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Presence or absence of a gallate moiety on catechins affects their cellular transport

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Abstract

The accumulation of (–)-epicatechin (EC), a non-gallate catechin, was significantly lower than that of (–)-epicatechin gallate (ECG), a gallate catechin, in Caco-2 cells. Using Caco-2 cell monolayers cultured in transwells, the transport of catechins in the basolateral-to-apical direction was much higher than that in the apical-to-basolateral direction, suggesting the involvement of an efflux transporter. Moreover, the results suggest that involvement of a transporter in EC efflux is greater than that for ECG. Treatment with transporter inhibitors MK571, quinidine or mitoxantrone, which inhibit MRP2, P-glycoprotein (P-gp) and BCRP, respectively, led to an increase in the accumulation of EC into Caco-2 cells and a decrease in the P_{app} ratio ($P_{app\ B\rightarrow A}/P_{app\ A\rightarrow B}$) for EC. These transporters seemed to be involved in EC efflux. BCRP was not an efflux transporter for ECG, and the influences of MRP2 and P-gp on ECG efflux were lower than for EC. Thus, efflux transporters appear to be responsible for the difference in cellular accumulation of EC versus ECG, suggesting that the presence or absence of a gallate moiety in the catechin structure influences the transporters.

Introduction

The major flavonoids of green tea, a beverage popular in many countries, are catechins. Consumption of catechins has been reported to have a variety of beneficial effects, including antioxidative, anti-tumour, anti-mutagenic and antihypertensive activity (Artursson & Karlsson 1991; Katiyar & Mukhtar 1997; Duffy et al 2001; Young et al 2002; Konishi & Shimizu 2003; Koo & Cho 2004). A cup of green tea contains 100–150 mg of catechins, consisting of 50% (−)-epigallocatechin gallate (EGCG), 15% (−)-epicatechin gallate (ECG), 15% (−)-epigallocatechin (EGC) and 8% (−)-epicatechin (EC). The daily intake of catechins is estimated to be several hundred milligrams in the case of people who drink green tea regularly, such as the Japanese (Nakagawa et al 1997; Tatsumura et al 2002). Determining the efficiency of intestinal absorption of catechins is important to assess their potential health benefits. The rate of intestinal absorption of catechins seems to be extremely low; for example, in one human study, only 2% of the ingested amount was detectable in blood after oral administration of 100 mg EGCG (Nakagawa et al 1997, 1999; Tatsumura et al 2002). However, few studies have addressed what factors may be responsible for the low oral bioavailability of catechins.

Various efflux transporters exist in the gastrointestinal tract, which is the first place of exposure to exogenous toxic substances after oral ingestion (Maliepaard et al 2001; Taipalensuu et al 2001; Albermann et al 2005; Takano et al 2006). Some reports have suggested that efflux transporters in the gastrointestinal tract may serve as a defence against toxic exogenous substances (Lechapt-Zalcman et al 1997; Bodo et al 2003; Schwab et al 2003; Sugihara et al 2006). The low oral bioavailability of catechins might be due to the presence of efflux transporters in the intestine.

The cellular accumulation of non-gallate catechins, such as EC, was reported to be very limited as compared with that of gallate catechins, such as ECG (Vaidyanathan & Walle 2003). It was considered that limited cellular EC content might be attributed to a lack of penetration of the apical membrane. However, EC that penetrated the apical membrane seemed to be more actively transported to the outside of the cell by efflux transporters than ECG. Some reports indicated that the efflux transport of EC was greater than that of ECG

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and the efflux of catechins decreased in the presence of MK-571, a competitive inhibitor of MRP2 transporter, in experiments using Caco-2 cells (Vaidyanathan & Walle 2001, 2003; Zhang et al 2004a). However, there have been no reports wherein the influence of MK571 was compared between EC and ECG in terms of differential cellular accumulation of EC versus ECG.

