Non-Involvement of the Human Monocarboxylic Acid Transporter 1 (MCT1) in the Transport of Phenolic Acid

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Phenolic acids such as p-coumaric acid and microbial metabolites of poorly absorbed polyphenols are absorbed by the monocarboxylic acid transporter (MCT)-mediated transport system which is identical to the fluorescein/H⁺ cotransport system. We focus here on the physiological impact of MCT-mediated absorption and distribution. We examined whether MCT1, the best-characterized isoform found in almost all tissues, is involved in this MCT-mediated transport system. The induction of MCT1 expression in Caco-2 cells by a treatment with sodium butyrate (NaBut) did not increase the fluorescein permeability. Moreover, the transfection of Caco-2 cells with an expression vector encoding MCT1 caused no increase in either the permeability or uptake of fluorescein. Furthermore, in the MCT1-expressing oocytes, no increase of p-coumaric acid uptake was apparent, whereas the uptake of salicylic acid, a substrate of MCT1, nearly doubled. Our data therefore establish that MCT1 was not involved in the MCT-mediated transport of phenolic acids.

Key words: fluorescein; phenolic acid; monocarboxylic acid transporter (MCT); MCT1

Research over the past decade into the human health effects of dietary polyphenols, especially flavonoids, has established a causal link with a reduction in cardiovascular diseases.¹ However, the biological activity of polyphenols must be reassessed in the light of new data concerning their bioavailability.² The absorption efficiency of polyphenols in humans is generally low, and the physiological significance of microbial metabolites of the parent polyphenols has been addressed. Hitherto, the partition coefficient (log octanol/water) of polyphenols has been considered to govern their absorption characteristics (i.e. transcellular passive diffusion was presumed to be the main mechanism).

Unlike flavonoids, phenolic acids have not been extensively studied and are not considered to be of great nutritional interest. Phenolic acids are present in many foods including grains, vegetables and fruits.³ We have recently elucidated the absorption characteristics of several phenolic acids (i.e. ferulic, p-coumaric, gallic and caffeic acids, and artepillin C) and related compounds (i.e. chlorogenic and rosmarinic acids) in terms of their affinity for the monocarboxylic acid transporter (MCT), and have demonstrated the diverse pathways of absorption for phenolic compounds in Caco-2 cells (i.e. MCT-mediated absorption, partial MCT-mediated absorption, paracellular diffusion, and transcellular passive diffusion).⁴–⁸ We have also established that the absorption characteristics of these compounds in the Caco-2 cell line are correlated with their absorption efficiency and bioavailability in vivo.⁹–¹¹ Furthermore, the microbial metabolites of poorly absorbed parent polyphenols are also thought to be absorbed and distributed by MCT, in a similar way to phenolic acids.¹²,¹³ These observations highlight the physiological impact of MCT-mediated absorption and distribution in humans, which involves specific transport systems that act not only on phenolic acids but also on microbial metabolites of poorly absorbed polyphenols or dietary fibers having biological activity, which are referred to as "metabo-nutrients."¹³

To date, 14 isoforms of MCT have been identified,¹⁴ but only MCT1 to MCT4 have been characterized in terms of their substrate and inhibitor kinetics. Each MCT isoform is likely to have a unique biological role which is related to the different tissue distribution. MCT1, the most well-characterized isoform, is found in almost all tissues of the human body (i.e. heart, skeletal muscle, small intestine, colon, liver, brain, spinal cord, testis, ovary, placenta and adrenal gland),¹⁵ which

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Abbreviations: MCT, monocarboxylic acid transporter; ECD, electrochemical detector; NaBut, sodium butyrate; CHC, α-cyano-4-hydroxycinnamic acid
would imply a physiological significance for humans. The aim of this study is to investigate whether the MCT1 isoform is involved in the transport of phenolic acids and "metabo-nutrients."[13]

Materials and Methods

Materials. The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Fetal calf serum, glutamine, non-essential amino acids, penicillin (10,000 units/ml in 0.9% NaCl), streptomycin (10 mg/ml in 0.9% NaCl), phosphate-buffered saline and Hank’s balanced salt solution (HBSS) were all purchased from Invitrogen Corp. (Carlsbad, CA, USA). Type I collagen was purchased from Nitta Gelatin (Osaka, Japan). Plastic dishes, plates and Transwell inserts were obtained from Corning (Corning, NY, USA). Fluorescein, p-coumaric acid, salicylic acid and sodium butyrate (NaBut) were from Sigma (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade.

