Cellular Uptake and Efflux of the Tea Flavonoid (−)-Epicatechin-3-gallate in the Human Intestinal Cell Line Caco-2

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ABSTRACT
(−)-Epicatechin gallate (ECG) is one of the flavonoids in green tea, which has been demonstrated to have cancer-preventive properties in many model systems. However, the extent and mechanisms of accumulation of these flavonoids in cells is unknown. The objectives of this study were to determine the accumulation of ECG by the intestinal epithelial cell Caco-2 and to characterize the transport mechanism involved. The cells were exposed to ECG at various transport inhibitors and incubated at 37°C. Absorbed flavonoids were extracted and quantified by high-performance liquid chromatography. The uptake of ECG included a nonsaturable initial rapid process as well as a much slower saturable process. The saturable ECG uptake by the Caco-2 cells was sodium-independent but clearly dependent on a pH gradient. Phloretin and benzoic acid, inhibitors of the monocarboxylate transporter (MCT), significantly reduced ECG uptake. The uptake of ECG in the Caco-2 cells increased 2-fold in the presence of 50 μM 3-{3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl}-{2-dimethylcarbamoylethylsulfanyl}methylsulfanyl propionic acid (MK-571), suggesting the involvement of multidrug-associated protein (MRP2) in efflux of ECG. This was confirmed using Madin-Darby canine kidney cells transfected with MRP2. Also P-glycoprotein was responsible for some ECG efflux. MK-571 also caused a dramatic increase in ECG accumulation in Chinese hamster ovary cells, suggesting that ECG was also a substrate for MRP1. Together, these observations demonstrate important roles of membrane transporters, i.e., MCT, MRP2, P-glycoprotein, and MRP1, in the cellular accumulation and potential effects of ECG.

Flavonoids are naturally occurring dietary compounds present in fruits, vegetables, tea, and wine (Hertog et al., 1993). Polyphenols, especially those belonging to the catechin type of flavonoids, are the most significant group of tea components. The major tea catechins are (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC) (Fig. 1), and (−)-epigallocatechin. These compounds have been suggested to have anticancer effects both in animals and in humans (Katiyar and Mukhtar, 1996). Epidemiological studies have advanced this notion by identifying an inverse association between tea consumption and the frequency of colon and rectal cancer as well as gastric and esophageal cancers (Bu-Tian et al., 1997; Su and Arab, 2002). Important biochemical mechanisms for inhibition of tumorigenesis by tea include inhibition of activities related to tumor promotion and cell proliferation (Lin and Lin, 1997; Yang et al., 2001).

In spite of convincing observations in animal and cell culture models, extrapolation of these in vitro findings for tea polyphenols to the in vivo situation is difficult, because the accumulation at target tissues is unknown. The oral bioavailability of tea flavonoids has been suggested to be low in rats (Chen et al., 1997; Zhu et al., 2000) as well as in humans (Yang et al., 1998; Baba et al., 2000; Chow et al., 2001; Warden et al., 2001).

In a direct examination of the transcellular absorption of EC, using the Caco-2 cell model of human intestinal absorption (Artursson and Karlsson, 1991; Yee, 1997; Walle et al., 2003), we found that there was very low absorption (Vaidyanathan and Walle, 2001). The main purpose of this study was to examine the Caco-2 cell uptake of ECG and EGCG, the tea flavonoids that have been shown to be the most biologically active, in comparison with EC and its geometric isomer.

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ABBREVIATIONS. EGGc, (−)-epigallocatechin-3-gallate; EGC, (−)-epicatechin-3-gallate; EC, (−)-epicatechin; BSO, buthionine-[S,R]-sulfoximine; FCCP, carboxylicyanide-p-trifluoromethoxyphenylhydrazone; MRP, multidrug-associated protein; MK-571, 3-{3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl}-{2-dimethylcarbamoylethylsulfanyl}methylsulfanyl propionic acid; HBSS, Hanks’ balanced salt solution; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; MDCK, Madin-Darby canine kidney; HPLC, high-performance liquid chromatography; TEER, transepithelial electrical resistance; GSH, glutathione; P_app, apparent permeability coefficient; SGLT1, sodium-dependent D-glucose transporter 1; MCT, monocarboxylate transporter.
Because extensive uptake was observed, we attempted to assess the nature of the apical membrane transporter responsible for the accumulation of ECG by the enterocytes. Our observations indicate that apical membrane uptake transporters as well as efflux pumps, such as the multidrug resistance-associated protein (MRP2) as well as MRP1 and P-glycoprotein, play a major role in cellular accumulation of ECG.