The difference in structure between EC and ECG is limited solely to the presence or absence of a gallate moiety (Figure 1). If transporters contribute to the low accumulation of EC, it may be that the presence or absence of a gallate moiety in the catechin structure influences the activity of transporters. The influence of other intestinal transporters, such as P-gp and BCRP, on efflux transport of catechins had not been studied simultaneously until now. Hence, there is little knowledge about which transporter influences the transport of catechins.

The Caco-2 cell, a human colonic adenocarcinoma cell line, is useful for in-vitro studies because it has many morphological and biochemical characteristics of human enterocytes. In this study using Caco-2 cells, the contribution of transporters to the difference in the cellular accumulation between EC and ECG was investigated in an experiment on the effects of various transporter inhibitors on the accumulation and the efflux transport of these catechins.

Materials and Methods

Materials

EC and ECG were purchased from Funakoshi Co. (Tokyo, Japan). MK571 and quinidine were obtained from Wako Pure Chemical Co. (Osaka, Japan). Mitoxantrone was purchased from LKT Laboratories (MA, USA).

Cell culture

Caco-2 cells in passage 40 were obtained from RIKEN (NO.RCB0988) and experiments were performed on cells in passages 43–63. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, 1% non-essential amino acids and penicillin–streptomycin–amphotericin B (Cambrex Bio Science Walkersville Inc, MD, USA) in an atmosphere of 5% CO_2–95% air at 37°C. Cells were seeded at a density of 2×10^5 cells/cm^2 on collagen-coated polycarbonate Petri dishes (35 mm diameter) and 5×10^4 cells/cm^2 on tissue culture-treated Transwell inserts (1.12 cm^2 growth area and 0.4 μm mean pore size polycarbonate membranes; Corning Costar Co., Cambridge, MA). Cells were incubated for 16–18 days after seeding and used for experiments.

Cellular accumulation of catechins

Before the addition of transporter inhibitors, cells were cultured in Petri dishes, rinsed twice with phosphate-buffered saline (PBS) and the culture medium was replaced with Hanks' balanced salt solution containing 25 mM HEPES and 25 mM glucose (HBSS buffer, pH 7.4). The cells were then treated by the addition of MK571, quinidine or mitoxantrone dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 μM or with DMSO alone as a control. EC or ECG was added to the medium at a final concentration of 50 μM 10 min after addition of the inhibitors. The cells were then incubated at 37°C for 3 h, washed in PBS and harvested in 1 mL 50 mM HCl–EtOH. After centrifugation at 10000 rev min^{-1} for 5 min, 100 μL of each supernatant was recovered and neutralized with 50 μL of Tris-HCl buffer 0.5 M, pH 7.4. Twenty-microlitre volumes of each neutralized supernatant were used for HPLC analysis.

The total amount of protein was determined by the method of Lowry et al (1951).

Transepithelial transport across Caco-2 cell monolayers

Transepithelial electric resistance (TEER) of Caco-2 cell monolayers cultured in a Transwell chamber was monitored before transport studies using a Millicell ERS testing device (Millipore, Bedford, MA, USA). Monolayers with a TEER of more than 250 Ω cm^{-2} were used in transport studies. The monolayers were treated by the addition of MK571, quinidine or

