Mechanism of the inhibition of the STAT3 signaling pathway by EGCG

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Abstract. Signal transducer and activator of transcription 3 (STAT3) is an oncogene that promotes cell survival, proliferation, and motility. In the present study, we explored the mechanism involved in the inhibition by epigallocatechin-3-gallate (EGCG) of STAT3 signaling as detected by surface plasmon resonance (SPR)-binding assays and in silico docking. Stat3-binding assay indicated that EGCG significantly interrupted Stat3 peptide binding at micromolar concentrations, and the docking experiments indicated that EGCG had a strong interaction with Arg-609, one of the key residues in the STAT3 SH2 domain that contributes greatly to Stat3 and phosphorylated peptide binding. Following treatment of the hepatocellular carcinoma cell lines BEL-7402 and QGY-7703 with EGCG, in vitro, EGCG significantly suppressed cell proliferation as detected by MTT assay, induced apoptosis as detected by flow cytometry, dramatically lowered the expression levels of phosphorylated Stat3 proteins (p-Stat3) as determined by immunoblot detection, and inhibited the expression of multiple genes including Bcl-xL, c-Myc, VEGF and cyclin D1 as demonstrated by RT-PCR analysis. In conclusion, our research data indicate that the anticancer function of green tea results from the inhibition of the STAT3 signaling pathway by EGCG.

Introduction

Signal transducer and activator of transcription 3 (STAT3) is one of the seven members of the Stat protein family that mediates the actions of many cytokines and growth factors. STAT3 shows constitutive activity in many different types of cancers, including breast, prostate, head and neck, lung, colon, liver and pancreatic cancers, and large granular lymphocytic leukemia and multiple myeloma (1-3). In addition, human tumor xenograft studies in mice have repeatedly demonstrated that inhibition of STAT3 signaling results in decreased tumor growth and improved animal survival by inducing apoptosis in tumor cells, inhibiting angiogenesis (4), and enhancing antitumor immune-mediated cytotoxicity (1,5). Thus, STAT3 has been identified as a potential high-yield target for pharmaceutical prevention in treating many types of cancers (6).

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide (7). The diagnosis of HCC is difficult due to the lack of early screening methods, and treatment is arduous due to its aggressive nature and the absence of therapeutic targets. Numerous studies regarding preventive and curative strategies for HCC have been conducted in recent years, leading to significant discoveries (6,8,9). HCC patients are found to have high levels of IL-6 that promote the survival of HCC cells through the upregulation of the STAT3 signaling pathway (10). Thus, abnormal levels of IL-6 have profound impacts on cancer occurrence, development and progression. Heightened expression of IL-6 may be blocked by disruption of the STAT3 pathway that in turn blocks cell transformation, inhibits angiogenesis and suppresses tumor growth (11); epigallocatechin-3-gallate (EGCG) can promote this disruption.

According to epidemiologic studies, the risk of HCC, along with that of many types of cancers, can be reduced through tea consumption. Although green tea is a promising dietary source of chemopreventive and chemoprotective chemicals (12-14), its mechanism is still not fully understood. However, many
The purpose of this study was to gain insight into the molecular mechanism involved in the effects of STAT3. Surface plasmon resonance (SPR) detection, in silico docking simulations, MTT assay, FACS-based apoptosis assay, immunoblotting, and RT-PCR were among the techniques used to validate our findings. EGCG was found to disrupt Stat3-phosphorylated peptide binding, inhibit the expression of phosphorylated Stat3 protein as well as many downstream genes regulated by STAT3, induce HCC cell apoptosis, and suppress HCC cell growth, by possibly inhibiting the STAT3 signaling pathway to directly interfere with the Stat3 protein.

Materials and methods

Reagents. EGCG was obtained from Catch Bio-Science & Technology Ltd. (Jiangsu, China) with a purity of >99.99%. All cell culture reagents were purchased from Biowest (USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] detection kit, Annexin V-FITC apoptosis detection kit and cell cycle detection kit were obtained from BestBio (Shanghai, China). Western blotting antibodies specific to p-Stat3, total-Stat3 and β-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Cell-based ELISA kit [human/mouse phospho-Stat3 (Y705) immuno-assay] was purchased from R&D Systems (Minneapolis, MN, USA).

