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Time-dependent intracellular trafficking of FITC-conjugated epigallocatechin-3-*O*-gallate in L-929 cells

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ABSTRACT

Many in vitro studies about green tea polyphenol, (−)-epigallocatechin-3-*O*-gallate (EGCG) focused on its pro-apoptotic and anti-proliferative effects on various types of cancer cells, while less attention has been paid to its incorporation into the cytoplasm and nuclear translocation. This study concentrated on the time-dependent intracellular trafficking of EGCG in L-929 cells. EGCG was conjugated with fluorescein-4-isothiocyanate (FITC) via the 3″-OH or 5″-OH group, as confirmed by NMR analysis, and then treated to either suspended or cultured cells. Confocal microscopic observations revealed that FITC-EGCG was clearly seen onto the membrane of suspended cells as well as into the cytoplasm and nucleus within 1 h. As an increase in treatment time, it concentrated on the nucleus and then was located at any places of the cells. The cellular uptake of FITC-EGCG in cultured cells was not observed until 1 h of culture, but started to be observed after at least 2 h. These results imply that although the cellular sensitivity and response to EGCG would be different from those of FITC-EGCG, it would be incorporated into the cytoplasm of cells and further be translocated into the nucleus in a time-dependent manner.

1. Introduction

Conventional studies have shown that green tea catechins have potent suppressive effects on cellular responses of various cancer cells and their intracellular signaling cascades.[1–3] and references therein Most of this anti-cancer activity of green tea are believed to be mediated by its major polyphenolic antioxidant constituent, (−)-epigallocatechin-3-*O*-gallate (EGCG).[4–6] Furthermore, some previous reports have demonstrated the incorporation of EGCG into the cytosol and/or even the nucleus of cells as well as its tissue distribution and metabolism in animals by using radioisotope-labeled EGCG, for example, [^{3}H]EGCG.[7–12] This cellular internalization of EGCG is considered to be involved in the mechanism of the anti-cancer activity of EGCG as a possible chemopreventive agent, since catechins exert pro-apoptotic and anti-proliferative activities on cancer cells.

Although the biological effects of EGCG have been extensively investigated and believed to be mainly due to its potent antioxidant activities, its time-dependent intracellular trafficking is still unclear. In these previous studies, EGCG was added to cell culture media, and the effects were examined 24, 48 or even 72 h later. It is not known whether the action of EGCG was exerted in the first few hours or throughout the experimental period. Our earlier study also demonstrated the suppressive effects of EGCG on vascular smooth muscle cells after 48 h.[13] In the present study, the binding of EGCG onto membranes, its incorporation into cytoplasm and subsequent translocation into nucleus in suspended and cultured L-929 cells for 4 or 8 h were demonstrated by FITC-conjugation, which would be exploited to give a clue to identify the primary targets and action mechanisms of EGCG.

2. Results

2.1. Characterization of EGCG conjugated with FITC

Figure 1 shows representative HPLC profiles of free FITC, EGCG and their conjugate, FITC-EGCG, submitted to HPLC analysis with UV detection at 280 nm.[14] One major peak and other minor were shown on the chromatogram of FITC-EGCG, which were named as peak 1 and 2, respectively (Fig. 1C). Peak 1 was identified as EGCG by comparison with the chromatogram of EGCG and its authentic standard.[15] Since the column used in HPLC was not

Abbreviations: EGCG, (−)-epigallocatechin-3-*O*-gallate; FITC, fluorescein-4-isothiocyanate; FITC-EGCG, FITC-conjugated EGCG; PBS, phosphate-buffered saline; PI, propidium iodide; UV, ultraviolet.