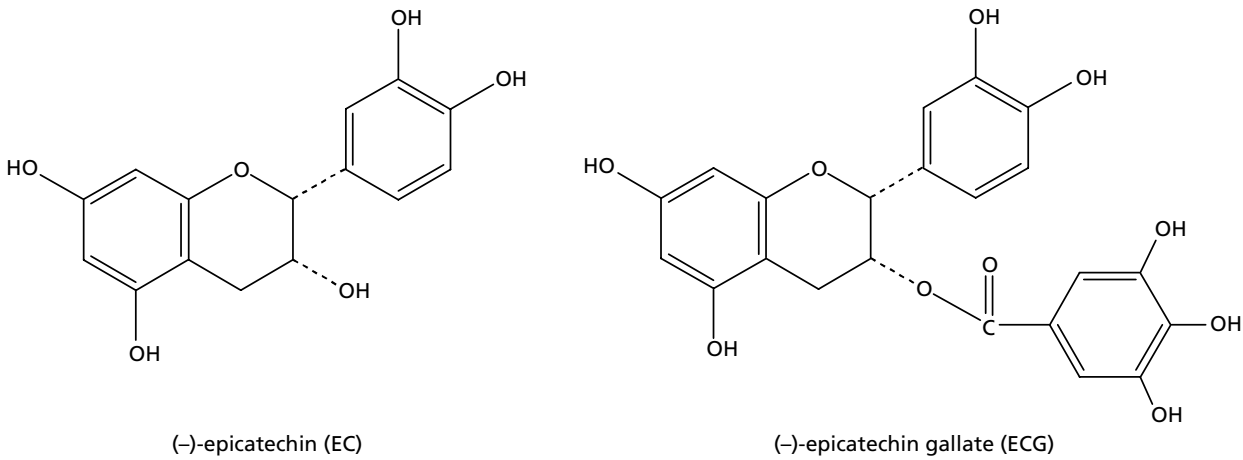

Figure 1 The structure of EC and ECG.

mitoxantrone at a concentration of 50 μM. Control monolayers were treated with DMSO. Before addition of transporter inhibitors and catechins, the monolayers were rinsed twice with PBS and re-suspended in HBSS buffer, pH 7.4. Catechins were placed either on the apical (0.5 mL) or basolateral (1.5 mL) side of the monolayer at a concentration of 50 μM and measured on the opposite side at 37°C periodically for 3 h. The transepithelial transport of catechins across cell monolayers was determined by HPLC analysis after preparation with 20 μL sample by the same method as the accumulation experiment.

The apparent permeability coefficient (P_{app}) values were calculated using the equation:

$$P_{app} = (dQ/dt)/(A \times C_0) \quad (1)$$

where dQ/dt is the Rho-123 permeation rate (mol s^{-1}), A is the filter/cell surface area (1.12 cm^2) and C_0 is the initial concentration of Rho-123 (mol mL^{-1}) (Tang et al 2004).

HPLC

A 20-μL volume of the sample was injected onto a 3.5-μm Symmetry C-18 semi-microcolumn (2.1 i.d. × 150 mm) adjusted to 30°C with a mixture of ethyl acetate (2%), acetonitrile (10%) and 0.5% phosphoric acid solution (88%) using a Waters Alliance HPLC System equipped with a 2690 Separation Module and a 2487 dual λ UV/VIS absorbance detector (Waters Co., Milford, MA, USA). The flow rate of the mobile phase was 0.2 mL min^{-1} and elution of catechins was detected based on UV absorption at 278 nm; EC or ECG was eluted at 9 and 10 min, respectively. Quantitations were based on the integration of absorbance peak areas.

Statistical analysis

The data are presented in the figures as the mean ± s.e.m. of five experiments. Differences among mean values were assessed by Dunnett's test using Stat-100 (BIOSOFT, UK) or Student's *t*-test. $P < 0.05$ was considered significant.

Results

Cellular accumulation of EC or ECG

The amount of catechins that accumulate into Caco-2 cells was assayed after 3 h of incubation with EC or ECG. EC, the non-gallate catechin, showed only limited accumulation; specifically, 0.067 nmol (mg protein)$^{-1}$, which is 0.17% of the total amount of EC added to the medium. In contrast, ECG, the gallate catechin, accumulated to the level of 2.31 nmol (mg protein)$^{-1}$, or 5.78% of the total amount of ECG added to the medium (Figure 2). Thus, cellular accumulation of ECG far exceeded accumulation of EC.

