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Stability, Cellular Uptake, Biotransformation, and Efflux of Tea Polyphenol (−)-Epigallocatechin-3-Gallate in HT-29 Human Colon Adenocarcinoma Cells[1]

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ABSTRACT

The biological effects of (−)-epigallocatechin-3-gallate (EGCG) have been extensively investigated in cell lines, but its stability and interactions with cells under culture conditions are unclear. In the present study, the stability, uptake, biotransformation, and efflux of [^{3}H]EGCG in HT-29 human colon adenocarcinoma cells were investigated. EGCG was unstable in McCoy's 5A culture media with a half-life of less than 30 min, and the half-life increased to 130 min in the presence of cells. The major oxidative products were theasinensin (M_r 914) and another dimer with M_r 884. Addition of EGCG (50 μM) to cell culture media caused the production of H_2O_2 (up to 25 μM), and the amount was lower and gradually decreased in the presence of cells. The uptake of EGCG was concentration dependent and did not plateau, even at 640 μM, suggesting a passive diffusion process. Approximately 75% of the [^{3}H]EGCG was found in the cytoplasmic fraction when the cells were incubated with 0.5–20 μM [^{3}H]EGCG for 15 min. The membrane-associated radioactivity increased with time, apparently because of the binding of dimers to the membrane. The accumulation of [^{3}H]EGCG in the cells was significantly higher at 4°C than at 37°C. Multidrug-resistant protein inhibitors, such as indomethacin and probenecid, effectively increased the accumulation of EGCG 4″-glucuronide and 4″-methyl EGCG in the cell. These results suggest that EGCG is metabolized in the cell and that the metabolites are pumped out by MRPs. The present study provides fundamental information on the stability, uptake, biotransformation, and efflux of EGCG under cell culture conditions and suggests the need for careful interpretation of related results on the biological activities of EGCG.

INTRODUCTION

Tea (*Camellia sinensis*) is one of the most popular beverages worldwide. Among the biological activities of tea, the cancer-preventive activity has attracted the greatest attention (1). In animal models, tea administration has been shown to inhibit tumor formation at different organ sites including the skin, oral cavity, esophagus, stomach, intestine, lung, liver, pancreas, mammary gland, urinary bladder, and prostate (2–11). The active constituents and molecular mechanisms of the inhibitory action, however, are not well understood. Many publications suggest that EGCG[3] is the active compound for the cancer preventive effects. EGCG, a major water-extractable constituent of tea, has been shown to inhibit tumor formation in certain animal models and to display many anticancer related activities *in vitro*. Nevertheless, the biological activities of other polyphenolic compounds and caffeine in tea could also significantly contribute to the inhibition of tumorigenesis (1).

Much of the mechanistic information on the possible biological activities of tea is derived from studies in cell lines with EGCG. For example, EGCG has been shown to inhibit the transformation of JB6 mouse epidermal cells and preneoplastic human mammary epithelial cells (12, 13), to inhibit cell proliferation and induce apoptosis in many cell lines (14, 15), to inhibit the activity of many protein kinases (16–18), and to block the activation of transcription factors such as activator protein-1 and nuclear factor-κB (19, 20). EGCG has been shown to modulate cell cycle progression by affecting the activity or expression level of cyclins, cyclin-dependent kinases, Rb, and other regulatory proteins (21–24). In certain cell lines, EGCG and other tea polyphenols have been shown to interfere with ligand binding and subsequent phosphorylation of receptors such as EGFR, PDGFR, and other receptor protein kinases (24–27). In other cell lines, EGCG has been shown to inhibit the release of vascular endothelial growth factor, the phosphorylation of vascular endothelial growth factor receptors, and angiogenesis (28, 29). Most of these studies involved the treatment of cancer cells in culture with 20–100 μM EGCG and other tea polyphenols. These concentrations are much higher than those observed in blood or tissues (usually lower than 1 μM) after ingestion of tea by animals and humans (30, 31).

In many of the experiments *in vitro*, measurements were usually made a few days after the addition of EGCG (or other tea polyphenols) to the culture media; the stability, cell uptake, and fate of these compounds are poorly understood. Without this knowledge, it is difficult to identify the primary targets of action of these compounds and to elucidate the mechanisms of their actions. EGCG consists of a meta-5,7-dihydroxyl substituted A ring and trihydroxy phenol structures on both the B and D rings. It is a potent antioxidant and is unstable under alkaline or even neutral conditions (32). EGCG binds to various biomolecules, but its transport properties are poorly understood. To understand the action and fate of EGCG in cell culture systems, we studied the stability and uptake of [^{3}H]EGCG, as well as its biotransformation and efflux under cell culture conditions with HT-29 colon cancer cells.