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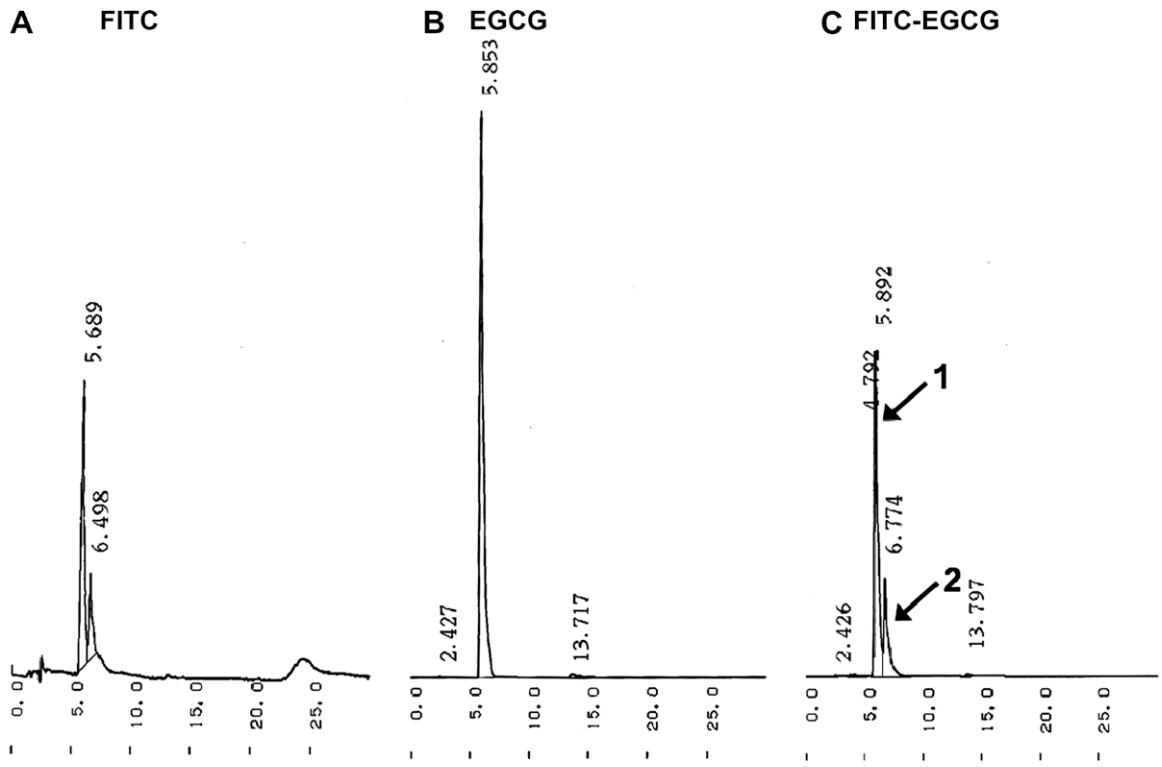

Figure 1. HPLC chromatograms of free FITC (A), EGCG (B) and FITC-EGCG (C). The HPLC conditions were described under Section 4.

suitable to differentiate free FITC from FITC-EGCG, it was not clear that peak 2 corresponded to the FITC-conjugate. From these results, it was concluded that the conjugation of EGCG with FITC could not be confirmed by HPLC analysis.

^{1}H NMR analysis was carried out to obtain structural information and confirm the conjugation of EGCG with FITC. Figure 2 shows the NMR results obtained for free FITC, EGCG and their conjugate, FITC-EGCG. A tentative assignment of free EGCG was carried out by comparison with literature data.[16,17] There was no significant variation in the chemical shift values of the protons in free FITC (Fig. 2A). A comparison between NMR spectrum of free EGCG (Fig. 2B) with that of its FITC-conjugate (Fig. 2C) allowed the assignment of the proton signals of the conjugate and showed the existence of newly formed signals upon going from the free compound to the conjugated form. New proton signals were located at 7.330 and 7.220 ppm in FITC-EGCG, suggesting that the conjugation to FITC might occur via the 3″-OH or 5″-OH groups, instead of the usual conjugation mode via the 3′-OH or 5′-OH positions.[18,19] On the other hand, the conjugation of EGCG with FITC leaded to split of C-2″,6″ signal as well as slight shift in the δ (ppm) values ranging from 0.05 to 0.15 ppm, although significant variations in the chemical shift values were not observed. Additionally, the signal of C-2′,6′ shifted from 6.557 ppm in free EGCG to 6.628 ppm in the conjugate. C-2, C-3 and C-4 signals in free EGCG disappeared after conjugation, but there were no significant changes in the spectral profile of EGCG. Therefore, when comparing Figure 2A–C, the formation of new proton signals in C-3″ or C-5″ of FITC-EGCG is evident and strongly suggests that the conjugation might occur via 3″-OH or 5″-OH group in the gallate ring of EGCG.

2.2. Cytotoxicity profiles

Cytotoxicity profiles of free FITC, EGCG and FITC-EGCG were determined in L-929 cells by WST-8 assay (Fig. 3). The cells exposed to free FITC for 24 h showed a dose-dependent decrease in relative cell viability with the IC_{50} value of 700 μM (Fig. 3A). In the cells treated with increasing concentrations of EGCG, the value of IC_{50} was found to be approximately 690 μM (Fig. 3B). However, FITC-EGCG was more cytotoxic to the cells and its IC_{50} value was about 325 μM (Fig. 3C). This result implies that the conjugation of EGCG with FITC induced a conformational change in EGCG and resulted in quite different property from EGCG, rather than a synergistic effect in cytotoxicity.

2.3. Intracellular trafficking of FITC-EGCG in suspended cells

Employing L-929 cells in suspensions (Fig. 4) treated with slightly toxic concentration (65 μM) of FITC-EGCG, the intracellular trafficking of FITC-EGCG, namely the incorporation of FITC-EGCG into cytoplasm and its subsequent nuclear translocation were observed by confocal microscopy. Compared with the non-treated controls (Fig. 4A), FITC-EGCG was seen mainly onto the membrane of the cells and into the nucleus after 0.5 h of treatment (Fig. 4B). After 1 h, it further concentrated on the nucleus while being slightly distributed into the cytoplasm (Fig. 4C). Then, it was located at any places of the cells including the cytosol after 4 h (Fig. 4D). This result could be more confirmed by Supplemental movies of suspended L-929 cells after 1 and 4 h of treatment with FITC-EGCG. It was found that FITC (50 μM) alone could not penetrate into the suspended cells (Fig. 4E).

