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Effects of folate cycle disruption by the green tea polyphenol epigallocatechin-3-gallate

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Abstract

We demonstrate that the tea polyphenol, epigallocatechin-3-gallate, is an efficient inhibitor of human dihydrofolate reductase. Like other antifolate compounds, epigallocatechin-3-gallate acts by disturbing folic acid metabolism in cells, causing the inhibition of DNA and RNA synthesis and altering DNA methylation. Epigallocatechin-3-gallate was seen to inhibit the growth of a human colon carcinoma cell line in a concentration and time dependent manner. Rescue experiments using leucovorin and hypoxanthine–thymine medium were the first indication that epigallocatechin-3-gallate could disturb the folate metabolism within cells. Epigallocatechin-3-gallate increased the uptake of [^{3}H]-thymidine and showed synergy with 5-fluorouracil, while its inhibitory action was strengthened after treatment with hypoxanthine, which indicates that epigallocatechin-3-gallate decreases the cellular production of nucleotides, thus, disturbing DNA and RNA synthesis. In addition to its effects on nucleotide biosynthesis, antifolate treatment has been linked to a decrease in cellular methylation. Here, we observed that epigallocatechin-3-gallate altered the *p16* methylation pattern from methylated to unmethylated as a result of folic acid deprivation. Finally, we demonstrate that epigallocatechin-3-gallate causes adenosine to be released from the cells because it disrupts the purine metabolism. By binding to its specific receptors, adenosine can modulate different signalling pathways. This proposed mechanism should help us to understand most of the molecular and cellular effects described for this tea polyphenol.

Keywords: Green tea; Epigallocatechin-3-gallate; Dihydrofolate reductase; Folic acid; DNA methylation

1. Introduction

Folic acid is a water-soluble B vitamin that plays a crucial role in DNA synthesis, its stability, integrity and repair. The active form of folic acid is 5,6,7,8-tetrahydrofolate (THF), which is formed in the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) by the enzyme dihydrofolate reductase (DHFR). THF serves as the principal component in folate metabolism, where it acts as a carrier of one-carbon units in its various cofactor forms. The synthesis of deoxythymidylate (dTMP) from deoxyuridylate (dUMP) is catalyzed by thymidilate synthase (TS) and uses 5,10-methylene-THF as the source of the methyl group of dTMP and as the reductant. This reaction requires DHF to be reduced by DHFR to THF. Thus, TS coupled with DHFR forms a crucial link responsible for the synthesis of dTMP and hence DNA. Folic acid may also modulate DNA methylation, which is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in

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chromosomal modifications, and in the development of mutations (Kim, 2003; Widschwendter & Jones, 2002).

Inhibitors of folic acid metabolism, also called antifolates, have provided important agents useful in cancer chemotherapy and antibiotics (Berman & Werbel, 1991). Based on the observation that classical (MTX) and nonclassical (TMP) antifolate compounds present similar chemical structures to some tea polyphenols, we hypothesised that tea catechins could well inhibit DHFR activity. Recently, we have shown that ester-bonded gallate catechins from green tea, such as epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG), are potent *in vitro* inhibitors of several DHFRs at concentrations found in the serum and tissues of green tea drinkers (0.1–1.0 μM) (Navarro-Martínez et al., 2005; Navarro-Perán, Cabezas-Herrera, García-Cánovas, et al., 2005; Navarro-Perán, Cabezas-Herrera, Hiner, et al., 2005). Interestingly, a recent publication shows that ECG and EGCG efficiently inhibit cellular folic acid uptake in Caco-2 cells (Alemdaroglu et al., 2007). These results suggested that catechins could act as antifolate compounds in the same way as MTX. Additional evidence that supports this observation can be obtained by examination of the related bibliography. Green tea has found similar clinical applications as antifolate compounds in the treatment of cancer, microbial and fungal infections, Crohn's disease, psoriasis or in chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis (Alic, 1999; Rasheed & Haider, 1998; Sueoka et al., 2001), and, surprisingly, EGCG and MTX show similar cellular and molecular effects against tumour development and progression (Seitz, 1999; Yang et al., 2000). To check our hypothesis that EGCG could act as an antifolate compound, we investigated the disruption of folate metabolism induced by this compound in Caco-2 cell monolayer, which acted as a model of the human intestinal epithelium. Colon cancer cells are highly sensitive to antifolate compounds (Singh et al., 2006) and the results obtained in this model could be of importance for understanding the epidemiological data that correlate the ingestion of green tea with the low risk of suffering gastrointestinal cancer (Mu et al., 2005).

2. Materials and methods

2.1. Materials

Tea polyphenols, EGCG (>95%), ECG (>98%), epigallocatechin (EGC >98%) and epicatechin (EC >98%) were from Sigma Chemical Co. (Madrid, Spain). Green tea extract was obtained as described elsewhere (Navarro-Perán, Cabezas-Herrera, Hiner, et al., 2005). Recombinant human DHFR (rHDHFR) was purchased from Sigma and its concentration was determined by MTX titration of enzyme fluorescence (Smith, Patrick, Stone, Phillips, & Burchall, 1979). DHF (90%) was obtained from Aldrich Chemical Co. (Madrid, Spain). NADPH, MTX, α,β-methylene adenosine-5′-diphosphate (APCP), thymine, hypoxanthine, 5-fluorouracil (5-FU), leucovorin (5-formyl-THF) and adenosine deaminase (ADA) were from Sigma.

2.2. DHFR assays

The activity of DHFR was determined at 25 °C by following the decrease in the absorbance of NADPH and DHF at 340 nm ($\varepsilon = 11800\,M^{-1}\,cm^{-1}$) (Stone & Morrison, 1986) in a Perkin-Elmer Lambda-35 spectrophotometer. Progress curves were obtained under ordered conditions ([NADPH] ≫ [DHF]). The calculation of rHDHFR inhibition constants by EGCG was performed as described elsewhere (Navarro-Perán, Cabezas-Herrera, Hiner, et al., 2005).

2.3. Fluorescence studies

The dissociation constant for the binding of EGCG to free rHDHFR was determined at 25 °C by fluorescence titration in an automatic-scanning Perkin-Elmer LS50B spectrofluorimeter. Fluorescence emission spectra were recorded when rHDHFR fluorescence was excited at 290 nm.

2.4. Cell culture and treatments

Caco-2 cells were purchased from the ATCC (Rockville, USA) and were cultured in EMEM (Gibco, Barcelona, Spain) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml of penicillin, 100 μg/ml streptomycin, 1 mM piruvate and non-essential aminoacids solution at 37 °C in a humidified atmosphere 95% air–5% CO_2. Cell injury was evaluated by a colorimetric assay for mitochondrial function using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. For this assay cells were plated in a 96-well plate at a density of 1000 cells/well and grown until they reached 50–60% confluence. Co-treatments of Caco-2 cells with EGCG and other reagents were carried out by supplementing medium cultures with HT medium (Sigma), thymine (0.1 mM), hypoxanthine (0.1 mM), leucovorin (0.1 mM), adenosine (10 μM), APCP (50 μM), 5-FU (50 μM) or ADA (10 μg/ml).