MATERIALS AND METHODS

Chemicals and Cell Lines. [^{3}H]EGCG (13 Ci/mmol in ethanol containing 8 mg/ml ascorbic acid) was synthesized as described previously (33). Unlabeled EGCG was a generous gift from Unilever Bestfoods (Englewood Cliffs, NJ). Indomethacin was purchased from Cayman Chemical Company (Ann Arbor, MI). McCoy's 5A media and HBSS were purchased from Mediatech Inc. (Herndon, VA). Probenecid, vinblastine, cyclosporin A, and all of the other chemicals were from Sigma Chemical Co. (St. Louis, MO). A standard of 4″-methyl EGCG was chemically synthesized in our laboratory (34). EGCG 4″-glucuronide was enzymatically synthesized and the structure was confirmed by nuclear magnetic resonance.[4] HT-29 cells (American Type Culture Collection, Manassas, VA) at 60–100 passages were used for this study. Cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine

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[3] The abbreviations used are: EGCG, (−)-epigallocatechin-3-gallate; MRP, multidrug-resistance protein; Pgp, P-glycoprotein; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; LC, liquid chromatography; HPLC, high-performance LC; MS, mass spectrometry; MS/MS, tandem MS.

[4] J. Hong, H. Lu, X. Meng, J-H. Ryu, Y. Hara, and C. S. Yang, unpublished observations.

serum, 100 unit/ml penicillin, and 0.1 mg/ml streptomycin, at 37°C in 95% humidity and 5% CO_2.

Analysis of EGCG Stability and H_2O_2 Formation under HT-29 Cell Culture Conditions. [^{3}H]EGCG (10 μM, 0.5 μCi/ml) was incubated in HBSS or serum-free McCoy's 5A medium at 37°C, 95% humidity and 5% CO_2. At different time points, aliquots were taken and added to a stabilizing solution (1% of ascorbic acid and 0.01% EDTA in final concentration). For studies in the presence of cells, $\sim 2 \times 10^5$ HT-29 cells in growth medium were plated into each well of a 24-well plate. When the cells reached 85% confluency, cells were treated with 0.5 ml of [^{3}H]EGCG (10 μM, 0.5 μCi/ml) in serum-free McCoy's 5A medium and incubated as above. At different time points, extracellular media were taken and stabilized. After centrifugation, the media were injected onto the HPLC system. For measuring H_2O_2 formation, 50 μM EGCG was added to McCoy's 5A medium in the presence or absence of cells. At different time points, the amounts of H_2O_2 in medium were analyzed using an Amplex Red Hydrogen Peroxide assay kit (Molecular Probes, Eugene, OR).

Cellular Uptake and Distribution of EGCG. Preparation of HT-29 cells was the same as described above. After 1-h preincubation with HBSS, cells were treated with [^{3}H]EGCG (5 μM, 0.5 μCi/ml) in 0.4 ml of HBSS. At different time points, cells were washed four times with ice-cold PBS. Washable EGCG was rarely detected in the third or fourth washing with PBS. Attached cells were lysed with 200 μl of 0.2 N NaOH, and wells were washed two times with 200 μl of distilled water. The radioactivity of the pooled cell lysates was measured by a scintillation counter (Beckman LS 3801) after mixing with 4 ml of scintillation cocktail. For analyzing concentration-dependent uptake, 0.5–640-μM concentrations of [^{3}H]EGCG (1, 0.5, or 0.25 μCi/ml) in HBSS were applied. After 15-min incubation, cells were washed and analyzed as described above. For fractionation of the cells, 0.5 ml of 0.1% acidic ascorbic acid solution (pH 4) was added to the cells. After three cycles of freezing and thawing, cells were harvested by repetitive pipetting, and the wells were washed two times with 0.4 ml of the 0.1% ascorbic acid solution. Pooled mixtures were sonicated three times in an ice-cold bath sonicator (VWR Aquasonic 75D; West Chester, PA) for 3 min each at level 7, then centrifuged at 10,000 $\times$ *g* for 20 min at 4°C. Precipitates were washed with 0.4 ml of the 0.1% ascorbic acid solution and centrifuged. Pooled supernatants and precipitates are referred to as the cytoplasmic and the membrane fractions, respectively. The residues in the wells were lysed by 300 μl of 0.2 N NaOH, washed two times with 200 μl of distilled water, and combined with the membrane fraction. Protein concentration in the cell lysates was analyzed by the method of Bradford (Bio-Rad, Hercules, CA).