2.4. Internalization of FITC-EGCG into cultured cells

The internalization of FITC-EGCG could be also observed in the mono-layered culture of L-929 cells treated with 130 μM FITC-EGCG (Fig. 5). The cellular uptake of FITC-EGCG was not observed until around 1 h of culture, but started to be observed between 2 and 4 h (Fig. 5A). Some cells showed severe alterations in the size and morphology due to cytotoxicity of FITC-EGCG, but others did not (Fig. 5B). As the progress in the incubation time, it was clearly

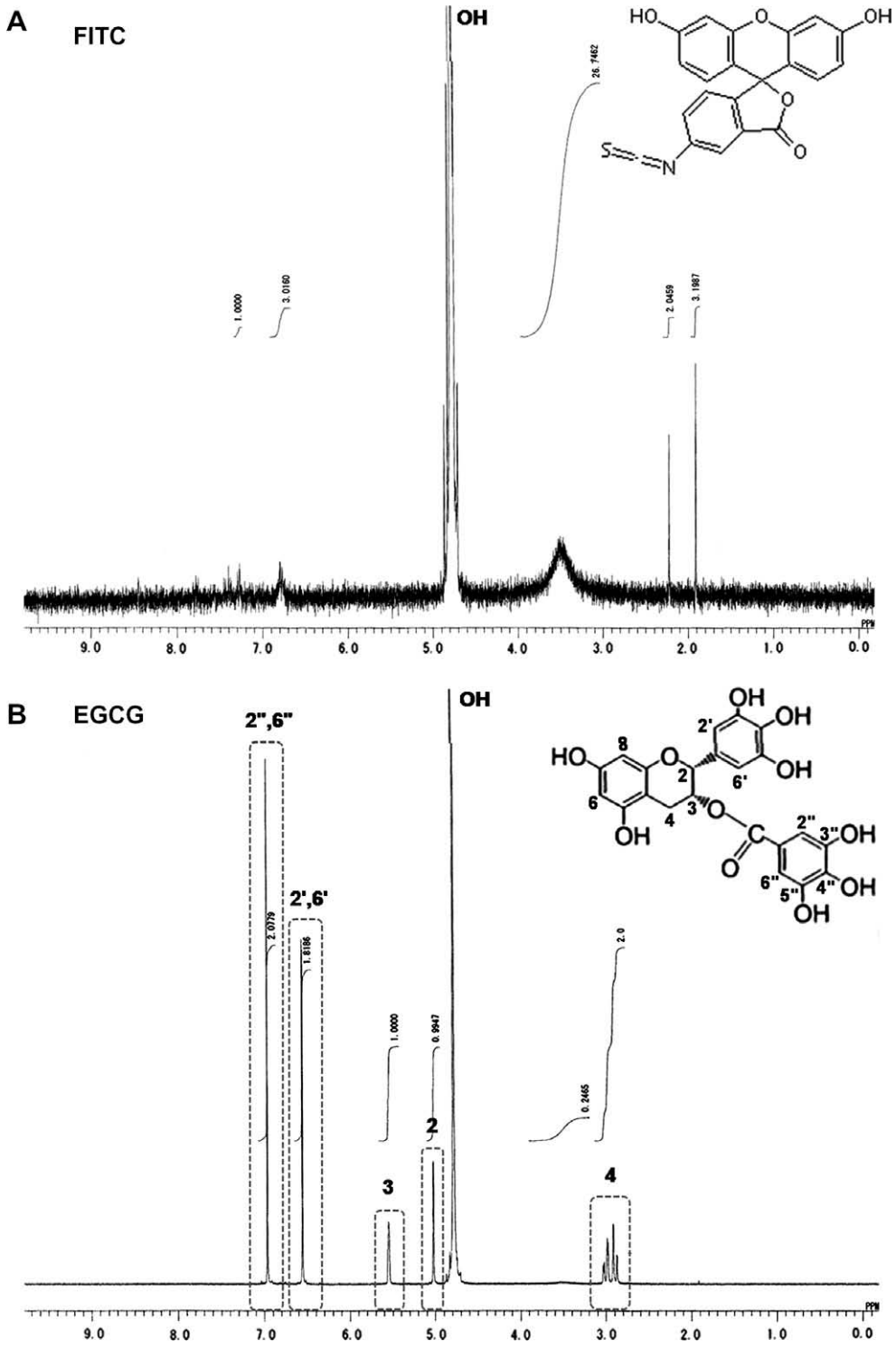

Figure 2. [1]H NMR spectra of free FITC (A), EGCG (B) and FITC-EGCG (C). The NMR analysis was performed under Section 4.

observed into the cytoplasm or even the nucleus, particularly with intensive bright green and red fluorescence (Fig. 5C). Among the cells incubated with FITC-EGCG for more than 8 h, some cells highly sensitive to FITC-EGCG showed typical apoptotic appearances, including blebbing, nucleus swelling and changes to the cell membrane such as loss of membrane asymmetry and attachment (Fig. 5D). After 24 h of treatment, FITC-EGCG was still incorporated into the cytoplasm of the detached cells, but the nucleus was not able to be delineated due to apoptosis (Fig. 5E). FITC (50 μM) alone could not only enter the cultured cells, but also penetrate into EGCG-pretreated cells as shown in the below fluorescence micrographs (Supplementary Fig. S1).

