E (**a**) or the TASK1 (**b**) cell line. The *Ddo*/*DDO* expression levels are expressed relative to those in the NRK-52E (**a**) or A431 (**b**) cell lines. Data are represented as the mean ± standard deviation ($n = 3$). There are significant differences ($P < 0.05$) among *bars* with *different letters* (*capital* or *lower-case letters* in the *Got1l1*/*GOT1L1* and *Ddo*/*DDO* expression levels, respectively), based on a Tukey–Kramer multiple comparison test. *ND* not detected

mRNA expression and the D-Asp content of these cell lines is consistent with the known role of DDO in the catabolism of free D-Asp. However, these results do not support the hypothesis that GOT1L1 is the primary enzyme responsible for the biosynthesis of D-Asp in cultured rat cells.

The relationships between the D-Asp content and the expression level of *GOT1L1* and *DDO* mRNAs in cultured human cells

The D-Asp content and the expression levels of the *GOT1L1* and *DDO* mRNAs were also determined in several human cell lines (NEC8, ECV304, A431, TASK1, Hep G2, and HeLa) via HPLC and real-time PCR. D-Asp was detected in NEC8, ECV304, TASK1, and HeLa cells, but not in A431 or Hep G2 cells. HeLa cells contained the highest level of D-Asp, followed by NEC8, TASK1, and then ECV304

Table 2 D-Asp and L-Asp levels in cultured human cells

Cell line	D-Asp (pmol/well)	L-Asp (pmol/well)	*D* %
HeLa	234 ± 48	1,409 ± 360	14.3 ± 0.6
NEC8	227 ± 18	2,173 ± 83	9.5 ± 0.6
TASK1	48.4 ± 11.5	4,434 ± 366	1.1 ± 0.4
ECV304	33.6 ± 9.1	527 ± 58	6.1 ± 2.0
Hep G2	ND	1,687 ± 98	ND
A431	ND	647 ± 92	ND

Data are represented as the mean ± standard deviation ($n = 3$). *D* % is the proportion of D-Asp to total Asp [D-Asp level/(D-Asp level + L-Asp level) × 100 %]

ND not detected

cells (Table 2). The L-Asp contents of the cell lines also varied widely, resulting in substantial differences between the proportions of D-Asp to total Asp among the cell lines (Table 2).

The *GOT1L1* mRNA expression level was highest in TASK1 cells, followed by Hep G2 and then A431 cells (Fig. 2b). Expression of this mRNA was extremely low in the ECV304, HeLa, and NEC8 cell lines (Fig. 2b), indicating a lack of correlation between the D-Asp content and *GOT1L1* mRNA expression in the human cells studied. On the other hand, *DDO* mRNA expression was highest in A431 and Hep G2 cells, markedly lower in HeLa and TASK1 cells, and extremely low in the other cell lines (Fig. 2b). Taken together, these data suggest that the undetectable D-Asp content of A431 and Hep G2 cells (Fig. 1e, f) is due to degradation of D-Asp by high levels of DDO, and that GOT1L1 is not the principal enzyme involved in D-Asp synthesis in human cells.