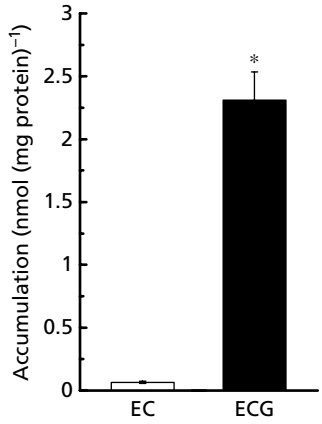

Figure 2 The cellular accumulation of EC or ECG in Caco-2 cells. Each catechin was added to medium replaced by an HBSS buffer at a concentration of 50 μM. After incubation at 37°C for 3 h, the cells were incubated and were harvested in 1 mL 50 mM HCl–EtOH. After centrifugation and neutralization, supernatants were analysed by HPLC. Each volume represents the mean ± s.e.m. of five trials. *$P < 0.05$ vs values of EC.

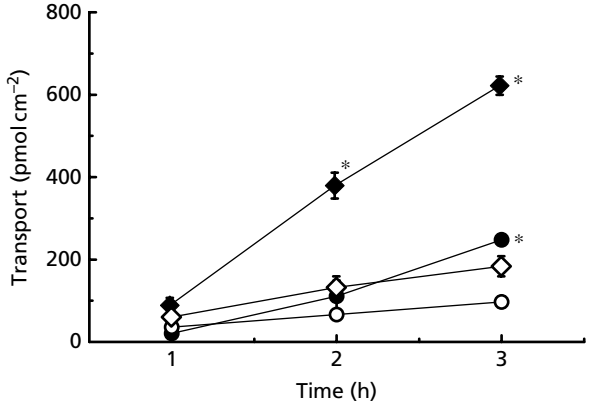

Figure 3 Transepithelial transport of catechins in the basolateral-to-apical direction (EC; ♦, ECG; ●) and in the apical-to-basolateral direction (EC; ◇, ECG; ○) across Caco-2 cell monolayers. Before the transport experiments, medium was replaced by an HBSS buffer. EC or ECG was added to medium at a concentration of 50 μM. Each value represents the mean ± s.e.m. of five trials. *$P < 0.05$, vs values of transport in the apical-to-basolateral direction.

Transepithelial transport of catechins across Caco-2 cell monolayers

Transport of both of EC and ECG in the basolateral-to-apical direction (B→A) was higher than that in the apical-to-basolateral direction (A→B; Figure 3). The P_{app} ratio ($P_{appB\to A}/P_{appA\to B}$), which is the ratio of efflux in the B→A direction versus the influx in the A→B direction, was determined. The P_{app} ratio of EC and ECG was 3.24 and 2.56, respectively, which suggests the involvement of an efflux transport for these catechins (Table 1). Although the ability of EC to penetrate the membrane appears to be greater than that of ECG, efflux transport of EC in the B→A direction was much greater than that of ECG.

Effect of transporter inhibitors on the accumulation of EC and ECG into Caco-2 cells

To determine the extent to which transporters are involved in efflux transport of EC and ECG, the effect of transporter inhibitors on the accumulation of the catechins into Caco-2

Table 1 The apparent permeability coefficient (P_{app}) of EC and ECG across Caco-2 cell monolayers in the absence or presence of MK571, quinidine or mitoxantrone

	EC P_{app} ($\times 10^{-7}$ (cm s^{-1}))			ECG P_{app} ($\times 10^{-7}$ (cm s^{-1}))		
	A→B	B→A	$P_{appB\rightarrow A}/P_{appA\rightarrow B}$	A→B	B→A	$P_{appB\rightarrow A}/P_{appA\rightarrow B}$
Control	3.53 ± 0.46	11.44 ± 0.41	3.24	1.79 ± 0.24	4.59 ± 0.15	2.56
MK571	6.56 ± 0.98*	6.57 ± 0.54*	1.00	2.64 ± 0.55	2.79 ± 0.30*	1.05
Quinidine	5.74 ± 1.08	5.99 ± 0.67*	1.04	3.26 ± 0.31*	2.45 ± 0.58*	0.75
Mitoxantrone	7.32 ± 0.88*	6.76 ± 1.29*	0.92	2.54 ± 0.34	5.78 ± 0.93	2.28

P_{app} was determined in the absence or presence of MK571 (50 μM), quinidine (50 μM) or mitoxantrone (50 μM). A→B is P_{app} in the apical-to-basolateral direction and B→A is P_{app} in the basolateral-to-apical direction. P_{app} entries are means ± s.e.m., n = 5. *$P < 0.05$ vs control.

cells was determined in the presence of MK571, quinidine or mitoxantrone, which are well-known inhibitors or substrates of transporters MRP2, P-gp or BCRP, respectively.