Materials and Methods

Materials. d-[1-14C]Mannitol (57.0 mCi/mmol) was purchased from Amersham Biosciences Inc. (Piscataway, NJ). EC, ECG, EGCg, (+)-catechin (Fig. 1), colchicine, meta-phosphoric acid, and BSO, chloridizin, glucose, phloretin, glycol-sarcosine, sodium azide, 2-deoxy-D-glucose, nigericin, FCCP, and benzoic acid were obtained from Sigma-Aldrich (St. Louis, MO). MK-571 was a gift from Dr. A. W. Ford-Hutchinson (Merck-Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, QC, Canada), and PSC833 (valspodar), was a gift from Novartis (Basel, Switzerland). EAG5, MRP2-specific antibody, was provided by Dr. Keppeler (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Glutathione assay kit was from Calbiochem (La Jolla, CA). Trifluoroacetic acid was of spectrophotometric grade from Aldrich Chemical Co. (Milwaukee, WI), fetal bovine serum from Atlanta Biologicals (Norrcross, GA), and Hanks’ balanced salt solution (HBSS) and other cell culture medium components were obtained from Cellgro Mediatech, Fisher Scientific (Pittsburgh, PA). Dulbecco’s phosphate-buffered saline (PBS) with 0.1 g/l calcium chloride was purchased from Invitrogen (Carlsbad, CA).

Cell Culture. Caco-2 cells from the American Type Culture Collection (Manassas, VA) were cultured in Eagle’s minimal essential medium with 10% fetal bovine serum, 1% nonessential amino acids, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C. The cells were used from passage 40 to 90.

Parental Chinese hamster ovary (CHO) cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium containing 1 mM sodium pyruvate, 2 mM l-glutamine, 10% fetal calf serum, 1% nonessential amino acids, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 25 mM β-mercaptoethanol in a humidified atmosphere of 10% CO2 at 37°C.

Parental Madin-Darby canine kidney (MDCK) cells and MDCK-MDR, i.e., MDCK cells transfected with multidrug resistance MDR1 gene (Pastan et al., 1988), were obtained from Dr. I. Pastan (National Cancer Institute, Bethesda, MD). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 1 mM sodium pyruvate, 5 mM l-glutamine, 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C.

To maintain selection of transfected cells, culture medium for MDCK-MDR cells contained 80 μg/ml colchicine.

Parental Madin-Darby canine kidney (MDCKII) cells and MDCK-MRP2, i.e., MDCKII cells transfected with MRP2 (Evers et al., 1998), were obtained from Dr. P. Borst (The Netherlands Cancer Institute, Division of Molecular Biology, Amsterdam, The Netherlands). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere of 10% CO2 at 37°C.

Preparation of Membrane-Enriched Cell Fractions and Immunoblot Analysis. Cell membrane fractions were prepared essentially as described previously (Almquist et al., 1995). Samples of membrane-enriched cell fractions were diluted 1:2 with sample treatment buffer and subject to immunoblot analysis as mentioned previously (Walle et al., 1999), using EAG5 MRP2 primary antibody (Cui et al., 1989).