Cell culture. The Caco-2 cells were routinely maintained in a humidified atmosphere of 5% CO2 at 37 °C in DMEM containing 10% fetal calf serum, 1% nonessential amino acids, 4 mM L-glutamine, 50 U/ml of penicillin and 50 μg/ml of streptomycin (pH 7.4). All the cells used were between passages 40 and 60. Upon reaching confluency, the culture was maintained for 7 days before adding NaBut to a final concentration 10 mM in the culture medium, this being re-supplemented daily for a further 2 days.

Western blot analysis. The cells were harvested and suspended in a lysis buffer (20 mM Tris–HCl at pH 7.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 1 μM pepstatin, and 5 mM EDTA) and Triton X-100 (1% final concentration). The suspension was incubated for 20 min on ice, and the soluble fraction was separated by centrifugation at 20,000 × g for 10 min at 4 °C. An aliquot of the solubilized protein fraction was separated by SDS–PAGE. The proteins were transferred on to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), which were then blocked by incubating with 5% (w/v) dried skimmed milk in TBS-T (20 mM Tris–HCl, 500 mM NaCl, and 0.1% Tween-20 at pH 7.5). The blots were probed with the anti-MCT1 polyclonal antibody (Biogenesis, NH, USA), then by the anti-mouse secondary antibody (Amersham Biosciences, UK) and developed by ECL®-Plus (Amersham Biosciences). The intensity of the bands was quantified by using an LAS 1000 plus luminescent image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Transcellular transport and uptake experiments in Caco-2 cells transfected with NaBut. The cells were seeded at a density of 1 × 105/cm2 and grown in Transwell inserts with a semipermeable membrane coated with type I collagen, as previously reported.[4-8,12,13] The integrity of the cell layer was evaluated by measuring the transepithelial electrical resistance (TER) with Millicell-ERS equipment (Millipore, Billerica, MA, USA). A monolayer with a TER value of more than 300 Ω·cm2 was used for the transepithelial transport experiments. To measure the permeability in the apical-to-basolateral direction, 2.6 ml of HBSS (pH 7.4, 37 °C) was added to the basal chamber of a 6-well Transwell insert and then 1.5 ml of the test solution (pH 6.0, 37 °C) containing fluorescein (7 nm) was added to the apical side. After 40 min of incubation at 37 °C, the basal solution was collected, and then the amount of fluorescein transported by the Caco-2 cells was determined with an F-4500 fluorescence spectrophotometer (HITACHI, Tokyo, Japan) at an excitation wavelength of 490 nm and emission wavelength of 514 nm. The results are expressed in terms of the permeation rate (nmol/min·cm2).

Construction of the human MCT1 (hMCT1) expression vector. The full-length hMCT1 cDNA fragment was amplified from Caco-2 cell total RNA by RT-PCR. The fragment containing the coding region was digested with Xhol and NotI, gel isolated, and subcloned into the mammalian expression vector (pME18S) at the corresponding restriction sites. The construct, pME18S/hMCT1, was confirmed by DNA sequencing.

Transfection of the Caco-2 cells. The cells were transfected with pME18S/hMCT1 by using the Polyfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Briefly, Caco-2 cells were cultured for 7 days after the monolayer had become confluent. A 2.5-μl amount of DNA was gently mixed with 150 μl of a serum-free medium and 15 μl of the Polyfect transfection reagent, and the mixture was incubated at room temperature for 10 min. 0.8-ml volume of a serum-containing medium was then added to the mixture which was transferred to each well. After a 48-h incubation at 37 °C, the cells were used for uptake experiments. The expression of hMCT1 mRNA was confirmed by RT-PCR with hMCT1-specific primers (forward primer, 5′-acactcgagatgccaccagcagttgg-3′; reverse primer, 5′-ataagggccgctgactggaccttctcttcc-3′). The cycle parameters were as follows: denaturing at 94 °C for 1.5 min, and then 30 cycles of 94 °C denaturing for 40 s, 60 °C annealing for 50 s, and 72 °C extension for 1 min.