HPLC Analysis of EGCG and Metabolites. EGCG and its oxidative products were analyzed by a reverse phase HPLC system consisting of a Waters automated gradient controller, Hitachi L-6200 Intelligence pump, Waters 717 autosampler, Supelcosil™ LC-18 reversed-phase column (150 $\times$ 4.6-mm inner diameter; particle size, 5 μm), and a Radiomatic Flo-One/Beta radioflow detector (Radiomatic Instruments and Chemical Co., Tampa, FL; Ref. 35). In brief, the column was eluted with a linear gradient from 100% solvent A ($CH_3CN/H_2O/CH_3COOH$, 20:1970:10 v/v/v) to 91% solvent A and 9% solvent B ($CH_3CN/H_2O/CH_3COOH$, 1400:590:10 v/v/v) over a 6-min period. The gradient was then changed to 86% solvent A and 14% solvent B over a 12-min period, followed by a linear gradient to 100% solvent B over 4 min, and then 8 min of 100% solvent B at a flow rate of 1.5 ml/min. The column was maintained at ambient temperature and the autosampler at 6°C. To detect methylated and glucuronide metabolites of EGCG, samples were analyzed on a HPLC system equipped with an ESA Model 465 refrigerated autosampler, two ESA Model 580 dual-piston pumps, and an ESA 5500 coulochem electrode array system (CEAS). The column was maintained at 35 $\pm$ 0.1°C, and the autosampler at 6°C. Two solvents (A, 100 mM sodium phosphate buffer containing 1.75% acetonitrile and 0.12% tetrahydrofuran at pH 4.0; and B, 15 mM sodium phosphate buffer containing 58.5% acetonitrile and 12.5% tetrahydrofuran at pH 4.0) were used for gradient elution. At a 1.0 ml/min flow rate, the gradient was initiated with 5-min isocratic segment (100% solvent A), then changed by increasing solvent B to 20% at 13 min, 35% at 25 min, and 100% at 26 min. It was maintained at 100% from 26 to 32 min and finally changed back to 100% solvent A at 32 min. The eluate was monitored by the CEAS with potential settings at -90, 100, 300, and 500 mV.

LC/Electrospray Ionization MS Analysis of Glucuronides and Methylated EGCG. The chemical identities of the EGCG glucuronides and methylated EGCG were determined by comparing their retention times and fragment patterns on LC/MS/MS with those of standards chemically synthesized in our laboratory. The glucuronides were analyzed using our previous method with slight modifications (36), including the addition of 0.2% acetic acid to the mobile phase without supplying extra methanol in the post-column eluent. For analyzing methylated EGCG, the column was eluted with 90% solvent A (10% aqueous methanol) and 10% solvent B (70% aqueous methanol), and the linear gradient was then changed to 31% solvent B at 3 min, and 33% solvent B at 17 min. It was changed to 90% solvent B at 30 min to clean the column and then re-equilibrated to 10% solvent B from 30 to 40 min for the next run. The LC eluate was introduced into the electrospray ionization interface after UV scanning from 200 to 400 nm.

Data Analysis. Statistical significance was evaluated by the Student *t* test. *P*s less than 0.05 in the two-tailed comparison were considered statistically significant.

RESULTS

Stability of EGCG and Formation of Oxidative Products. EGCG is rather stable in the acidic pH range of 2.0–5.5. In neutral pH, however, EGCG is easily autooxidized. In HBSS (pH 7.4) at 37°C, oxidative products formed in a time-dependent manner and were readily detectable within 10 min. In a reverse phase HPLC system, EGCG was eluted at 16.3 min, and its major oxidative product peak had a retention time of 21 min, suggesting that the products are more hydrophobic or have higher molecular weights (Fig. 1*A*). The EGCG oxidative products consisted of two major compounds with Mrs of 884 and 914. On the basis of LC/MS/MS, they were tentatively identified as theasinensin (M_r 914) and another product, P2 (M_r 884) that was more abundant (Fig. 1, *B* and *C*). Both of these compounds are dimers of EGCG, which have been reported to be formed in mild alkaline fluids or after reaction with the 1,1-diphenyl-2-picryl-hydrazyl radical (37–39). The dimers displayed increased absorbance at a broad range of 350–400 nm, showing a yellow brown color.

Stability of EGCG under Culture Conditions for HT-29 Cells. The stability of EGCG under cell culture conditions was analyzed (Fig. 2). In McCoy's 5A culture medium (pH 7.4) at 37°C, EGCG (10