Cellular Uptake Studies. For all cellular uptake studies, confluent monolayers of Caco-2, CHO, MDCK, MDCK-MDR, MDCKII, and MDCKII-MRP2 cells were grown in six-well plastic plates. Culture medium was replaced three times a week, and cells were used 7 to 10 days post seeding for Caco-2 and 3 to 4 days post seeding for all other cells. Fresh culture medium was added 24 h before transport experiments. In experiments with Caco-2 and MDCK-derived cells, HBSS was used, whereas with CHO-derived cells, PBS was used. One hour before uptake experiments, culture medium was aspirated and monolayers were incubated twice for 30 min in warm buffer. Where applicable, uptake inhibitors were included in the final 30-min preincubation, except with BSO, which was added 24 h before the experiment. In experiments designed to examine sodium dependence of flavonoid uptake, PBS (140 mM Na+) was replaced with sodium-free PBS brought to equal osmolarity with choline chloride (140 mM). In experiments with ionophores, the Caco-2 cells were ATP-depleted by pretreatment with 10 mM sodium azide and 10 mM 2-deoxy-D-glucose in a glucose-free buffer for 2 × 30 min. During the second wash, nigericin or FCCP (10 μM/gl) was added to buffer pH 7.4 or 5.5, respectively. The preincubation buffer was then replaced with 1 ml of buffer containing flavonoid substrate ± inhibitors. Stock solutions of flavonoids, PSC833, and verapamil were in dimethyl sulfoxide and were diluted with transport buffer before experiments. The resulting final concentration of dimethyl sulfoxide, 0.5%, did not affect the transport. All other compounds were dissolved in the buffer. After the specified incubation time, the cells were rinsed three times with ice-cold buffer.

The absorbed flavonoid was extracted from the monolayer with methanol. The cells were scraped off the dishes and extracted twice with 1 ml of methanol and centrifugation at 14,000 g for 2 min. The combined supernatants were evaporated to dryness under N2 gas and reconstituted in 300 μl of mobile phase and analyzed by reverse-phase HPLC.

Transepithelial Transport Experiments. For transport studies, Caco-2 cells were seeded in 12-mm i.d. Transwell inserts (polycarbonate membrane, 0.4-μm pore size; Costar, Cambridge, MA) in 12-well plates at a density of 105 cells/cm2. Caco-2 cells in Transwells at passage 50 to 95 were used for experiments 20 to 30 days post seeding. Transepithelial electrical resistance (TEER) values across the cell monolayers were measured using a Millicell-ERS volthometer (Millipore Corporation, Bedford, MA). Inserts with TEER values >300 Ω cm2 in culture medium were selected for transport experiments. The inserts were washed twice for 30 min with warm transport buffer, HBSS containing 25 mM HEPES, pH 7.4. TEER values were also obtained after completion of transport experiments. The paracellular transport marker [14C]mannitol was added to the apical side of all inserts for the assessment of monolayer integrity. Aliquots

Fig. 1. Chemical structures of tea flavonoids studied.
Uptake of ECG was measured at 50 and 200 μM concentrations. Transport buffer containing ECG (0.5 ml) was added to the apical side of the inserts, whereas the receiving basolateral chamber contained transport buffer (1.5 ml). Inhibitors were added in the last 30-min preincubation as well as with the flavonoid. At the end of the 3-h incubation at 37°C, samples were collected from both sides of the cell monolayer and analyzed for flavonoid content by HPLC.

**HPLC Analysis.** ECG, EGCG, EC, and (+)-catechin were analyzed by reversed-phase HPLC of 200-μl samples on a Millennium HPLC system (Waters, Milford, MA) with a Symmetry C18 column (3.9 × 150 mm) and a model 996 photodiode array detector. The mobile phase for ECG consisted of 30% methanol in 0.3% trifluoroacetic acid, whereas the mobile phase for EC, EGCG and (+)-catechin consisted of 20% methanol in 0.3% trifluoroacetic acid. The flow rate was 0.9 ml/min with detection at 278 nm. Quantitation was done by peak area measurements in comparison with standard curves for ECG, EGCG, EC, and (+)-catechin.