Cell lines. The BEL-7402 and QGY-7703 human HCC cell lines were provided by Shanghai Institute of Biochemistry and Cell Biology. They were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 1X penicillin-streptomycin solution in a humidified 5% CO₂ atmosphere at 37°C.

Stat3/pY-peptide binding assay. Stat3 binding assays were performed at 25°C with a BIAcore 3000 biosensor using 20 mM Tris-buffer pH 8.0 that contained 2 mM β-mercaptoethanol and 5% DMSO as running buffer. Phosphorylated and non-phosphorylated control biotinylated EGFR-derived dodecapeptides based on the sequence surrounding Y1068 were immobilized on a streptavidin-coated sensor chip (BIAcore Inc., Piscataway, NJ, USA). The binding of Stat3 was conducted in 20 mM Tris-buffer pH 8.0 containing 2 mM β-mercaptoethanol at a flow rate of 10 µl/min for 1-2 min. Aliquots of Stat3 at 500 nM were premixed with compound to achieve a final concentration of 1-1,000 µM, and incubated at 4°C prior to being injected onto the sensor chip. The chip was regenerated by injecting 10 µl of 100 mM glycine at pH 1.5 at 4°C. Cells were then resuspended in 1X Annexin V binding buffer. Five microliters of Annexin V-FITC solution was added to each tube. All tubes were incubated for 15 min at 4°C in darkness. Fifteen microliters of PI solution was added to each tube. All tubes were incubated for another 5 min at 4°C in darkness. Cells were then analyzed using flow cytometry (Accuri C6; BD Biosciences, USA).

Western blotting and ELISA. To detect protein expression and modification in response to treatment with EGCG, HCC cells, which were treated with various concentrations of EGCG, were plated onto 6-well plates at a density of 2x10⁵ cells/ml. After incubation for 24 h, cells were lysed in cold RIPA lysis buffer. The lysate was then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was collected and the protein concentration was determined using a bicinchoninic acid (BCA) assay. Western blotting and ELISA were performed using commercially available antibodies against p-Stat3 and total Stat3, respectively. The blots were probed with secondary antibodies conjugated to horseradish peroxidase and visualized using an enhanced chemiluminescence (ECL) system.
buffer. Total protein was extracted with high-salt buffer (0.5% sodium deoxycholate, 1% SDS, 1 mM sodium orthovanadate, 1 mM β-glycerol phosphate, 1 mM sodium fluoride, 2.5 mM sodium pyrophosphate) containing a protease inhibitor cocktail (Roche, Nutley, NJ, USA). Protein samples were separated by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with the corresponding antibodies. The signals were visualized with Enhanced Chemiluminescence Plus (ECL Plus) detection system (Dingguo, China). The ELISA procedure was used according to the manufacturer’s instructions (Cell Signaling Technology, Inc.). Briefly, 100 µl of 15,000 BEL-7402 cells was seeded into each well of a black 96-well microplate with a clear bottom, and incubated overnight at 37˚C. Cells were then treated with different concentrations of EGCG in complete medium for 24 h. Subsequently, cells were stimulated with interleukin 6 (IL-6) (50 ng/ml) to induce Stat3 phosphorylation. Following the treatments, cells were treated and tested with the cell-based ELISA kit.

RT-PCR. The BEL-7402 and QGY-7703 cells were treated with EGCG at 40, 80 and 160 µM for 48 h as previously described. Total RNAs were extracted from the cells using a commercially available RNA-Bee isolation kit (Tel-Test). Standard reverse transcription was performed with 500 ng of total RNA using TIANScriptRT kit (Tiangen Beijing, China). Reverse transcription-PCR was performed using 1 µl of cDNA template, 10 pmol of primers, and a PCR premix (1 U Taq DNA polymerase, 250 mM dNTPs, 10 mM Tris-HCl, 40 mM KCl and 1.5 mM MgCl2; Tiangen). The following primers were used in the PCR reactions: Bcl-xL forward, 5'-agctggtggtgactttctc-3' and reverse, 5'-ccggaagtaggtcctcctac-3'; c-Myc forward, 5'-ctaccctctcaacgacagcag-3' and reverse, 5'-gtgtgtctcgctgtcgctg-3'; VEGF forward, 5'-gcagaatcatcacgaagtggt-3' and reverse, 5'-cattgtgtgctgtaggaagc-3'; cyclin D1 forward, 5'-actacgcacacactcactcactc-3' and reverse, 5'-gcatttttgagaagaagttc-3'; β-actin forward, 5'-agagctacgagctgcctgctg-3' and reverse, 5'-agtacttgcgctcaggaga-3'.