Biosynthesis of D-Asp in HeLa and GH_3 cells

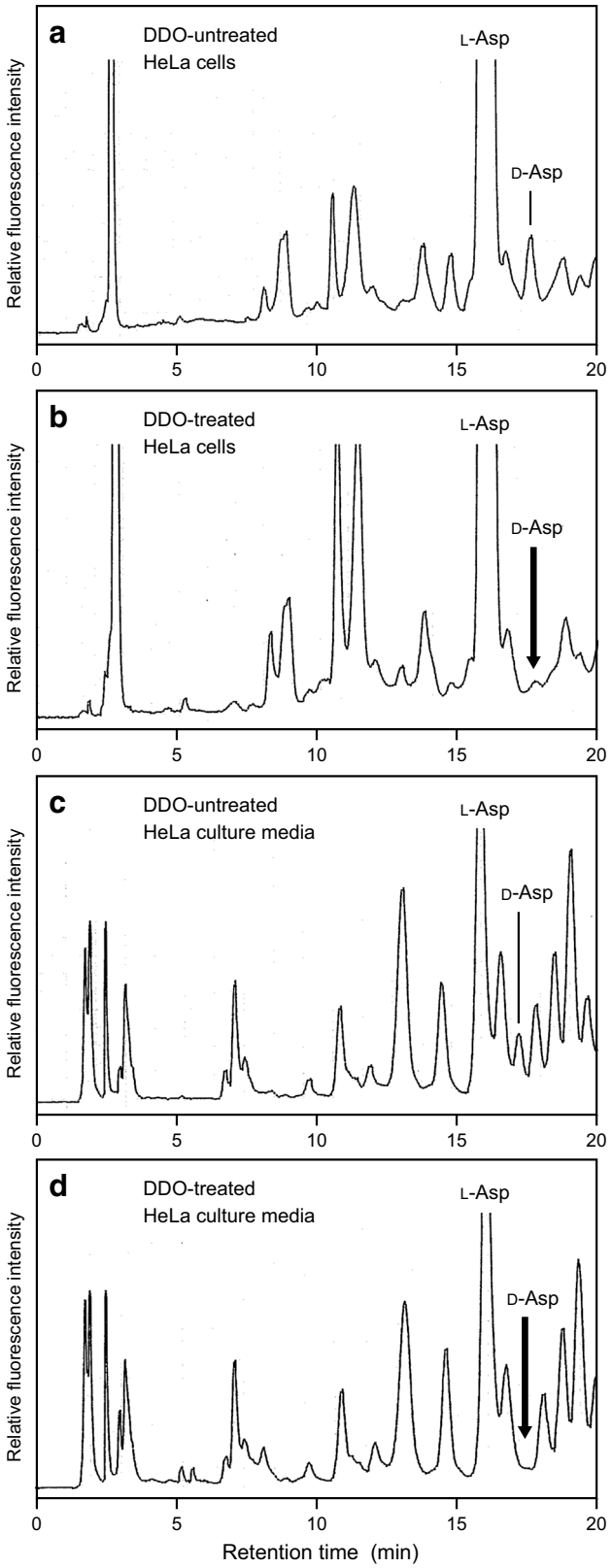
In our previous studies, we demonstrated that GH_3 and PC-12 cells are able to biosynthesize D-Asp (Long et al. 1998, 2000). To confirm and extend these findings, HPLC was employed to determine the D-Asp contents of both GH_3 and HeLa cells and the culture media in which they were grown (Fig. 3). In the HPLC analysis, the OPA/Boc-L-cysteine precolumn derivatization technique was used (see details in "Materials and methods"), since the D-Asp contents in the culture media were accurately determined by this method. As expected, the levels of D-Asp in both the cells and the culture medium increased progressively during culture of GH_3 cells (Fig. 4a). Since D-Asp was not added to the culture medium, this observation confirms that D-Asp is indeed synthesized in GH_3 cells, as described previously (Long et al. 2000). Similarly, the levels of D-Asp in both the cells and the culture medium increased during the culture of HeLa cells (Fig. 4b), confirming that D-Asp was also synthesized by this cell line. Since the expression level of *GOT1L1* mRNA in HeLa cells was lower than that in other human cell lines such as TASK1, Hep G2, and A431 (Fig. 2b), it is questionable whether GOT1L1 contributes to the biosynthesis of D-Asp in human cells.

The effect of *Got1l1* gene knockdown on the D-Asp content of GH_3 cells

To examine its potential involvement in D-Asp biosynthesis further, knockdown of the *Got1l1* gene in GH_3 cells was performed using two different siRNA expression plasmids. Hairpin-type siRNA expression sequences (siGOT1L1-1 and siGOT1L1-2) were designed to target two regions of the rat *Got1l1* gene (+587 to +605, and +669 to +687, respectively). In addition, scrambled control siRNA expression plasmids were also generated. The cells were stably transfected with the *Got1l1*-specific or scrambled control siRNA expression plasmids, and the expression level of *Got1l1* mRNA was determined in the clonal lines by real-time PCR. Four GH_3 clonal lines (GH_3.si-1-20, GH_3.si-1-21, GH_3.si-2-10, and GH_3.si-2-11) with significantly lower *Got1l1* mRNA expression levels than those in the wild-type cell line and two control clonal lines (GH_3.si-1-Ctrl and GH_3.si-2-Ctrl) with *Got1l1* mRNA levels similar to those in the wild-type cell line were established (Fig. 5a).