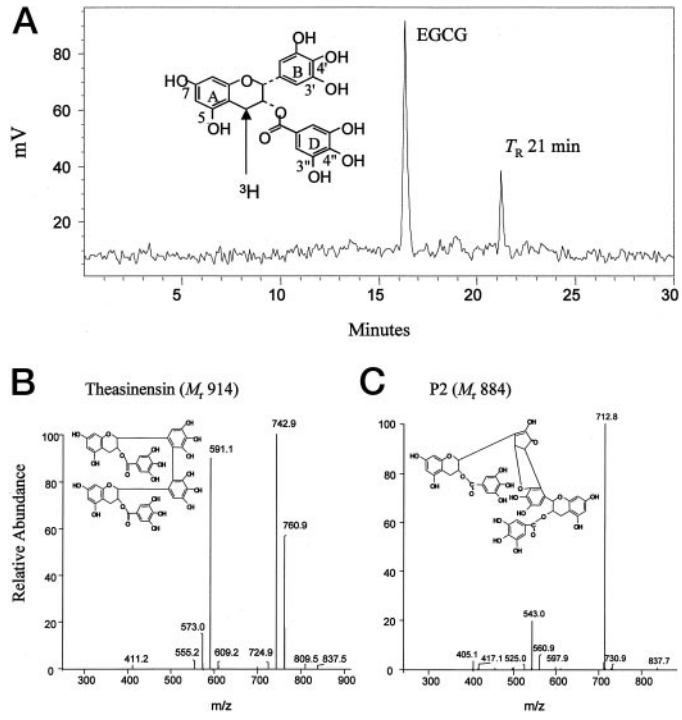

Fig. 1. HPLC chromatograms of [^{3}H]EGCG and its major oxidative products. In *A*, EGCG (5 μM) was incubated in HBSS for 15 min at 37°C and was analyzed by HPLC. In *B* and *C*, the peak with T_R (retention time) 21 min consisted of two products *Theasinensin* and *P2*) and was analyzed by LC/MS/MS.

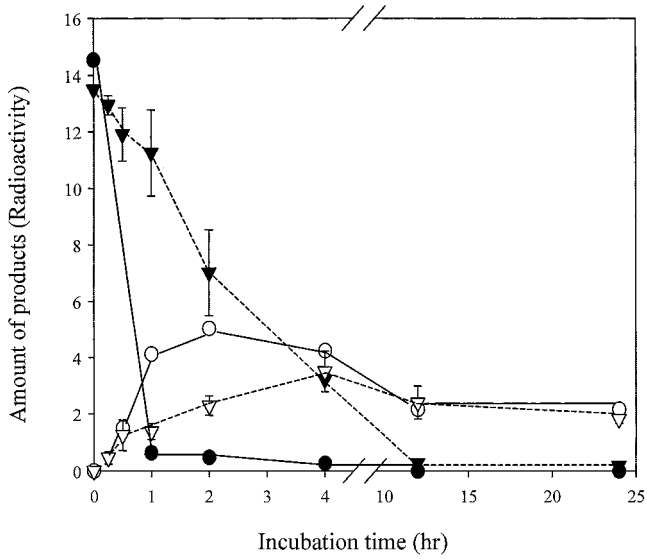

Fig. 2. Stability of EGCG under cell culture conditions. [^{3}H]EGCG (10 μM, 0.5 μCi/ml) was incubated with serum-free McCoy's 5A medium with or without HT-29 cells. At different time points, aliquots of extracellular medium were taken and analyzed by HPLC. ●, EGCG in McCoy's 5A medium; ○, compounds with T_R 21 min in McCoy's 5A medium; ▼, EGCG in the presence of cells; ▽, compounds with T_R 21 min in the presence of cells. The results in the absence and in the presence of cells are the mean of duplicated assays and average ± SD (n = 3), respectively.

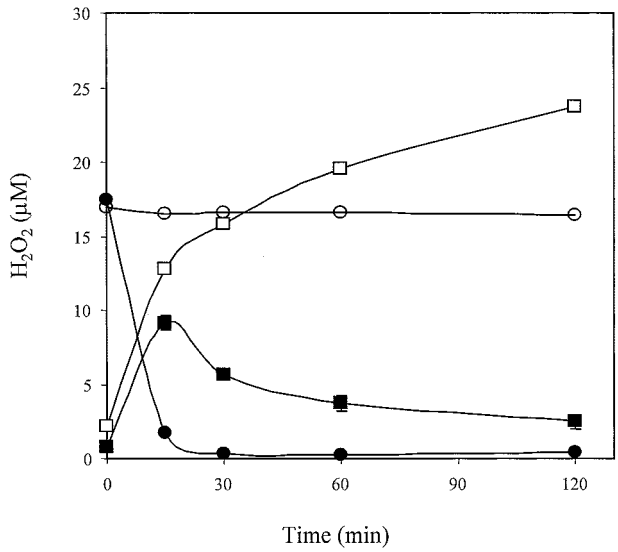

Fig. 3. Time-dependent changes of H_2O_2 under cell culture conditions. 50 μM of EGCG or 20 μM of H_2O_2 were incubated with/without HT-29 cells in McCoy's 5A medium. At different time points, medium was taken, and the amount of H_2O_2 was analyzed. ●, 20 μM of H_2O_2 in the presence of cells; ○, 20 μM of H_2O_2 in the absence of cells; ■, 50 μM of EGCG in the presence of cells; □, 50 μM of EGCG in the absence of cells. The results in the case of EGCG incubation are the mean ± SD (n = 3), and the results in the case of H_2O_2 are the mean of duplicate values.

μM) was not stable with a half-life <30 min, and more than 90% of EGCG disappeared within 1 h. The autooxidation of EGCG in McCoy's 5A medium was similar to that in HBSS with the formation of two major oxidative products as described above, except that the rate was slightly lower than in HBSS (data not shown). Formation of the oxidative products with retention time (T_R) of 21 min was also found in another cell culture medium, Ham's F-12, with a slower rate than McCoy's 5A medium (data not shown). The presence of HT-29 cells partially stabilized EGCG in McCoy's 5A culture medium to display a half-life of 130 min. Under these conditions, the two major EGCG oxidative products were also produced. The levels of these dimers peaked at 2 h in the absence of cells and at 4 h in the presence of cells. These dimers were not stable, but significant amounts were still present at 24 h.