Accumulation of EC increased in the presence of each of the inhibitors tested as compared with untreated controls (0.067 nmol (mg protein)$^{-1}$). EC levels were: 0.172 nmol (mg protein)$^{-1}$ in the presence of MK571; 0.107 nmol (mg protein)$^{-1}$ in the presence of quinidine; and 0.234 nmol (mg protein)$^{-1}$ in the presence of mitoxantrone. Among the inhibitors, mitoxantrone had the strongest effect, resulting in an increase of 249% in cellular accumulation of EC relative to cells that did not receive the inhibitor. In Caco-2 cells treated with MK571 and quinidine, the percent increases in cellular accumulation of EC were 158% and 60% respectively (Figure 4).

The influence of inhibitors on cellular accumulation of ECG was significant only in Caco-2 cells treated with MK571. ECG content increased by 49% in MK571 treated cells compared with the control (Figure 4). Accumulation of ECG in cells treated with quinidine appeared to increase but the results were not significantly different from controls. Treatment with mitoxantrone did not influence the cellular accumulation of ECG.

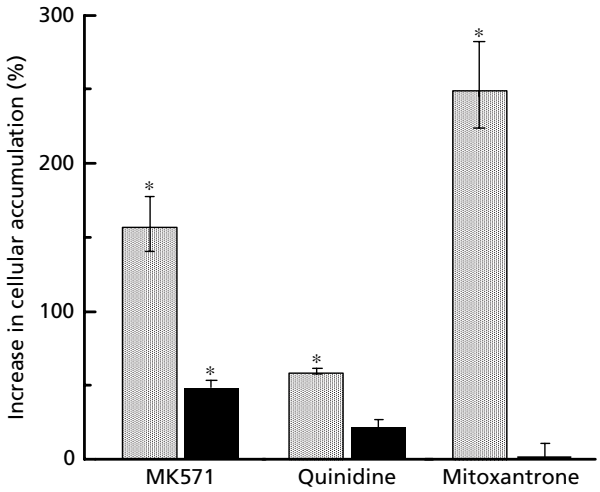

Figure 4 Percent increase of the cellular accumulation of catechins in the presence of transporter inhibitors. Transporter inhibitors, MK571, quinidine or mitoxantrone, were added to medium at a concentration of 50 μM, respectively, before the addition of EC or ECG at a concentration of 50 μM. The cells were incubated at 37°C for 3 h and were harvested in HCl–EtOH. After centrifugation and neutralization, EC or ECG in supernatants was analysed by HPLC. The % increase was calculated from the equation: $\% = (C_i - C_c)/C_c \times 100$, where C_i and C_c are the cellular content of catechin in the presence or absence of each inhibitor, respectively. EC, ▨; ECG, ■. Each volume represents the mean ± s.e.m. of five trials. *$P < 0.05$, vs value in the absence of transporter inhibitor.

Effect of transporter inhibitors on the transepithelial transport of catechins across Caco-2 cell monolayers

The effect of transporter inhibitors on transepithelial transport of EC is shown in Figure 5. Transport of EC in the B→A direction decreased in the presence of all transporter inhibitors tested. The value for $P_{appB\rightarrow A}$ in the presence of MK-571, quinidine or mitoxantrone was 6.57, 5.99 and 6.76, respectively, as compared with a value of 11.44 in the absence of inhibitor (Table 1). Transport of EC in the A→B direction tended to increase in monolayers treated with these inhibitors, but no significant increase was observed in monolayers treated with quinidine. The $P_{appB\rightarrow A}/P_{appA\rightarrow B}$ ratio was 1.00, 1.04 and 0.92 in Caco-2 cells with MK571, quinidine or mitoxantrone, respectively.