The amplified products obtained from the β-actin-specific primers served as internal controls. PCR was conducted using Bio-Rad T-100 (Bio-Rad, Hercules, CA, USA) with a 5-min denaturation step at 94˚C; 30 cycles of 94˚C for 30 sec, 62˚C for 30 sec and 72˚C for 30 sec; and a final extension at 72˚C for 10 min. PCR amplifications were verified to be in the linear range.

Statistical analysis. Data are presented as means ± SD for 3 separate experiments. One-way ANOVA was employed for statistical analysis using SPSS 17.0. P<0.05 was considered to indicate a statistically significant result.

Results

EGCG blocks Stat3 binding to its phosphopeptide ligand. EGCG was tested for its ability to block Stat3 binding to its phosphopeptide ligand using SPR binding assay (15). SPR experiments showed that EGCG was able to directly compete with pY-peptide for binding with Stat3 at an IC50 value of 10-30 µM (Fig. 1).

Molecular docking between EGCG and Stat3. Fig. 2A is a computer model image of EGCG on Stat3. According to the figure, EGCG is located in a phosphopeptide binding pocket formed by the STAT3 SH2 fold. Fig. 2B shows the spatial matching results of EGCG and Stat3; the 3-D structure of EGCG matches perfectly with the phosphopeptide binding site of STAT3 SH2. Fig. 2C and D depict the specific
interactions between EGCG and STAT3 SH2. According to these two figures, the -NH3 group of LYS591 is located between the 2 aromatic rings of EGCG and the 2 hydrogen atoms from the -NH3 group, resulting in the formation of cation-π bonds. The other hydrogen atom in the -NH3 group forms a hydrogen bond with the -O- in EGCG. On the other hand, the -C=O group functions as a hydrogen receptor in EGCG, forming hydrogen bonds with -NH in GLU612 and -OH in SER613, respectively. While the 2 aromatic rings both function as hydrogen donors in EGCG, one also forms hydrogen bonds with the -NH2 group in ARG60, while the other forms hydrogen bonds with the -NH2 group in Glu638. The other part of EGCG forms a van der Waals interaction with the phosphopeptide binding pocket of STAT3 SH2 (LYS591, GLU594, ARG609, SER611, GLU612, SER613, THR620, VAL637, GLU638, PRO639) (Fig. 2C). These multiple interactions result in a steady locked relationship (15-18).

**EGCG suppresses BEL-7402 and QGY-7703 cell growth, and induces apoptosis in QGY-7703 cells.** To determine the potential cytotoxic and anti-proliferative effects of EGCG, we cultured the human HCC cell lines BEL-7402 and QGY-7703 with different concentrations of EGCG for 48 h, and the apoptotic fraction of cells was detected by Annexin V staining (x-axis)/PI (y-axis) staining using flow cytometry. The lower right quadrant of the plot indicates early apoptotic cells that are Annexin V-positive and PI-negative.
EGCG inhibits Stat3 phosphorylation and suppresses the expression of STAT3-regulated genes. (A) QGY-7703 cells were treated with EGCG (0-80 µM) for 48 h. The expression levels of p-Stat3 and T-Stat3 were detected by western blotting. β-actin was used as a loading control. (B) The relative intensities of p-Stat3 in QGY-7703 cells were measured by cell-based ELISA. Cells treated with AG490 were used as positive control. Data represent the means ± SD. *P<0.05 or **P<0.001 indicates a significant difference from the respective control. (C) mRNA levels of Bcl-xL, c-Myc, VEGF and cyclin D1 in QGY-7703 cells treated with EGCG were detected by RT-PCR. Housekeeping gene β-actin was used as the control.