The proliferative capacities of the GH_3.si-1-20, GH_3.si-1-21, GH_3.si-2-10, and GH_3.si-2-11 cells were then assessed and compared with those of the GH_3, GH_3.si-1-Ctrl, and GH_3.si-2-Ctrl cells. The results indicated that knockdown of *Got1l1* had no effect on proliferation of the GH_3 cells (data not shown). Next, the D-Asp contents of these cells and their culture media were determined. Interestingly, the D-Asp contents of the *Got1l1*-knockdown GH_3 cells were significantly higher than those of wild-type and control siRNA-treated GH_3 cells (Fig. 5b). Conversely, the D-Asp levels in the culture media of the *Got1l1*-knockdown cells were significantly lower than those of the wild-type and control siRNA-treated cells (Fig. 5c). In fact, the D-Asp level in the media of the knockdown cells was 34.4 ± 7.7 to 83.4 ± 29.4 pmol/well (Fig. 5c), whereas the level in fresh culture medium (before culture) was 226 ± 21 pmol/well (mean ± standard deviation, $n = 3$). The total D-Asp contents of the *Got1l1*-knockdown cells (D-Asp in the cells plus D-Asp in the media) were equal to or only slightly but significantly higher than those of the wild-type and control cells (Fig. 5d), suggesting that knockdown of *Got1l1* did not reduce D-Asp synthesis in the GH_3 cells.

The L-Asp contents of the wild-type and siRNA-treated GH_3 cells and their culture media were also determined. The L-Asp contents of the GH_3.si-1-20 and GH_3.si-1-21 cells were equal to or slightly but significantly lower than those of the wild-type and corresponding control



◀ **Fig. 3** Typical examples of the measurement of D-Asp and L-Asp contents of HeLa cells and their culture media by HPLC using the OPA/Boc-L-cysteine precolumn derivatization technique. Chromatograms of samples prepared from HeLa cells (**a**, **b**) and their culture media (**c**, **d**) are shown. Details of the analytical conditions are provided in "Materials and methods". Peaks corresponding to D-Asp were detected in the samples (**a**, **c**). When the samples were treated with DDO, these peaks disappeared almost completely (**b**, **d**), confirming the identity of D-Asp