Formation of H_2O_2 by EGCG under Cell Culture Conditions. After the addition of EGCG (50 μM) to McCoy's 5A medium, H_2O_2 formation was readily observed at 15 min (13 μM) and approached a peak of ~25 μM H_2O_2 at 2 h (Fig. 3). In the presence of cells, the apparent rate of formation of H_2O_2 was lower, reaching a plateau at 15 min (9 μM) and then gradually decreasing, but H_2O_2 still maintained extracellular levels of 4–3 μM during the period of 1–2 h. Exogenously added H_2O_2 was rapidly decomposed in the presence of cells, whereas it was relatively stable without cells. H_2O_2 was also produced when EGCG was added to other solutions, such as RPMI 1640, PBS, and distilled water (data not shown).

Uptake and Distribution of EGCG in HT-29 Cells. After adding [^{3}H]EGCG to the cell culture system, radioactivity was readily taken up by HT-29 cells, and then it was taken up at a lower rate (Fig. 4*A*). The uptake pattern was similar when the cells were in either McCoy's 5A or HBSS. The uptake of EGCG by HT-29 cells increased in a concentration-dependent manner and did not plateau, even in the range of 320–640 μM (Fig. 4*B*), suggesting a passive diffusion process. To analyze the distribution of EGCG in the cells, cytoplasm and membrane/particulate fractions were prepared by centrifugation at 10,000 × g. The EGCG associated with the membrane/particulate fractions gradually increased during a 2-h incubation period, whereas EGCG associated with cytoplasm fractions increased only slightly after 30 min (Fig. 5*A*). Membrane-bound EGCG also increased in a concentration-dependent manner, and it accounted for 24% of the EGCG associated with cells when the cells were incubated with 0.5–20 μM EGCG for 15 min (Fig. 5*B*). The portion of membrane-

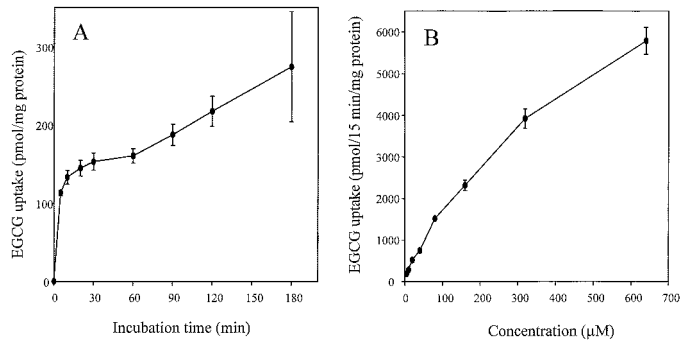

Fig. 4. Time- and concentration-dependent uptake of EGCG by HT-29 cells in HBSS. The results are the mean ± SD (n = 4). Time-dependent [^{3}H]EGCG (5 μM, 0.5 μCi/ml) uptake (*A*) and concentration-dependent uptake of [^{3}H]EGCG after 15 min (*B*).

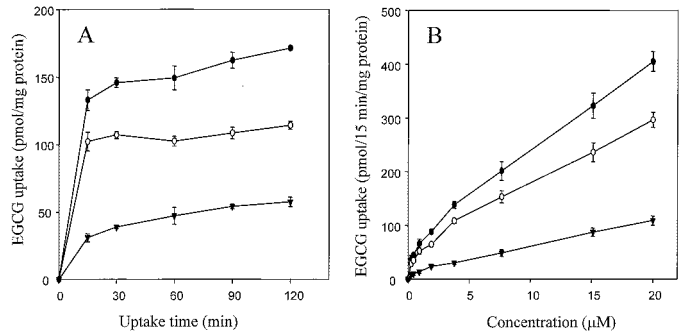

Fig. 5. Time- and concentration-dependent uptake and distribution of EGCG in HT-29 cells in HBSS. Time-dependent uptake of [^{3}H]EGCG (5 μM, 0.5 μCi/ml; *A*) and concentration-dependent uptake of [^{3}H]EGCG after 15 min (*B*) were conducted as described in "Materials and Methods." The results of total cell-associated EGCG (●), cytosolic EGCG (○), and membrane-associated EGCG (▼) are the mean ± SD (n = 4).