3. Discussion

As a great deal of attention has been focused on various beneficial activities of EGCG, exact information about the time-dependent intracellular trafficking of this compound is required in

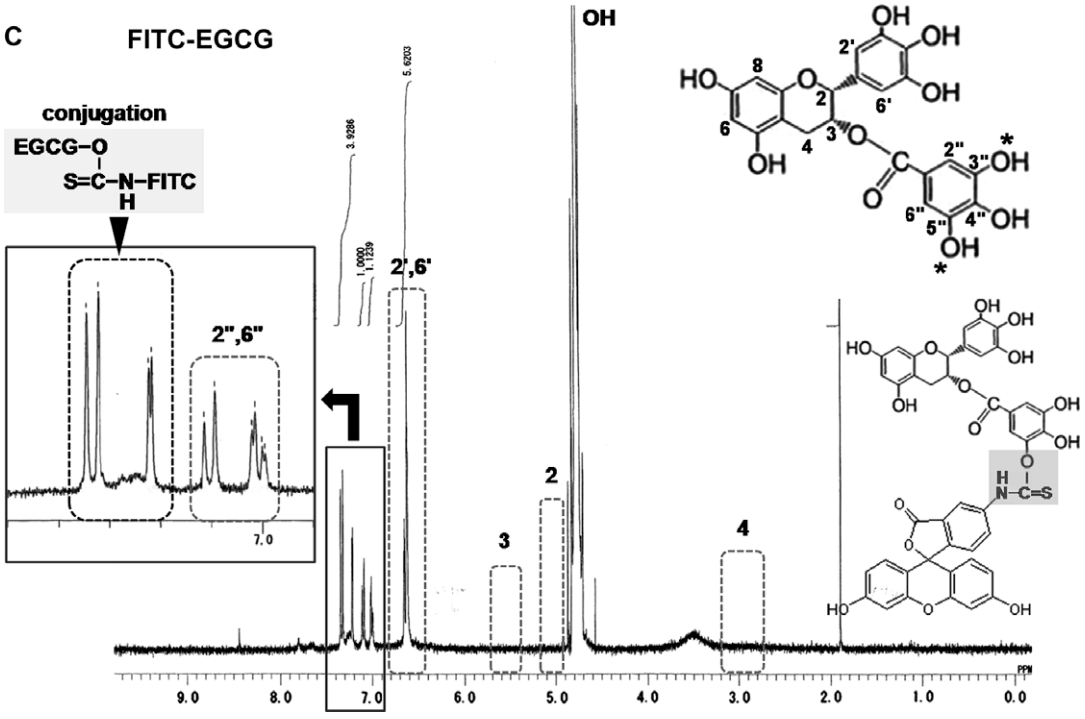

Fig. 2 (*continued*)

order to elucidate its primary targets of action and precise mechanisms of its actions. For this purpose, EGCG was conjugated with FITC via 3″-OH or 5″-OH group in the gallate ring of EGCG, which was confirmed by NMR analysis (Fig. 2).

The binding of FITC-EGCG onto membranes, its incorporation into cytoplasm and subsequent translocation into nucleus were clearly observed in L-929 cells. In suspended cells (Fig. 4), FITC-EGCG was incorporated into the cytoplasm via binding of the membrane and further translocated into the nucleus of cells in a time-dependent manner. Interestingly, a colored cell surface was observed and possibly attributable to the binding of EGCG to membranes. The cellular uptake of FITC-EGCG in the cultured cells started to be observed after at least 2 h (Fig. 5). As an increase in treatment time, internalized FITC-EGCG triggered apoptosis of the cells. However, the incorporation of FITC-EGCG into cells was appreciably decreased by pretreatment of free EGCG (data not shown), suggesting that the conjugate will less accumulate within cells by virtue of competition with unlabeled EGCG for membrane binding sites. Moreover, it has been shown that the cell membrane is not permeable for FITC, a frequently used derivatizing reagent for laser-induced fluorescence detection and not able to penetrate into viable cells by itself.[20,21] These reports coincide with our results showing that FITC alone could enter neither the suspended nor cultured cells (Fig. 4E and Supplementary Fig. S1). These results imply that although the cellular response to EGCG and its binding pattern to membrane receptors would be different from those of FITC-EGCG, it might penetrate into the cytoplasm of cells thru binding to specific receptors onto the membrane and forming complexes with the receptors, and further be translocated into the nucleus in a time-dependent manner. These phenomena might be related to the intrinsic characteristics of polyphenolic compound, EGCG. It has already been shown that this compound, due to its amphipathic properties, readily binds to extracellular matrices, lipid membranes and any types of intracellular proteins.[22–24] Therefore, this compound would be allowed to penetrate into cell cytoplasm and further to be translocated into nucleus, leading to modulation of the exogenous signals directed to genes required for the survival or apoptosis of cells.