Transport of ECG in the B→A direction across the monolayer decreased significantly in the presence of MK517 and quinidine (Figure 6B, Table 1). However, the decrease for ECG was less than that for EC. Furthermore, mitoxantrone seldom influenced the transport of ECG in the B→A direction. Quinidine had an effect on transport of ECG in the A→B direction but for the other inhibitors. The $P_{appB\rightarrow A}/P_{appA\rightarrow B}$ ratio was 1.05, 0.75 and 1.98 in Caco-2 cells treated with MK571, quinidine or mitoxantrone, respectively (Table 1). In the presence of mitoxantrone, the level of transport of ECG was not influenced in either direction.

Discussion

This study showed that accumulation of EC, the non-gallate catechin, in Caco-2 cells was much lower than that of ECG, the gallate catechin. Cellular accumulation of EC and ECG increased with time and after 3 h of incubation was 0.17% and 5.78%, respectively, of the total amount of each catechin that was added to the medium.

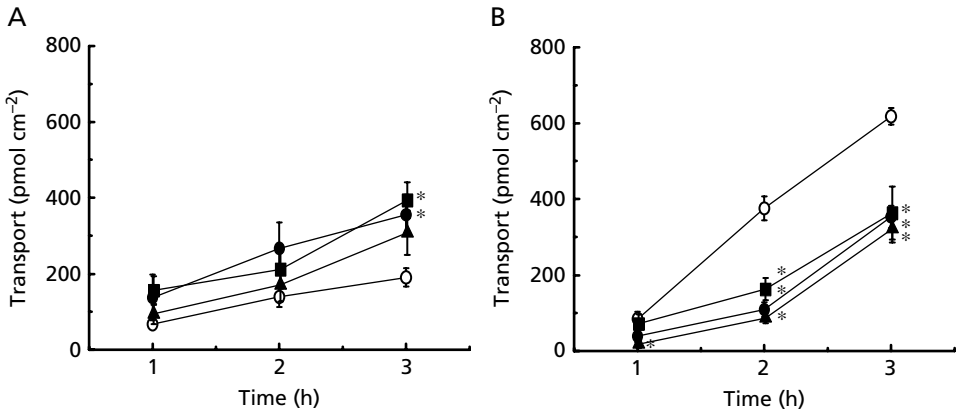

Figure 5 Effect of transporter inhibitors on the transepithelial transport of EC in the apical-to-basolateral direction (A) and in the basolateral-to-apical direction (B) across Caco-2 cell monolayers. The monolayers were incubated with EC (50 μM) in the absence (○) or presence of MK571 (●), quinidine (▲) or mitoxantrone (■) at a concentration of 50 μM. Other experimental conditions were the same as in Figure 3. Each volume represents the mean ± s.e.m. of five trials. *$P < 0.05$, vs values in the absence of transporter inhibitor.