**Glutathione Assay.** The total cellular reduced glutathione levels in MDCKII-MRP2 cells were measured colorimetrically using a kit obtained from Calbiochem (San Diego, CA) as directed. Briefly, cells grown in six-well plastic dishes were pretreated with BSO (5 mM) or 5-

**Calculation and Statistics.** The apparent permeability coefficient (P_app), expressed in centimeters per second, was determined as P_app = dC/dt x V/A x C0, where dC/dt is the change in concentration on the receiving side over time (micromolar per second), V is the volume of the solution in the receiving compartment (cubic centimeters), A is the surface area of the membrane (square centimeters), and C0 is the initial concentration in the donor chamber (micromolar) (Artursson and Karlsson, 1991). The effect of transport inhibitors on P_app was expressed relative to control.

The statistical significance of differences between treatments was evaluated by using two-tailed paired student t tests with a significance level of P < 0.05.

**Results**

**ECG Uptake by Caco-2 Cells.** The Caco-2 cells, being epithelial cells, are polarized with well distinguishable apical and basolateral membranes (Artursson and Karlsson, 1991). When the cells are grown to confluence in regular six-well plates, only the apical membrane is accessible. In the uptake experiments, the cells were incubated with tea flavonoids for 60 min. The cell content of flavonoids was determined by HPLC after methanol extraction. We have previously shown that this is an effective approach for lysing the cells and extracting the flavonoids (Walgren et al., 2000b). Similar to our previous study on transcellular transport of EC (Vaidyanathan and Walle, 2001), there was no accumulation of EC by the Caco-2 cells (Fig. 2), i.e., EC could not penetrate the apical membrane. Also (+)-catechin, an isomer of EC, had a very limited accumulation. In contrast to EC and (+)-catechin, both gallated tea flavonoids, i.e., ECG and EGCG, accumulated in the Caco-2 cells in significant amounts, 3037 ± 311 and 2335 ± 446 pmol/mg protein, respectively, during a 60-min incubation period (Fig. 2). This uptake was molecularly specific, as shown by HPLC (data not shown), and not the result of metabolites of ECG or EGCG. All subsequent studies were conducted with ECG, which was stable under the study conditions used. EGCG, in contrast, had a very low stability, as recently noted (Hong et al., 2002), making it difficult to study under these conditions.

Figure 3A shows the time-dependent uptake of ECG (50 μM) by the Caco-2 cells. The time course had a unique pattern with an extremely rapid initial uptake in less than 1 min followed by a more gradual further increase, approaching equilibrium at 60 min. Figure 3B shows the initial rapid

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**Fig. 3.** A, effect of time on Caco-2 cell uptake of ECG (50 μM). Mean ± S.E.M. values are shown (n = 9). B, concentration dependence of the initial 15-s rapid uptake of ECG. Mean ± S.E.M. values shown (n = 4). C, concentration dependence of the Caco-2 cell uptake of ECG at 5 min. Initial rapid uptake at 15 s was subtracted for each concentration (n = 4).
Involvement of SGLT1 in ECG Uptake. ECG has been shown to inhibit glucose uptake in rabbit brush-border membranes via SGLT1 (Kobayashi et al., 2000). Also, certain flavonoid glucosides have been shown to be transported by SGLT1 (Walgren et al., 2000b). To test the hypothesis that ECG is a substrate for SGLT1, ECG uptake by Caco-2 cells was examined for sodium dependence and inhibition by both the SGLT1 substrate glucose and the SGLT1 competitive inhibitor phloridzin (Toggenburger et al., 1982). The uptake of ECG in the absence of sodium was 2206 ± 561 pmol/mg protein. This was not significantly different from that seen in the presence of sodium (2282 ± 571 pmol/mg protein) or in the presence of 30 mM glucose (2813 ± 466 pmol/mg protein) or 0.5 mM phloridzin (2553 ± 679 pmol/mg protein) in at least three experiments.