Although the exact mechanism of the internalization of EGCG into cells had not been elucidated yet, some evidence has been reported using various cancer cell lines treated with [^{3}H]EGCG. Okabe et al. demonstrated by microautoradiography that [^{3}H]EGCG was bound to the membrane as well as incorporated into the cytoplasm and the nucleus of PC-9 cells and that the radioactivity of the cells was increased time-dependently.[7] Furthermore, it was shown that the uptake of [^{3}H]EGCG by HT-29 cells displayed a concentration-dependent increase and did not plateau, suggesting that EGCG uptake mainly occurs by a passive diffusion process.[8] Those quantitative results using [^{3}H]EGCG well agreed with our qualitative results showing that the intracellular trafficking of EGCG in L-929 cells took place time-dependently.

Consistently, recent study has demonstrated that expression of the metastasis-associated 67 kDa laminin receptor confers EGCG responsiveness to cancer cells at physiologically relevant concentrations, suggesting that the gallate moiety of EGCG may be critical for receptor binding and subsequent activity.[3,25] Moreover, the observation that nucleic acids extracted from catechin-treated cells were colored implied that because both galloyl and catechol groups of EGCG were essential for DNA binding, both groups seemed to hold strands of DNA via their branching structure.[26] Nevertheless, it would remain to be determined whether conjugation with FITC via –OH group in the gallate ring of EGCG adversely affects the receptor binding of EGCG and its subsequent activity or not.

Collectively, the findings provide support to a scenario in which the intracellular trafficking of EGCG takes place according to such cascades as follows. First, EGCG freely binds to membrane receptors on cells, resulting in the formation of EGCG-receptor complexes. Next, it becomes to be internalized into cytoplasm thru unknown mechanism, which would affect various signal factors responsible for regulating transcription. Evidence that EGCG causes internalization of the epidermal growth factor receptor in human

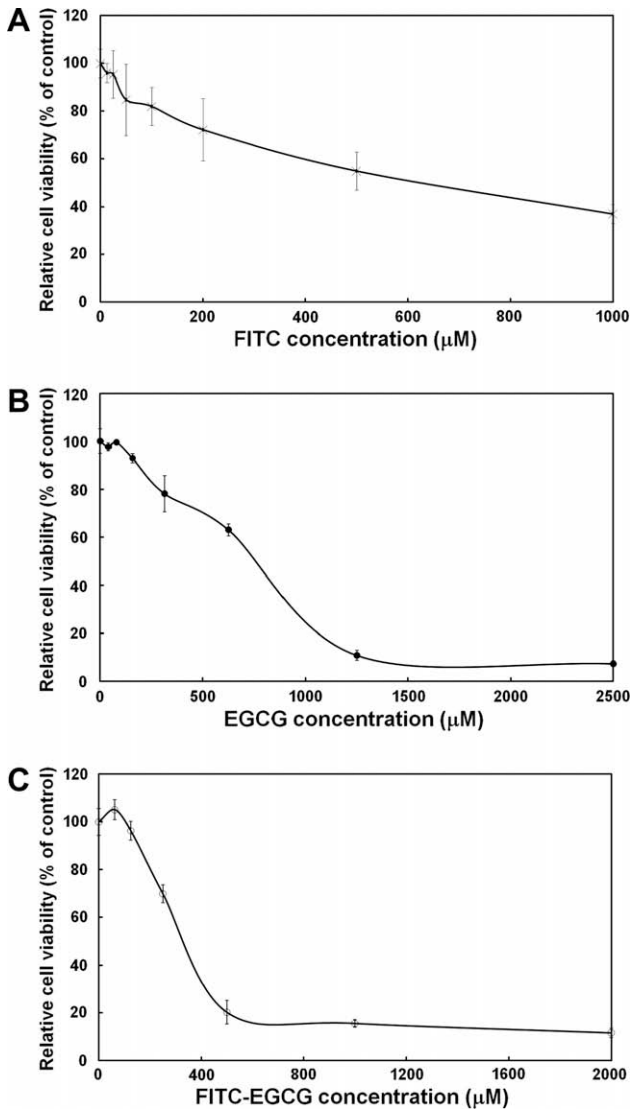

Figure 3. Cytotoxicity profiles of free FITC (A), EGCG (B) and FITC-EGCG (C) to L-929 cells. The relative cell viability (%) was measured by WST-8 assay, as described in Section 4.

colon cancer cells may explain, at least in part, that mechanism.[27] Afterwards, its translocation into nucleus occurs via unknown pathways, which might be partly explained by the fact that a phytoestrogen molecule, structurally related with catechin, binds to an estrogen receptor and then moves to the nucleus thru nuclear pores.[28] Finally, it regulates various genes related to cellular responses including proliferation and cell cycle progression. Further study about what signal transduction pathways are involved in regulating cell cycle-related genes is required, and the exact mechanism needs to be elucidated. Since this is the first observation to visualize the intracellular trafficking of EGCG, it is believed that the results may be exploited to understand the mechanism of anti-cancer effects of EGCG as well as to craft strategies for chemoprevention and/or therapy against cancer by EGCG.