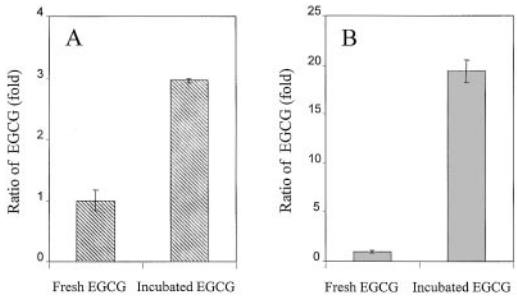

Fig. 6. Binding of EGCG and its oxidative products to cells. EGCG oxidative products were generated by 2-h incubation of [^{3}H]EGCG (5 μM, 1 μCi/ml) in McCoy's 5A medium at 37°C. The EGCG oxidative products or fresh [^{3}H]EGCG (5 μM, 1 μCi/ml) were incubated with HT-29 cells in McCoy's 5A medium containing 100 μM ascorbic acid. After 1-h incubation, cells were lysed with cell lysis buffer [1% ascorbic acid, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium PP_i, 1 mM β-glycerolphosphate, and 1 mM Na_2VO_4, in 20 mM Tris (pH 7.2)] and were sonicated. Total cell-associated radioactivity (*A*) was counted from the total cell lysates and the radioactivity in membrane fraction (*B*) was analyzed after centrifugation (10,000 $\times$ *g*). The results are shown as a ratio of the amount of incubated:fresh EGCG. The results are the mean $\pm$ SD (n = 3).

bound EGCG slightly increased to 33% at 2 h of incubation, possibly because of the binding of EGCG dimers to the membrane.

To investigate the uptake of the dimers by the cells, 5 μM [^{3}H]EGCG was incubated in McCoy's 5A medium for 2 h at 37°C, resulting in the loss of more than 95% of the [^{3}H]EGCG. About 40% of radioactivity was recovered in the dimers as the major products (Fig. 2). When the cells were incubated with this mixture, total cell uptake was about three times higher, and the membrane-bound radioactivity was 20 times higher, in comparison with results from incubation with fresh [^{3}H]EGCG (Fig. 6). The results suggest that the binding of EGCG dimers to the cell membrane is an important factor in the cell-uptake experiment. In subsequent experiments, 56 μM ascorbic acid was added to culture media to stabilize EGCG.

Effects of Temperature and Efflux Pump Inhibitors. [^{3}H]EGCG accumulation in HT-29 cells was significantly higher at 4°C than at 37°C (by over 40%), and the increase of the accumulation became more significant after 1 h than after 30 min of incubation (data not shown). This is apparently not caused by the influx of EGCG because the influx should be faster at 37°C than at 4°C. Because many foreign compounds can be pumped out by MRPs or Pgp, the effects of several inhibitors against those efflux pumps on EGCG accumulation were investigated. Indomethacin and probenecid, known MRP inhibitors, significantly increased the accumulation of [^{3}H]EGCG in HT-29 cells, whereas vinblastine, a known Pgp inhibitor, failed to increase EGCG accumulation (Fig. 7*A*). Indomethacin showed a dose-dependent effect to increase EGCG accumulation up to 10 μM (data not shown), and its effect became more obvious with the increase of incubation time (Fig. 7*B*). The results suggest that MRPs, but not Pgp, are involved in the efflux of EGCG or its metabolites formed in HT-29 cells.

Identification of EGCG Metabolites Affected by MRP Inhibitors. Glucuronides and glutathione-conjugated forms of xenobiotics have been reported to be substrates for MRPs (40). To determine the nature of the action of MRP inhibitors, the intracellular EGCG and EGCG metabolites after incubation in the presence or absence of indomethacin were analyzed by HPLC. In the presence of indomethacin, the amount of EGCG in cell lysates was not significantly affected, but the heights of the three metabolite peaks were increased (Fig. 8*A*). Peaks B and C (Fig. 8*A*) had the same HPLC retention times as EGCG 4″-glucuronide (T_R, 19.5 min) and 4″-methyl EGCG (T_R, 23 min), respectively. The structure of the metabolite with T_R of 19.5 min was confirmed by comparing the LC/MS/MS with biosynthesized EGCG 4″-glucuronide (formed after incubation of EGCG with microsomal fraction from HT-29 cells, data not shown). The results suggest that EGCG 4″-glucuronide was formed in the HT-29 cells and that its efflux was inhibited by MRP inhibitors. The metabolite with T_R of 23 min (which was eluted at 22.5 min by the current LC/MS/MS gradient) with a Mr of 472 was identical to the enzymatically synthesized 4″-methyl EGCG in LC/MS/MS (Fig. 8B). The amount of 4″-methyl EGCG in the cell lysate was more than five times higher in the presence of 10 μM indomethacin and was also significantly increased in the presence of 1 mM probenecid (data not shown). When the cells were incubated with 4″-methyl EGCG in the presence of indomethacin, accumulation of the metabolite in cells was significantly increased by 100% (data not shown), suggesting that indomethacin inhibits the efflux of 4″-methyl EGCG.