Involvement of a pH-Dependent Mechanism in ECG Uptake. To determine whether the transport of ECG involved a proton cotransporter, we determined the effect of pH on its uptake by Caco-2 cells. As shown in Fig. 4A, with a decrease in the pH from 7.4 to 5.0, there was a 2.2-fold increase in the cellular accumulation of ECG. Also treatment of ATP-depleted Caco-2 cells with a protonophore, FCCP, significantly decreased ECG uptake in pH 5.5 (1640 pmol/mg protein) compared with control cells (2631 pmol/mg protein). On the other hand, treatment of ATP-depleted cells with nigericin (an ionophore for K+ and H+) significantly increased the uptake in the absence of a pH gradient. The uptake in the presence of nigericin was 1938 ± 230 pmol/mg protein compared with 1486 ± 228 pmol/mg protein in control cells at pH 7.4. Nigericin acts as a Na+ (or K+)-H+ exchanger and generates an inwardly directed H+ gradient across the membranes. This effect of ionophores is very similar to that recently shown for lovastatin uptake in mesangial cells (Nagasawa et al., 2002). The increase in transport with a decrease in pH has been shown to occur for the peptide transporter PEPT1 and the monocarboxylate transporter MCT (Garcia et al., 1994; Tamai et al., 1999).

To further characterize this proton-dependent mechanism of uptake of ECG, inhibitors of the PEPT1 and MCT, both of which are expressed in the Caco-2 cells (Brandsh et al., 1994; Hadijagapiou et al., 2000), were used. To test the involvement of PEPT1, the model dipeptide substrate and inhibitor glycel-sarcosine (Sugawara et al., 2000) was used. The cellular accumulation of ECG by the Caco-2 cells at pH 5.0 in the presence (2877 ± 435 pmol/mg protein) or absence (2879 ± 349 pmol/mg protein) of 20 mM glycel-sarcosine, a concentration well known to inhibit PEPT1, was not significantly different.

To determine the potential role of MCT in the transport of ECG, the MCT transport inhibitors phloretin (Garcia et al., 1994) and benzoic acid (Tamai et al., 1999) were used. As shown in Fig. 4B, the uptake of ECG by the Caco-2 cells decreased in a concentration-dependent manner with increasing phloretin concentration. With 0.3 mM phloretin the decrease in the uptake was 44% compared with control (P < 0.01). Higher concentrations of phloretin could not be used because of noticeable cell toxicity (lifting off from the plastic). Benzoic acid also inhibited the uptake of ECG. As shown in Fig. 4C, the uptake in the absence of benzoic acid was 2645 ± 173 pmol/mg protein, which was reduced to 1788 ± 196 pmol/mg protein in the presence of 20 mM benzoic acid (P < 0.05).
and Walle, 2001), showed an increase in transepithelial absorption in the presence of MK-571, an MRP1/MRP2 inhibitor (Jedlitschky et al., 1994; Leier et al., 1994). To test the hypothesis that ECG may be a substrate of MRP2, we examined the effect of MK-571 on its accumulation by the Caco-2 cells. The uptake at 60 min in the absence of MK-571 was 810 ± 141 pmol/mg protein. In the presence of 50 μM MK-571, this significantly increased (P < 0.05) to 1698 ± 308 pmol/mg protein (Fig. 5).

**ECG Uptake in MDCKII and MDCKII-MRP2 Cells.** To confirm this observation, the uptake of ECG was also determined in another polarized epithelial cell, MDCKII as well as MDCKII-MRP2 cells. The latter cells are stably transfected with human MRP2 (Evers et al., 1998; Cui et al., 1999) and have been extensively used to determine the involvement of MRP2-mediated transport. In agreement with the original study (Evers et al., 1998), the transfected cells had overexpression of MRP2 protein, as shown in Fig. 6A. As shown in Fig. 6, B and C, the uptake of ECG by the MDCKII-MRP2 cells, 2265 ± 257 pmol/mg protein, was significantly less than in the parental MDCKII cells, 5651 ± 371 pmol/mg protein (P < 0.05). In the presence of 50 μM MK-571, cell uptake increased somewhat in the parent MDCKII cells to 9393 ± 1519 pmol/mg protein (P < 0.05), consistent with a low level of expression of MRP2 (Fig. 6A). In the MDCKII-MRP2 cells, this effect of MK-571 was as expected more marked, 14,757 ± 1095 pmol/mg protein (P < 0.01).