4. Materials and methods

4.1. Conjugation of EGCG with FITC

EGCG, the major polyphenolic component of green tea, was purchased from DSM Nutritional Products Ltd. (Teavigo™, Basel, Switzerland).[29] In order to visualize the intracellular trafficking of EGCG, it was conjugated with fluorescein-4-isothiocyanate (FITC, Dojindo Lab., Kumamoto, Japan) by using a modified labeling method as reported previously.[30] In brief, EGCG (1 g) was dissolved in 100 ml of 0.2 M K_2HPO_4 (pH 9.2) and incubated for 72 h at 37 °C with FITC (60 mg). After incubation, the conjugate was extracted with 100 ml of ethyl acetate and then evaporated at 50 °C. It was then dissolved in excess double distilled water and recovered by freeze-drying. The resultant product was regarded as FITC-conjugated EGCG (FITC-EGCG) and quite stable, confirming that it did not exchange with water or phosphate-buffered saline (PBS, pH 7.4) over a period of 24 h. The purity of the product was found to be more than 85% by analytical HPLC and LC/MS (Supplementary Fig. S2). The analytical HPLC was performed in a JASCO liquid chromatograph apparatus with a JASCO 870 ultraviolet (UV) detector. Following are the conditions: column, CAPCELL PAK C18 UG120 (Shiseido Co., Ltd., 250 mm × 4.6 mm i.d., 5 μm, 110 Å); mobile phase, 50% aqueous acetonitrile containing 0.01% acetic acid; flow rate, 1.0 ml/min; injection volume, 20 μl; temperature, 35 °C; detection, UV 280 nm.

4.2. HPLC analysis

FITC-EGCG was detected by HPLC analysis. The analysis was performed on a Shimadzu (Kyoto, Japan) LC-10ADvp equipped with an SPD-10Avp UV detector, a DGU-12A degasser, an SCL-10Avp system controller, a C-R&A chromatopac data recorder and a CTO-20A column oven. A Shim-pack VP-ODS column (150 mm × 4.6 mm i.d., 5 μm, 110 Å) protected by a pre-column GVP-ODS (10 mm × 4.6 mm i.d.) both from Shimadzu, was used at 40 °C throughout this study. The mobile phase was composed of 10 mM phosphate buffer: acetonitrile (85:15 v/v), and the aqueous phase was adjusted to pH 2.5 with phosphoric acid. A flow rate was 1.0 ml/min, an injection volume was 20 μl, and peaks were detected at 280 nm.

4.3. NMR analysis

Conjugation of EGCG with FITC was further confirmed by NMR analysis. ^{1}H NMR spectra were obtained at 300 MHz with a JEOL JNM-LA400 spectrometer (Tokyo, Japan) at 25 °C in DMSO-d6. Chemical shift values are reported relative to tetramethyl silane (TMS, δ = 0.00 ppm) in carbon tetrachloride as an external standard inserted into an NMR tube (ϕ = 5 mm) with a coaxial cell. The digital resolution of the ^{1}H NMR spectral data was 0.1 Hz.

4.4. Cell culture and conditions

A murine fibroblast cell line (L-929 cells from subcutaneous connective tissue) was obtained from American Type Culture Collection (CCL-1, Rockville, MD). The cells were routinely maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma-Aldrich Co.) and 1% antibiotic antimycotic solution (including 10,000 U penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml, Sigma-Aldrich Co.) at 37 °C in 95% humidity and 5% CO_2. Studies were performed with the cells at 30–50 passages.

4.5. Cytotoxicity assay

The number of viable cells was quantified indirectly using a highly water soluble tetrazolium salt [WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono-sodium salt] (Dojindo Lab., Kumamoto, Japan), reduced to formazan dye by mitochondrial dehydrogenases. Cell viability was found to be directly proportional to the metabolic