DISCUSSION

In many previous studies, EGCG was added to cell culture media, and the effects were examined 24, 48, or 72 h later. It is not known whether the action of EGCG was exerted in the first few hours or throughout the experimental period. In the present study, EGCG was found to be unstable in McCoy's 5A culture medium or in HBSS. Several factors, including pH, concentration of proteins, antioxidant levels, and the presence of metal ions, could affect the stability of EGCG, of which pH is probably the most critical. The stability of EGCG was increased in the presence of cells. It may be caused by stabilizing factors from cells such as proteins and antioxidants. In typical cell culture conditions at pH 7.2–7.4, EGCG is rapidly autooxidized leading to the formation of two major products with dimeric structures. Our results are similar to those in previous reports in that EGCG is oxidized to dimers in mild alkaline conditions (38). It was surprising that high quantities of these dimers are formed under cell culture conditions. Under our experimental conditions, the products peaked at 2–4 h, and significant amounts still remained, even after almost all of the EGCG disappeared. Because the formation of these oxidative products is believed to be a common phenomenon in cell culture conditions, their formation and biological effects should be considered in future cell culture studies.

Our results suggest that the dimers bind mainly to the cell membranes (Fig. 6). Our preliminary experiments indicate that these dimers were less effective in the inhibition of the release of arachidonic acid (and its metabolites) from HT-29 cells, in contrast to EGCG that has potent inhibitory activity in this assay. On the other hand, binding of these dimers to the cell membrane may affect

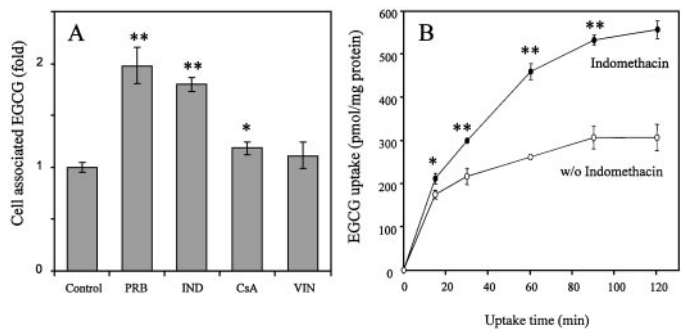

Fig. 7. Effects of efflux pump inhibitors on EGCG uptake by HT-29 cells. [^{3}H]EGCG (10 μM, 0.5 μCi/ml) was incubated with HT-29 cells in HBSS containing 56 μM ascorbic acid for 1 h in the presence of various inhibitors (*PRB*, 1 mM probenecid; *IND*, 10 μM indomethacin; *CsA*, 10 μM cyclosporin A; or *VIN*, 50 μM vinblastine) for 1 h (*A*). Time-dependent effects of indomethacin (10 μM) on EGCG uptake by HT-29 cells (*B*) were conducted under the same conditions. At different time points, radioactivity in cell lysates was counted by a scintillation counter. The results in *A* and *B* are the mean $\pm$ SD (n = 4). *, $P < 0.05$; **, $P < 0.01$.

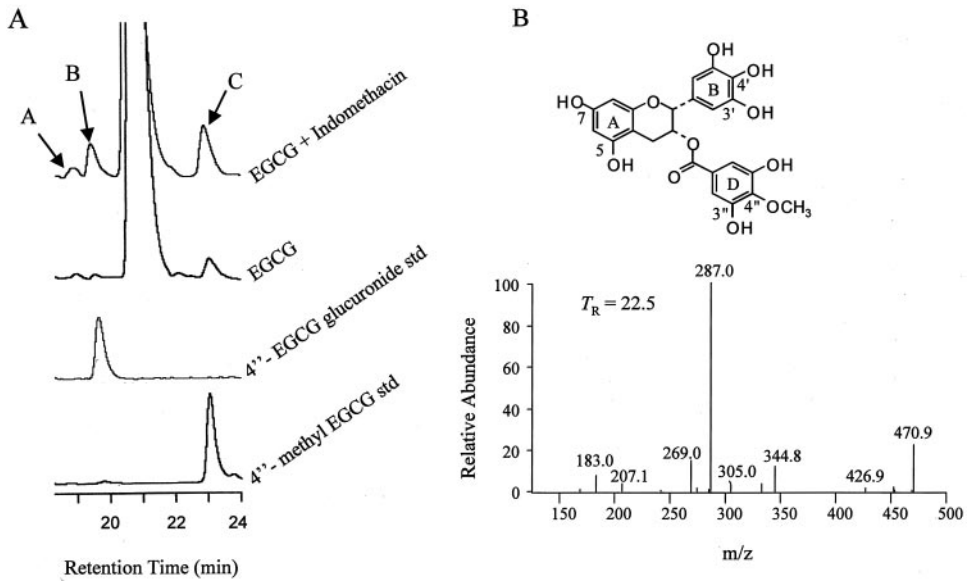

Fig. 8. Identification of accumulated EGCG metabolites in the presence of indomethacin. Unlabeled EGCG (10 μM) was incubated with HT-29 cells in the presence of indomethacin (10 μM) in HBSS. After 2-h incubation, the cell homogenates were prepared as described in "Materials and Methods," and injected onto HPLC. The HPLC results (*A*) were a representative of three independent experiments that showed the same pattern. For the identification of accumulated metabolites, the same preparations were analyzed by LC/MS/MS (*B*).