**ECG Uptake in CHO Cells.** Previous studies have shown that CHO cells have a high expression of MRPs1 (Barnoun et al., 1998). MRP1 and MRP2 have 49% homology and are the best-characterized MRPs transporters with respect to function and tissue localization. Furthermore, MRP1 and MRP2 have been demonstrated to have overlapping substrate specificity (for review, see Borst et al., 2000; Leslie et al., 2001). To test the involvement of MRPs1 in ECG transport, cellular uptake studies were done in CHO cells in the presence and absence of the MRP1/MRP2 inhibitor MK-571. Parallel studies in our laboratory, using MRP1-selective antibodies (Hoffman et al., 1996) show the expression of MRP1 protein in CHO cells (T. Walle and U. K. Walle, manuscript submitted for publication). As shown in Fig. 7, the uptake in the CHO cells (849 ± 251 pmol/mg protein) dramatically increased in the presence of 50 μM MK-571 to 14,951 ± 566 pmol/mg protein (P < 0.05).

**Involvement of Glutathione in MRP2-Mediated Transport.** Glutathione has been shown to be cotransported by MRPs1 and MRP2 along with nonionic compounds such as vincristine and vinblastine (Loo et al., 1998). To test the hypothesis of a possible mechanism of cotransport of ECG with glutathione, ECG uptake was studied in MDCKII-MRP2 cells. After pretreatment with BSO (5 mM) for 24 h, the uptake was 23867 ± 127 pmol/mg protein, which was not significantly different compared with the untreated cells (2492 ± 118 pmol/mg protein). The cellular glutathione levels were reduced to 30% of control levels after BSO treatment (24 ± 8 versus 78 ± 11 nmol/mg protein; n = 3).
ment of P-glycoprotein in the cellular uptake of ECG, a known inhibitor of P-glycoprotein, PSC833 was used. The uptake of ECG in the Caco-2 cells (2687 ± 179 pmol/mg protein), increased in the presence of PSC833 (1 μM) to 4923 ± 762 pmol/mg protein (P < 0.05). The uptake was also examined in MDCK-MDR (P-glycoprotein-transfected) cells in the presence (15,600 ± 1010 pmol/mg protein) and absence (11,240 ± 1660 pmol/mg protein) of the inhibitor. There was a small but significant increase in ECG accumulation in the presence of PSC833 (P < 0.05). At a concentration of 1 μM, PSC-833 is specific for P-glycoprotein and should not affect MRP2 (Böhme et al., 1993).

**Transcellular Transport of ECG in Caco-2 Cells.**

With both absorptive and efflux transporters for ECG identified, it became of importance to examine not only Caco-2 cell uptake but also potential apical to basolateral transcellular absorption. Thus, the absorptive transport of ECG (both 50 and 200 μM) across Caco-2 cells grown on permeable membranes was measured. As shown in Table 1, there was a very low P_app of ECG at 50 μM, which significantly increased at 200 μM (P < 0.0001). In the presence of inhibitors of both MRP2 and P-glycoprotein, i.e., 50 μM MK-571 and 1 μM PSC833, there was no increase in the P_app of ECG at 50 μM but a significant increase at 200 μM (P < 0.0005). The P_app of [14C]mannitol increased significantly at higher ECG concentration (P < 0.005) and was also higher in the presence of both inhibitors along with ECG 50 μM (P < 0.005) (Table 1). There was also a significant reduction in TEER values in the presence of inhibitors (P < 0.001) and at higher ECG concentration (P < 0.05). There was a simultaneous increase in the apparent permeability of [14C]mannitol under these conditions compared with control (Table 1).

**Discussion**

It is important to know the bioavailability of flavonoids when attempting to extrapolate from in vitro to the in vivo situation. We have used human Caco-2 cell monolayers, a well accepted model of human intestinal absorption (Artursson and Karlsson, 1991; Yee, 1997; Walle et al., 2003), to study the mechanisms underlying the absorption of these flavonoids. In a previous study, we have demonstrated that there was no transepithelial absorption of one of the major tea flavonoids, EC; however, in the presence of the MRP1/MRP2 inhibitor MK-571, there was some absorption, although low (Vaidyanathan and Walle, 2001).