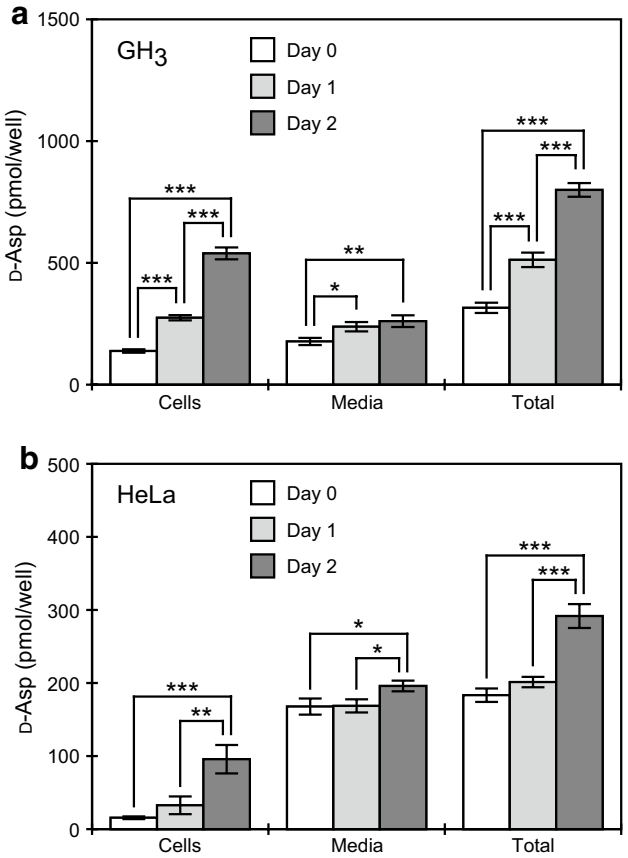


Fig. 4 Time-dependent changes in the D-Asp contents of GH_3 (**a**) and HeLa (**b**) cells and their culture media. GH_3 (1×10^6) and HeLa (2×10^5) cells were seeded into six-well plates and cultured as described in "Materials and methods". The following day, the medium was replaced and the cells were cultured for 0, 1 or 2 days. The total D-Asp content of the cells and media is also shown for each cell line. Data are represented as the mean ± standard deviation ($n = 3$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, based on a Tukey–Kramer multiple comparison test

siRNA-treated cells, while the L-Asp contents of the GH_3.si-2-10 and GH_3.si-2-11 cells were significantly higher than those in the wild-type and corresponding control siRNA-treated cells (Fig. 6a). By contrast, the L-Asp levels in the culture media of all of the *Got1l1*-knockdown cells were significantly lower than those in the culture media of the wild-type and control siRNA-treated cells (Fig. 6b). In fact, the L-Asp level in the media of the knockdown cells was 0.55 ± 0.10 to 1.93 ± 0.11 nmol/well (Fig. 6b), while the level in fresh culture medium was 11.7 ± 0.4 nmol/well (mean ± standard deviation, $n = 3$). The total L-Asp