receptor functions. It would be interesting to determine whether the previously reported EGCG effect on ligand binding to EGFR and PDGFR (24, 25) is caused by the action of EGCG or the EGCG dimers formed in the incubation. These dimers may also disrupt the membrane structure or cause membrane fusion similar to that we proposed for the action of theaflavin 3,3′-digallate (16). Because theasinensin, one of the dimers formed, has been reported to induce apoptosis through activation of caspase-3 and -9 (41), the biological effects of dimers should be clarified in mechanistic studies with EGCG. Kitano *et al.* (42) have suggested that EGCG causes "a cell sealing effect." Presumably, this was attributable to the observation of a colored cell surface (also observed in our previous experiment) and was possibly caused by the binding of the dimers. It remains to be determined whether their observed biological effect is attributable to the binding of dimers to the cell membrane or attributable to EGCG.

EGCG produced substantial amounts of H_2O_2 under cell culture conditions. This result is similar to the previous observations that H_2O_2 was formed after the addition of EGCG to several culture media such as DMEM, RPMI 1640, and McCoy's 5A (43). Because exogenously added H_2O_2 was rapidly decreased in the presence of cells, the H_2O_2 formed in the presence of EGCG is believed to be decomposed in cells through the action of glutathione peroxidase and catalase. H_2O_2 formed by EGCG, however, may exert biological effects on cells. Our previous results indicated that 30 and 100 μM EGCG induced apoptosis in H661 lung cancer cells and that this effect was abolished by the addition of catalase (50 units/ml) in the culture medium (14). In a second study, formation of H_2O_2 was measured in the presence of 30 μM EGCG under cell culture conditions for Ha-*ras*-transformed 21BES human bronchial epithelial cells, and the apoptotic effect of EGCG was partially eliminated by catalase (44). Therefore, results from *in vitro* studies with EGCG, especially at high concentrations, should be interpreted with caution to clearly distinguish between the direct effects of EGCG- and H_2O_2-mediated effects.

Uptake of [^{3}H]EGCG by HT-29 cells displayed a concentration-dependent increase and did not plateau, even at 640 μM, suggesting that EGCG uptake mainly occurs by a passive diffusion process. Inside the cells, EGCG appears to be more stable chemically and is biotransformed to glucuronidated and methylated products. The biological activities of these compounds remain to be determined. These metabolites are pumped out of the cells. The involvement of efflux pumps in determining the accumulation of [^{3}H]EGCG is supported by two observations: (*a*) the accumulation of EGCG in HT-29 cells was significantly higher at 4°C than at 37°C, suggesting the involvement of an energy-dependent efflux; and (*b*) the MRP inhibitors indomethacin and probenecid significantly increased the accumulation of EGCG. HT-29 cells have been reported to express MRPs (45), but the expression of Pgp is not conclusive (46, 47). Recently, it was reported that green tea catechins, including EGCG, inhibited Pgp (48). The lack of an effect of vinblastine, a Pgp inhibitor, in our experiment may be because of the absence of Pgp, but this point needs to be investigated further.

EGCG metabolites such as 4″-EGCG glucuronide could be substrates of MRPs. 4″-methyl EGCG was also accumulated in the presence of indomethacin and probenecid (Fig. 8). It remains to be determined whether 4″-methyl EGCG is a substrate of MRP or whether EGCG is accumulated by other mechanisms. If EGCG metabolites are substrates of MRPs, they may be expected to serve as competitive substrates and increase the efficacy of therapeutic agents the efflux of which is mediated by MRPs.

On the basis of the present results, several factors need to be considered in understanding the action of EGCG *in vitro*. EGCG is not stable in cell culture systems, and its oxidation leads to the formation of dimers and H_2O_2; both could have significant biological effects. EGCG appears to be transported into the cell by passive diffusion and is subsequently converted to the methylated metabolites and glucuronides. EGCG could exert its activity by binding to receptors such as EGFR and PDGFR (24, 25) or, intracellularly, by inhibiting protein kinases such as extracellular signal-regulated kinases (16, 19). EGCG metabolites may also be biologically active; they are pumped out by MRPs. We hope that this information will provide a base for understanding the molecular mechanisms of the biological effects of EGCG and, perhaps, other polyphenolic compounds in cell culture systems. Because of the low oxygen tension *in vivo*, the formation of H_2O_2 and dimer may not be significant, but this issue needs to be investigated.

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Stability, Cellular Uptake, Biotransformation, and Efflux of Tea Polyphenol (–)-Epigallocatechin-3-Gallate in HT-29 Human Colon Adenocarcinoma Cells

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