Transcellular transport and uptake experiments in Caco-2 cells transfected with human MCT1. After the Caco-2 cells had reached confluence (1 × 105 cells/cm2) in 12-well Transwell inserts, the culture was continued for 7 days. pME18S/hMCT1 or pME18S
Oocytes and injections. Xenopus laevis females were purchased from Copacetic (Aomori, Japan). Oocytes were isolated by a collagenase treatment as described previously. The oocytes were microinjected with either 41 nl of water or MCT1 cRNA at a concentration (0.5 mM) at 37°C incubation in HBSS (pH 6.0) containing fluorescein (0.5 mM) at pH 7.0) before incubating in a test solution containing HEPES at pH 7.0. The fluorescein uptake was quantified by extracting the cells with methanol/50 mM sodium acetate containing 5% methanol (pH 3.0; 10:1, v/v) for 10 min. The amount of fluorescein in the extract was then determined by fluorescence spectrophotometer as already described.

Uptake experiments on oocytes expressing human MCT1 (hMCT1). For each determinant, groups of 2 oocytes injected with cRNA or water were washed twice with 3 ml of OR2+ (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, and 5 mM HEPES at pH 7.0) before incubating in a test solution containing p-coumaric or salicylic acid (1 mM) at room temperature. After incubating for 15 min, the oocytes were washed three times with 3 ml each of ice-cold OR2+, and then were sonicated and extracted with methanol/50 mM sodium acetate containing 5% methanol (pH 3.0)/10% SDS (5:4:1, v/v) for 10 min. The amount of p-coumaric or salicylic acid in this extract was estimated by using HPLC-EC detection equipment fitted with an ESA coulometric detection system (ESA, Boston, MA, USA) as described in previous studies. In brief, to measure p-coumaric acid, chromatographic separation was performed in a C18 column (ODS150, MC Medical, Tokyo, Japan) with mobile phase A (solvent A) of 50 mM sodium acetate containing 5% methanol (pH 3.0) and mobile phase B (solvent B) of 50 mM sodium acetate containing 40% acetonitrile and 20% methanol (pH 3.5). Eight electrode detector potentials (from 0 to 700 mV in increments of 100 mV) were used. The level of p-coumaric acid and salicylic acids were respectively 14.7 min and 600 mV, and 19.4 min and 800 mV (Figs. 3A and B). The peak (RT of about 9 min) is considered to have originated from an unknown compound derived from oocytes, because that peak could not be observed in the chromatograms of authentic p-coumaric and salicylic acids. The uptake rate of salicylic acid, a substrate of MCT1, increased significantly in response to the injection of MCT1 encoding cRNA (Table 1A). Furthermore, the increase in salicylic acid uptake in oocytes injected with cRNA encoding MCT1 was inhibited by α-cyano-4-hydroxycinnamic acid (CHC), a specific inhibitor of MCT1 (Table 1B). In contrast, the uptake of p-coumaric acid was no different between the control and MCT1-expressing oocytes (Table 1A).

Discussion

We have reported in a previous study that fluorescein...
was transported by intestinal MCT in Caco-2 cells, and established that measuring the competitive inhibition of fluorescein transport in this cell line by various substances would be a useful means of identifying compounds that are transported by MCT.\textsuperscript{17,19} MCT-mediated transport of phenolic acids was first determined by this method.\textsuperscript{4–6} The MCT isoform responsible for fluorescein transport is presumed to be identical to that responsible for the transport of phenolic acids in Caco-2 cells. It has also been reported that nateglinide, a novel oral hypoglycemic agent, was taken up by the intestinal fluorescein/H\textsuperscript{+} cotransport system in Caco-2