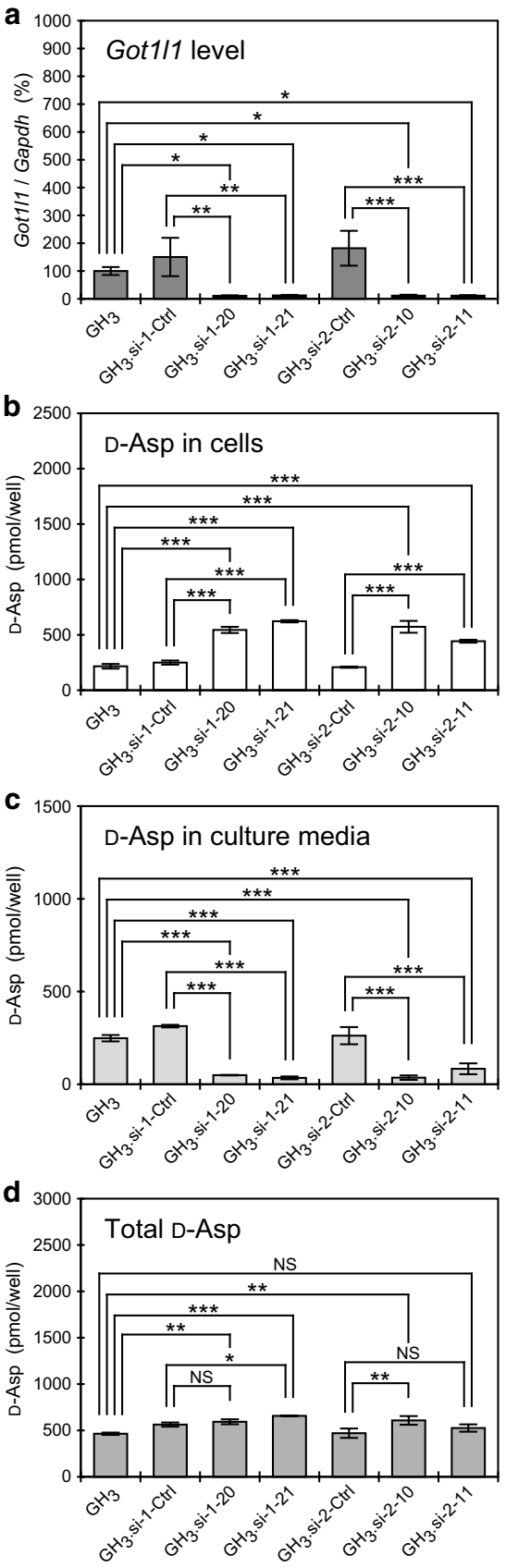


Fig. 5 The effect of *Got1l1* gene knockdown on D-Asp synthesis by ▶ GH_3 cells. **a** Real-time PCR analyses of *Got1l1* mRNA levels, normalized to those of the mRNA encoding GAPDH, in wild-type GH_3 cells and GH_3 cells treated with *Got1l1*-specific or scrambled control siRNAs. The *Got1l1* expression levels in each cell are expressed relative to that in wild-type GH_3 cells. Data are represented as the mean ± standard deviation ($n = 3$). **b–d** HPLC analyses of D-Asp synthesis by wild-type GH_3 cells and GH_3 cells treated with *Got1l1*-specific or scrambled control siRNAs. The cells (1×10^6) were seeded into six-well plates and cultured as described in "Materials and methods". The following day, the medium was replaced and the cells were cultured for 24 h. The D-Asp contents of the cells (**b**) and the culture medium (**c**), and the total D-Asp content (D-Asp contents in the cells plus media) (**d**) are shown. Data are represented as the mean ± standard deviation ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, based on a Tukey–Kramer multiple comparison test. *NS* not significant ($P > 0.05$)

contents of the *Got1l1*-knockdown cells (L-Asp in the cells plus L-Asp in the media) were equal to or significantly lower than those of the wild-type and control siRNA-treated cells (Fig. 6c).

Discussion

The aim of this study was to determine whether the rat and human homologs of murine Asp racemase (GOT1L1) are involved in the biosynthesis of D-Asp. First, the relationship between the cellular D-Asp content and the expression level of the mRNA encoding GOT1L1 was determined in several rat and human cell lines. The expression levels of DDO, the enzyme responsible for catabolism of D-Asp in mammals (Katane and Homma 2010; Ohide et al. 2011), were also examined. The data presented here suggest that GOT1L1 is not primarily involved in the biosynthesis of D-Asp in rat and human cells. For example, the ratio of the D-Asp contents of TASK1 and HeLa cells was approximately 1:5 (Table 2), whereas the corresponding ratios of the expression levels of *GOT1L1* and *DDO* mRNAs were >100:1 and approximately 1:4, respectively (Fig. 2b), suggesting that the D-Asp content is not related to the expression level of GOT1L1, even if the expression level of DDO is considered. Likewise, D-Asp contents were of almost no relevance to the expression levels of GOT1L1 in the other rat and human cell lines examined.

To examine the contribution of GOT1L1 to the biosynthesis of D-Asp further, we determined the effect of *Got1l1* gene knockdown on the D-Asp contents of GH_3 cells, which are known to synthesize D-Asp (Fig. 4a; Long et al. 2000). Knockdown of *Got1l1* did not reduce the total D-Asp content of the cells and their culture medium (Fig. 5d), confirming that GOT1L1 contributes little, if at all, to the biosynthesis of D-Asp in rat cells and potentially human cells.