The results from the present study demonstrate that ECG and EGCG are extensively taken up by the Caco-2 cells. Consistent with our previous transport studies, there was little, if any, uptake of EC or its isomer (+)-catechin. The uptake of ECG was specific for the intact flavonoid, as seen by HPLC. Furthermore, the uptake was found to be time- and concentration-dependent. ECG showed a very high initial rapid binding, most likely due to adsorption to the cell surface, very similar to that shown recently for fluorescein (Kuwayama et al., 2002). The slower saturable uptake is evidence of involvement of an active membrane transporter in this process.

We further characterized the saturable uptake of ECG as sodium-independent but pH-dependent, i.e., accelerated at acidic pH. The uptake was not affected by glycyrrhizin, ruling out PEPT1 as the active transporter. The monocarboxylate transporter MCT1 was originally found as a pH-dependent lactate and pyruvate transporter in CHO cells and has been shown to be present in a variety of tissues, including the intestine (Poole and Halestrap, 1993; Garcia et al., 1994). At least five MCT isoforms have been shown in the Caco-2 cells, with MCT1 being the most abundant (Hadjigapiou et al., 2000). The ECG uptake by Caco-2 cells was inhibited by phloretin and benzoic acid, previously characterized as inhibitors of MCT1 (Garcia et al., 1994). However, the only about 50% inhibition of ECG transport observed with phloretin and benzoic acid may be closer to complete inhibition of MCT when considering that about one-third of cellular accumulation of ECG seems to involve cell surface binding (Fig. 4, B and C). The nature of the latter process deserves further investigations.

The finding that ECG is a substrate for an MCT transporter, presumably MCT1 in the Caco-2 cells, was unexpected. The multiple MCT isoforms, particularly in the colon, are well known to transport low molecular weight short-chain fatty acids, such as butyrate, propionate, and lactate (Garcia et al., 1994; Tamai et al., 1999; Hadjiagapiou et al., 2000; Stein et al., 2000). MCTs have also been implicated in the transport of carboxylic acid type of drugs such as lovastatin acid (Nagasawa et al., 2002) and fluorocein (Kuwayama et al., 2002). However, ECG is not a carboxylic acid but rather a polyphenol with a high pKₐ value of 8.39. Thus, it is present at the apical site of absorption predominantly in the undissociated nonionized form. This study may have identified a novel structural feature, e.g., the galacto moiety, important for recognition by this transport family of proteins. This is currently under further investigation.

Based on our previous findings with EC, the accumulation of ECG inside the enterocytes may be modulated by efflux by MRP2 and possibly P-glycoprotein. We first focused on MRP2, which has been shown to be involved in the transport of other flavonoids (Walgren et al., 2000a). In Caco-2 cells, which express MRP2 in the apical membrane (Walle et al., 1999; Walgren et al., 2000a), there was a significant increase in ECG accumulation in the presence of MK-571, an MRP1/MPR2 inhibitor (Jedlitschky et al., 1994; Leier et al., 1994) (Fig. 5).

As additional evidence for transport of ECG by MRP2, we examined transport of ECG in MDCK-MRP2 cells. These cells have been transfected with human MRP2 and the cellular expression and localization demonstrated and transport characterized (Evers et al., 1998; Cui et al., 1999). We con-
firmed the expression of MRP2 protein at very low levels in the parental MDCKII and high levels in the transfected MDCKII-MRP2 cells (Fig. 6A). Consistent with our Caco-2 cell observations, the accumulation of ECG in the MDCKII-MRP2 cells was significantly lower than in the parent MDCKII cells. Furthermore, MK-571 resulted in a significant increase in the ECG accumulation in both cells (Fig. 6, B and C). The relative increase in ECG accumulation in the presence of MK-571 was consistent with the expression of MRP2 in these cells (Fig. 6A).