![Diagram A](image)

![Diagram B](image)

![Diagram C](image)

**Fig. 1.** Permeability of Fluorescein in Caco-2 Cells Up-Regulated with Human MCT1 mRNA by a Treatment with NaBut.
A, Caco-2 cell monolayers were pre-incubated with 10 mM NaBut or a vehicle for 48 h and then subjected to total RNA extraction. RT-PCR was performed with hMCT1 and \( \beta \)-actin specific primers. B, Quantitative analysis of hMCT1 mRNA expression. Densitometric data for the signals were quantified by using Scion Image and normalized to the \( \beta \)-actin signals. C, Confirmation of NaBut-induced expression of hMCT1. A western blot analysis was performed with the anti-MCT1 antibody. D, Permeability of fluorescein across Caco-2 cell monolayers pre-treated with 10 mM NaBut (filled bar) or a vehicle (unfilled bar) for 48 h. *\( P < 0.01; \) for control versus 10 mM NaBut. Each column presents the mean \( \pm \) SD of three experiments. hMCT1, human MCT1.

![Diagram D](image)

**Fig. 2.** Permeability and Uptake of Fluorescein in Caco-2 Cells Transfected with Human MCT1.
A, Caco-2 cell monolayers were transfected with pME18S/hMCT1 or pME18S and then subjected to total RNA extraction. RT-PCR was performed with hMCT1 and \( \beta \)-actin specific primers. B, Quantitative analysis of hMCT1 mRNA expression. Densitometric data for the signals were quantified by using Scion Image and normalized to the \( \beta \)-actin signals. C, Confirmation of NaBut-induced expression of hMCT1. A western blot analysis was performed with the anti-MCT1 antibody. D, Permeability of fluorescein across Caco-2 cell monolayers pre-treated with 10 mM NaBut (filled bar) or a vehicle (unfilled bar) for 48 h. *\( P < 0.01; \) for control versus 10 mM NaBut. Each column presents the mean \( \pm \) SD of three experiments. hMCT1, human MCT1.
was apparent. These results suggest the fluorescein/H uptake profile of salicylic acid. A significant (2-fold) increase in salicylic acid uptake by MCT1-expressing oocytes was observed (Table 1). Furthermore, the addition of CHC, a typical MCT1 inhibitor, reduced the uptake of salicylic acid to the same level as that of the control oocytes (Table 1). In contrast, no increase in the uptake of p-coumaric acid was apparent in MCT1 expressing oocytes (Table 1). This, together with the results obtained for the Caco-2 cells, clearly indicates that MCT1 did not participate in the MCT-mediated transport of phenolic acids.

Transepithelial transport studies of fluorescein or phenolic acid (e.g. ferulic acid or p-coumaric acid) have shown lactic acid, which is a typical substrate for MCT1–MCT4, to have no effect on their transport. Further studies are required to establish the physiological impact of the MCT-mediated absorption and distribution in humans involving specific transport systems not only for phenolic acids, but also for microbial metabolites of poorly absorbed polyphenols or dietary fibers. We have focused on the physiological roles of each MCT subtype. Further studies to characterize the various subtypes of MCTs participated in the absorption of phenolic acids in Caco-2 cells: MCT on the apical side appeared to be different from MCT1–MCT4. Thus, it was tentatively proposed that Caco-2 intestinal cells might possess a novel MCT responsible for phenolic acid transport which is distinct from MCT1–MCT4. The results described in this paper confirm this proposal. It has been reported that various subtypes of MCT such as MCT1, MCT3, MCT4, MCT5 and MCT6 were expressed in Caco-2 cells. Furthermore, it has recently been reported that different subtypes of MCTs participated in the absorption of phenolic acids in Caco-2 cells: MCT on the apical side appeared to be different from MCT on the basolateral side in terms of the affinity for Artepillin C. We have focused on the physiological impact of the MCT-mediated absorption and distribution in humans involving specific transport systems not only for phenolic acids, but also for microbial metabolites of poorly absorbed polyphenols or dietary fibers.

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References