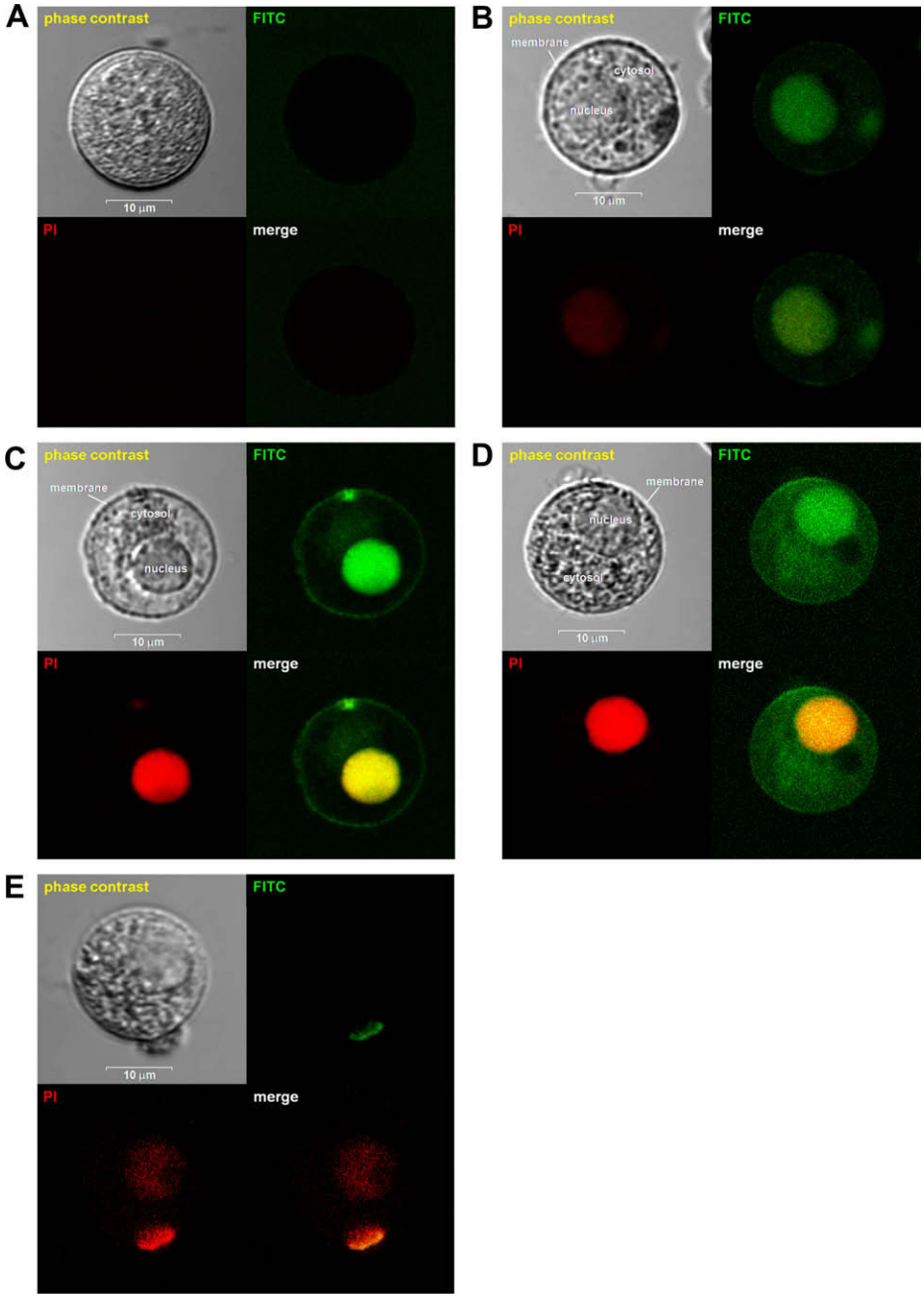

Figure 4. Confocal microscopic photographs of suspended L-929 cells 0 h (A), 0.5 h (B), 1 h (A) and 4 h (D) after treatment with 65 μM FITC-EGCG or over 4 h after 50 μM FITC treatment (E). All the photographs shown in this figure are representative of four independent experiments, showing similar results (original magnification, 1200×).

reaction products obtained in WST-8. Briefly, WST-8 assays were conducted as follows. L-929 cells were incubated with WST-8 for the last 4 h of the culture period (24 h) at 37 °C in the dark. In order to avoid a direct reaction between antioxidant, FITC-EGCG and WST-8 to be reduced, the excess antioxidant was completely removed and the medium was exchanged before adding WST-8. For comparison, free FITC and EGCG were also assayed for their cytotoxicity. Parallel sets of wells containing freshly cultured non-treated cells were regarded as the controls. Absorbance was determined at 450 nm using an ELISA reader (Spectra Max 340, Molecular Device Co., Sunnyvale, CA). The relative cell viability was determined as percentage of the optical densities in the medium containing serially diluted concentrations of FITC-EGCG to the optical densities in the fresh control medium. The LD_{50}, the concentration (%) inhibiting growth of cells by 50%, was estimated from the relative cell viability.

4.6. Confocal laser scanning microscopy

Preparation of L-929 cells was the same as described above. After 20–30 min of preincubation in PBS, suspended cells were treated with FITC-EGCG (65 μM) in 0.5 ml of PBS for 0.5–4 h. At different time points, the cell suspension was washed three times with PBS and then centrifuged at 250g for 5 min at 4 °C. After centrifugation, the cell pellets were re-suspended in PBS and fixed with 3.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7) for 5 min at room temperature. The incorporation of FITC-EGCG into the cytoplasm of the cells and its subsequent nuclear translocation