MRP2 is the second member identified in the now nine-member family of MRP membrane transporters (Dean et al., 2001). Of these, MRP1 and MRP2 are best characterized. MRP2 is localized mainly in the liver, kidney, and gut, whereas MRP1 is distributed ubiquitously throughout the body. Human MRP1 and MRP2 have a high degree of similarity in substrate recognition (Borst et al., 2000; Leslie et al., 2001). Both confer resistance to several structurally unrelated cytostatic drugs and have been shown to transport organic anions and conjugates to glutathione, glucuronide or sulfate, for example, leukotriene C₄, estradiol-17β-glucuronide, and sulfated bile salt sulfatalithocholate (Borst et al., 2000; Leslie et al., 2001).

Based on this similarity, we also determined whether ECG is a substrate of MRP1, using CHO cells. These cells express the 190-kDa hamster homolog of MRP1 and mediate ATP-dependent transport of monogluthathionyl chlorambucil and LTC₄, both high-affinity substrates of MRP1 (Barnouin et al., 1998). The accumulation of ECG dramatically increased in the presence of MK-571 (Fig. 7), indicating that ECG is also an excellent substrate of MRP1.

These findings that ECG, a neutral molecule (Fig. 1), is a substrate of both MRP1 and MRP2 is unexpected based on the known substrate specificity for these transporters. Apart from anionic compounds and conjugates, both MRP1 and MRP2 have been shown to transport cytotoxic drugs, for example vinca alkaloids and anthracyclines for which no negatively charged conjugates are known to exist. This transport was found to require glutathione and was associated with GSH export (Loe et al., 1998). To determine whether ECG transport follows a similar mechanism, we depleted the intracellular glutathione in the cells by pretreatment with BSO (5 mM) for 24 h. This treatment reduced the cellular accumulation of ECG dramatically increased in the presence of MK-571 (Fig. 7), indicating that ECG is also an excellent substrate of MRP1.

To address this question it became important to consider the concentrations of the tea flavonoids that can actually be expected to be present in the intestinal lumen. The concentration (50 μM) of the flavonoids used in this and previous transport studies is likely in the lower range of what can be attained physiologically. Of the naturally occurring flavonoids, the catechins comprise 30% of the total solids per cup of tea (500 mg) (Balentine et al., 1997). Based on the amount of each flavonoid in this fraction, the possible concentration that can be reached in the lumen can be calculated. Assuming complete extraction, with gastric fluid volume of 100 to 500 ml, the concentration of ECG in the lumen may range between 75 and 300 μM and this can be even higher for EGCG.

When determining the apical to basolateral transport of ECG across the Caco-2 cell monolayer, we found concentration-dependent absorption, but very low and comparable with the paracellular transport marker mannitol transport (Table 1). In the presence of MRP2 and P-glycoprotein inhibitors, this transport increased, but only to a small extent. At the same time the TEER values decreased and the mannitol values increased, consistent with opening of the tight junctions. Maybe this is what actually happens in vivo, providing the low extent of systemic absorption of the tea flavonoids reported (Baba et al., 2000; Chow et al., 2001; Warden et al., 2001). The balance between absorption and efflux may also shift depending on the presence of other flavonoids or components in the diet. The most important finding in this study of the tea flavonoids may be their high accumulation in epithelial cells such as Caco-2 or epithelial cells along the aerodigestive tract in general. These seem to be the major sites for biological activities of tea flavonoids (Lin and Lin,
Vaidyanathan and Walle

1997; Yang et al., 2001). The value of the Caco-2 cell model as a strong predictor of the bioavailability of other flavonoids in humans has recently been reported (Walle et al., 2003).

In summary, this study demonstrates high and specific accumulation of the gallated tea flavonoid EGCG in the epithelial Caco-2 cells. This accumulation seems to be mediated by the proton cotransporter MCT. Such accumulation should encourage further studies of the gallated tea flavonoids as chemopreventive agents against tumors of the aerodigestive tract. This study also demonstrates that EGCG is a substrate of multiple efflux transporters, i.e., MRP2, MRP1, and P-glycoprotein. These can play important roles in modifying the accumulation of the tea flavonoids in epithelial cells. This also could be important in drug-drug or drug-food interac-
tions.

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References


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