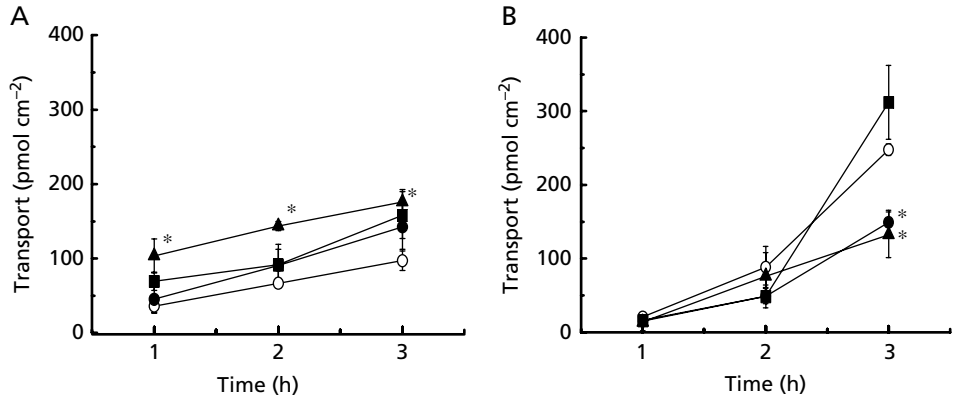

Figure 6 Effect of transporter inhibitors on the transepithelial transport of ECG in the apical-to-basolateral direction (A) and in the basolateral-to-apical direction (B) across Caco-2 cell monolayers. The monolayers were incubated with ECG (50 μM) in the absence (○) or presence of MK571 (●), quinidine (▲) or mitoxantrone (■), at a concentration of 50 μM. Other experimental conditions were the same as in Figure 3. Each volume represents the mean ± s.e.m. of five trials. *$P < 0.05$, vs values in the absence of transporter inhibitor.

Similar results have been reported by Vaidynathan & Walle (2003) but, in that study, it was assumed that EC could not penetrate the apical membrane because the authors did not detect transport or accumulation of EC in the Caco-2 monolayer. As shown in this study, transport of EC in a Caco-2 monolayer was detected in both directions (basolateral-to-apical and apical-to-basolateral), as well as the cellular accumulation of EC. Transport of catechins in the basolateral-to-apical direction was higher than that in the reverse direction, suggesting that that catechins that penetrate the apical membrane are easily transported to the outside of the cell via the activity of efflux transporters. The very limited accumulation of catechins in cells seems to be due to higher efficiency for efflux as opposed to influx. Furthermore, the $P_{appB \rightarrow A}/P_{appA \rightarrow B}$ value for EC was larger than that for ECG.

After oral administration, major amounts of catechins were reported to be metabolized to sulfate or glucuronate conjugates (Lee et al 1995; Donovan et al 2001). Catechin metabolism may thus be related to the limited catechin accumulation and the different amounts of EC and ECG accumulated. Drug metabolite activity of Caco-2 cells seems to be lower than that of hepatocytes. In this study using HPLC, non-metabolized catechins were detected in samples extracted from Caco-2 cells, showing a major peak after 3 h of incubation. Zhang et al (2004a) demonstrated that EC metabolites were mainly sulfate conjugate, methylated EC and methylated sulfate conjugate of EC in the basolateral-to-apical transport experiment using Caco-2 cells. They detected a higher concentration of EC than these metabolites in the apical side. The glucuronate conjugates of EC seem to be the minor metabolites in the human intestine (Kuhnle et al 2000; Vaidyanathan & Walle 2002). EC was reported to be the most stable among all the tea flavonoids at 37°C and pH 7.4 (Vaidyanathan & Walle 2001). The lower level of cellular accumulation of EC could be attributed to a more rapid transport of EC, than ECG, to the outside of the cell.

Higher levels of efflux transport of EC as compared with ECG were also reported by Zhang et al (2004a). According to

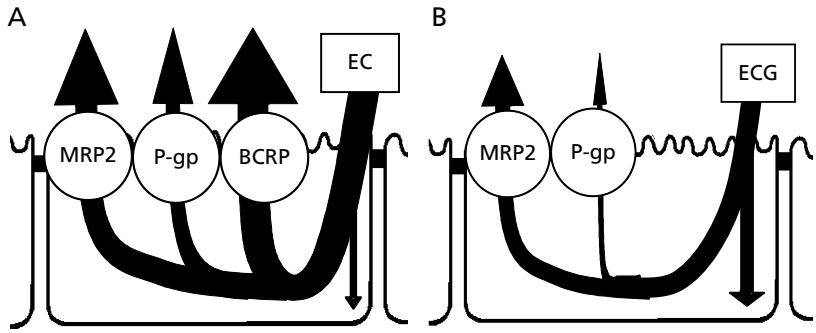

Figure 7 The transport model of EC (A) and ECG (B) mediated by transporters in the enterocyte.

their study, the $P_{appB\rightarrow A}/P_{appA\rightarrow B}$ value for EC decreased in the presence of MK571; hence, efflux transport of EC was functionally linked to the transporter MRP2. The influence of MRP2 on efflux transport of ECG was not investigated in that study, and there have been no previous reports on the different effects of MK571 on EC versus ECG. The results of our study indicated that efflux transport of EC by MRP2 may be attributed to the limited cellular accumulation of EC. In addition to MRP2, the other transporters we tested helped to explain the difference in cellular accumulation of EC versus ECG.