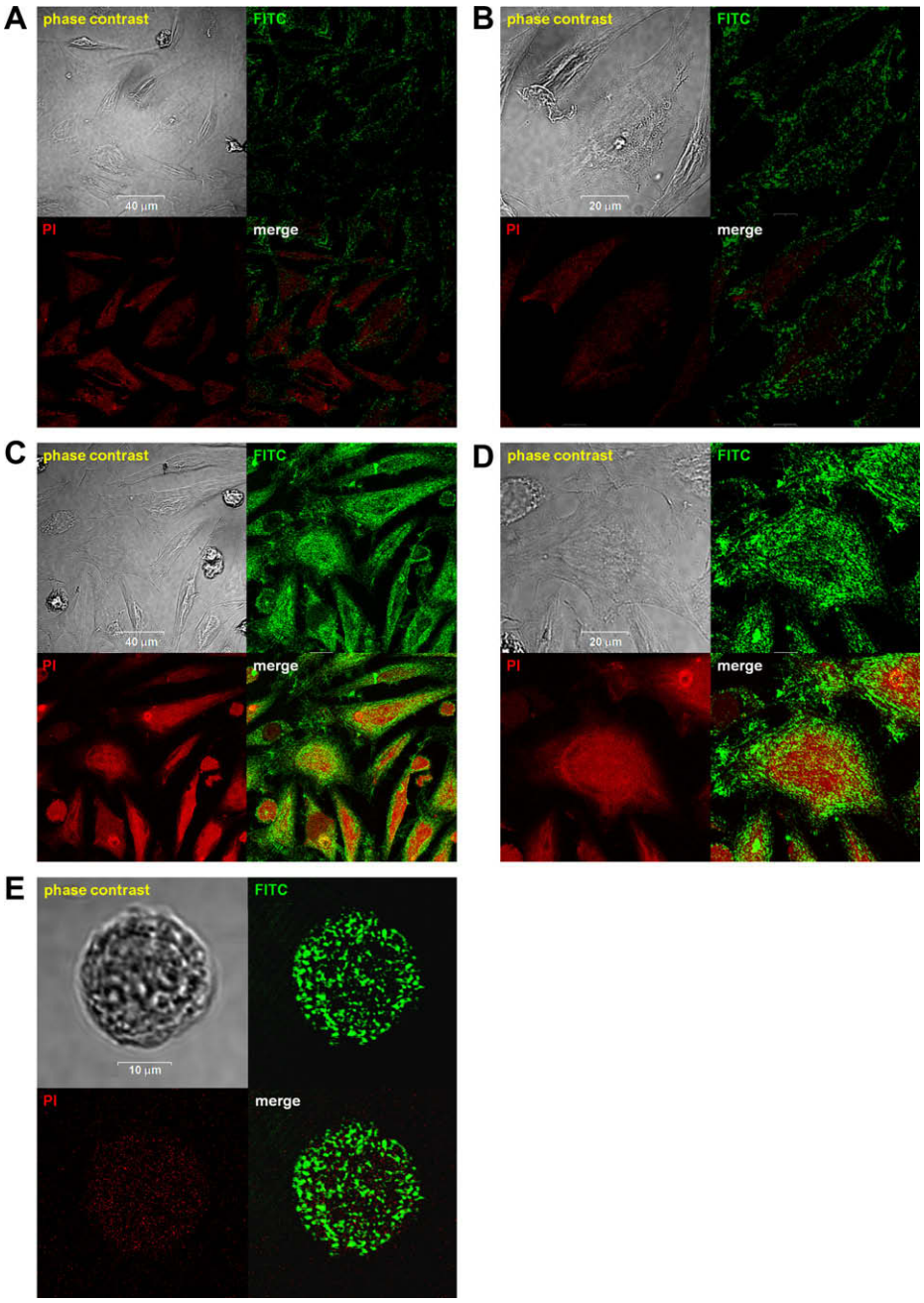

Figure 5. Confocal microscopic photographs of cultured L-929 cells 2–4 h (A and B), 8 h (C and D) and 24 h (E) after treatment with 130 μM FITC-EGCG. All the photographs shown in this figure are representative of four independent experiments, showing similar results (original magnification, 400× in (A) and (C); 800× in (B) and (D); 1500× in (E)).

were observed under a confocal laser scanning microscope (LSM 510, Carl Zeiss Advanced Imaging Microscopy, Jena, Germany). For comparison, the suspended cells were treated with free FITC (50 μM) and then observed. Cell nuclei were counterstained with 5 μM propidium iodide (PI, Sigma-Aldrich Co.) directly before 3–5 min of observation. For studies in cultured cells, $\sim 2 \times 10^5$ L-929 cells in growth medium were plated into each well of a 24-well plate. When the cells reached 80–85% confluency, they were treated with 0.5 ml of FITC-EGCG (130 μM in PBS), incubated for 2–24 h and fixed with 3.5% paraformaldehyde as described above. At different time points, the cell cultures were examined for the time-dependent intracellular trafficking of FITC-EGCG by confocal microscopy. Cell nuclei were counterstained with 5 μM PI as described above. The concentration of FITC-EGCG treated to either suspended or cultured cells was predetermined from its cytotoxicity profile.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.10.009.

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