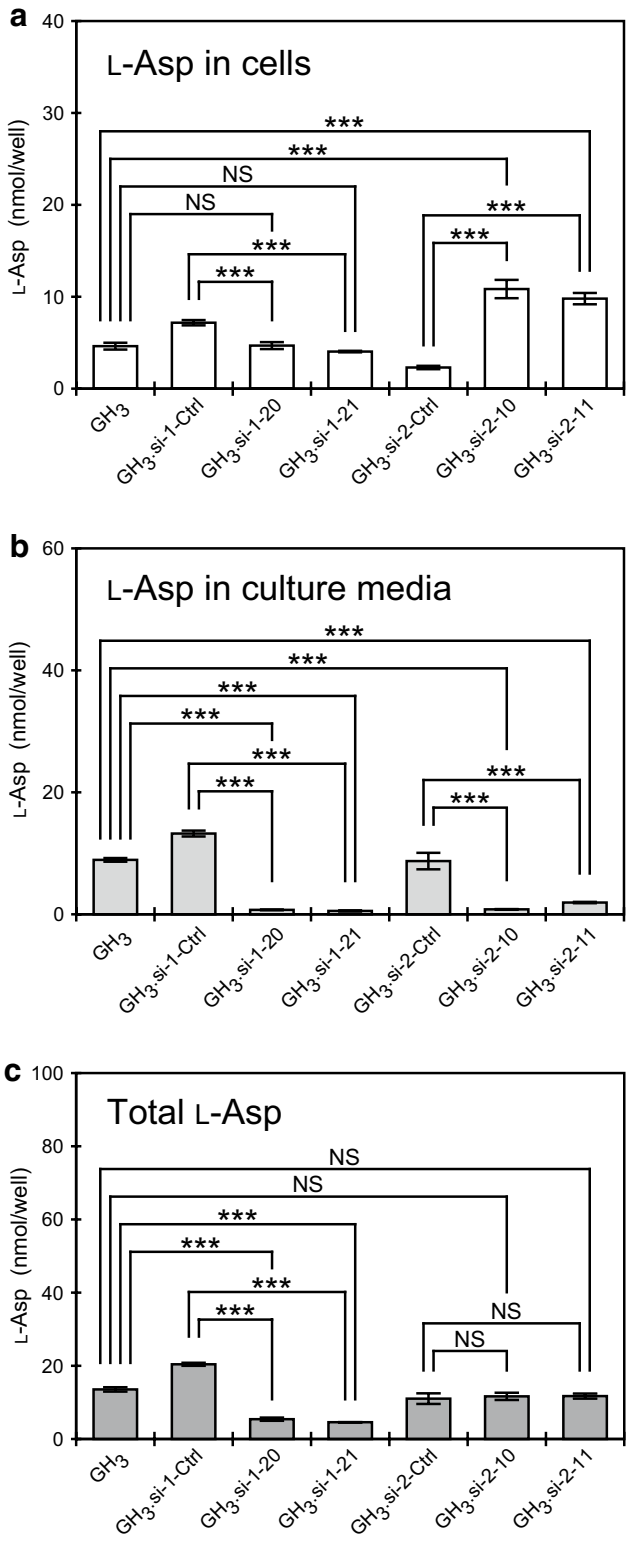


Fig. 6 The effect of *Got1l1* gene knockdown on L-Asp synthesis by GH_3 cells. HPLC analyses of L-Asp synthesis by wild-type GH_3 cells and GH_3 cells treated with *Got1l1*-specific or scrambled control siRNAs. The cells (1×10^6) were seeded into six-well plates and cultured as described in "Materials and methods". The following day, the medium was replaced and the cells were cultured for 24 h. The L-Asp contents of the cells (**a**) and the culture media (**b**), and the total L-Asp content (L-Asp contents in the cells plus media) (**c**) are shown. Data are represented as the mean ± standard deviation ($n = 3$). ***$P < 0.001$, based on a Tukey–Kramer multiple comparison test. *NS*, not significant ($P > 0.05$)

The molecular functions of the rat and human GOT1L1 proteins are currently unknown. These enzymes show moderate amino acid sequence identity with rat (38.1 %) and human (40.3 %) GOT1; therefore, they may function as isoforms of GOT1. The D-Asp and L-Asp levels in the culture media were reduced significantly by knockdown of *Got1l1* in GH_3 cells (Figs. 5c, 6b), suggesting that uptake of D-Asp and L-Asp was enhanced by *Got1l1* knockdown. Extracellular D-Asp and L-Asp can be taken up into cells via L-Glu transporters, and the affinities of these transporters for these amino acids are similar to that for L-Glu (Arriza et al. 1997; Kanai and Hediger 1992; Pine et al. 1992). It is unclear whether knockdown of GOT1L1 activity upregulates transport of D-Asp and L-Asp by the L-Glu transporter, and additional studies are required to understand the physiological roles of GOT1L1 in mammalian cells fully.

In conclusion, the results reported in this study suggest that the rat and human homologs of mouse GOT1L1 contribute little, if at all, to the biosynthesis of D-Asp. The biosynthetic pathway of D-Asp in the mammalian body is still an urgent issue to be resolved.

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Conflict of interest The authors declare that they have no conflict of interest.

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