EC appears to be transported by all efflux transporters investigated in this study (MRP2, P-gp and BCRP). Involvement of these transporters in EC efflux was greater than that observed for ECG. In particular, there was a large difference in the influence of BCRP on transport of EC and ECG. Treatment of Caco-2 cells with mitoxantrone induced an increase in cellular accumulation of EC and, accordingly, a decrease in efflux transport of EC was observed in the experiment using Caco-2 cell monolayers. Mitoxantrone did not appear to affect cellular accumulation and monolayer transport of ECG.

Regarding the transporter P-gp, its inhibitor quinidine did not have as great an influence on ECG or EC as was found for MK571 and mitoxantrone. Among the inhibitors that were tested in this study, quinidine had the least influence on cellular accumulation of EC. Cellular accumulation of ECG did not increase significantly after the treatment with quinidine. Hence, the difference in cellular accumulation between EC and ECG may be attributed to the efflux transporters in general, and to the BCRP transporter in particular. Other reports suggest that gallate catechins such as ECG and EGCG inhibit P-gp-mediated transport and lead to an increase in cellular accumulation of rhodamine-123, a substrate of P-gp (Kitagawa et al 2004; Mei et al 2004). Moreover, not only gallate catechins but also gallate esters caused an increase in cellular accumulation of rhodamine-123 (Jodoin et al 2002; Kitagawa et al 2005). The gallate moiety in the catechin structure seems to be important for P-gp inhibition. Lower transport of ECG by P-gp than of EC might be due to inhibition of P-gp caused by the gallate moiety that is present in the ECG structure but not in EC. The effect of the gallate moieties on other transporters, such as MRP2 or BCRP, has not been apparent. Similar to P-gp, MRP2 and BCRP might be inhibited by the gallate moiety. A previous report suggested that cellular accumulation of mitoxantrone increased after treatment with EGCG in BCRP-overexpressing human breast cancer cells (MCF-7 cells), but that EGC was not similarly affected (Zhang et al 2004b).

The only difference between the structures of EC and ECG is the presence or absence of a gallate moiety. The hydrophobicity of ECG is higher than that of EC due to the presence of the gallate moiety. The increase in hydrophobicity is speculated to make it a more favourable substrate for the efflux transporter because transporters investigated in this study penetrate into the membranes. However, EC seems to be a more favourable substrate than ECG for efflux transporter in Caco-2 cells. The presence of a gallate moiety might cause transporters to lose the capability to recognize catechins as substrates or the gallate moiety itself may directly influence the activity of transporters of catechins. That is, inhibition of the efflux transporter activity by the gallate moiety in ECG may increase the cellular accumulation of ECG as compared with EC. It is proposed that the very limited accumulation of EC as compared with ECG can be attributed to the differences in the response elicited by efflux transporters.

The conclusions of the present study are summarized in Figure 7. Efflux transport of EC was observed to be involved in all the transporters investigated—MRP2, P-gp and BCRP. BCRP did not appear to function as an efflux transporter for ECG and the involvement of the other transporters, MRP2 and P-gp, was much lower than that observed for efflux transport of EC.

Taken together, the results suggest that lower levels of cellular accumulation of EC compared with ECG may be the result of the difference in efflux transport of the two catechins. The gallate moiety might influence the activity of efflux transporters, particularly the BCRP efflux transporter, because its presence is the only difference in the structures of these two catechins.

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