EGCG at various concentrations (0-320 µM). Cell viability was then determined by MTT assay. Results showed that treatment with EGCG led to a significant dose-dependent inhibition of HCC cell growth in vitro (Fig. 3A). The half maximal (50%) inhibitory concentrations (IC_{50}) for BEL-7402 and QGY-7703 cells were ~55 and 35 µM, respectively. Induction of cell apoptosis was confirmed by Annexin V-FITC staining in QGY-7703 cells. Results showed that treatment with EGCG led to significant dose-dependent apoptosis-inducing effects on HCC cell growth in vitro. According to Fig. 3B, the upper right quadrant represents late apoptosis, while the lower right quadrant represents early apoptosis. Increasing concentrations of EGCG at 20, 40, 80 and 160 µM, respectively, were added to the QGY-7703 cell line for 48 h. As a result, the rates of cell apoptosis were 11.7, 18.7, 42.6 and 73.6%, respectively. Thus, as the concentration of EGCG increased, the rate of apoptosis of the QGY-7703 cells also increased. The standard deviations were calculated based on 3 independent experiments.

**Discussion**

Tea is one of the most popular beverages in the world and has been well known to promote good health in numerous ways for over two thousand years. Daily consumption of tea may reduce cholesterol and the incidence of heart disease, boost immunity and benefit human skin. Particularly, tea may lower the risk of various types of cancers, including gastric, pancreatic and colorectal, in the human population (19-21). EGCG, which contributes to more than 40% of the total polyphenol mixture in tea, plays an essential role in its chemotherapeutic and chemopreventive effects. In fact, the anti-oxidative activity and metal chelating functions of EGCG may contribute to the inhibitory activity of tea against carcinogenesis (22). Additionally, there is considerable evidence that EGCG has an anticancer nature by modulating the intracellular signaling network.

To study the mechanism of the inhibitory effects of EGCG on carcinoma cells, we conducted molecular binding computation and related experiments. Based on our study from the BLACore binding assay in micromoles, EGCG blocked Stat3 binding to its phosphopeptide ligand on SPR testing. Furthermore, the EGCG molecule had major interactions with two key residues, R609 and K591, localized in the STAT3 SH2 domain, which we found through docking simulation analysis. We then confirmed that EGCG significantly inhibited carcinoma cell growth in vitro in two human HCC cell lines, BEL-7402 and QGY-7703, in a dose-dependent trend by MTT assay. Additionally, EGCG interrupted Stat3 phosphorylation on tyrosine 705 in a dose-dependent manner (Fig. 4A). The p-Stat3 relative fluorescence intensity was significantly reduced following EGCG treatment. When QGY-7703 cells were treated with EGCG at concentrations of 10, 20, 40, 80 and 160 µM, respectively, the p-Stat3 relative average fluorescence intensities were 7,400, 6,600, 4,500, 3,400 and 2,200, respectively. Statistical analysis showed a P-value of <0.05 for EGCG at 10, 20 and 40 µM in relation to their corresponding fluorescence intensity; EGCG at 80 and 160 µM had a P-value of <0.001 in relation to their corresponding fluorescence intensity (Fig. 4B).

**EGCG downregulates the expression of cancer-related genes.** Exposure to EGCG resulted in a dose-dependent decrease in cyclin D1 mRNA expression in both BEL-7402 and QGY-7703 cells, as demonstrated by RT-PCR analysis. Furthermore, the expression levels of Bcl-xL, c-Myc and VEGF were also significantly reduced at the transcriptional levels (Fig. 4C).
as determined by RT-PCR analysis. This in turn led to HCC cell apoptosis, as demonstrated by flow cytometry.

Our research data support that the anticancer function of green tea is the result of the inhibition of the STAT3 signaling pathway by EGCG. However, additional studies suggest that EGCG is not only a multiple effector that regulates cell signaling such as STAT1 and ERK1/2 (23), but is also a general binder that binds to STAT1 and other bio-molecules, including RNA. Based on our conclusion, EGCG is a STAT3 signaling inhibitor that competitively binds to the STAT3 SH2 domain, contributing to the regulation of the cellular signaling network and the anticancer effects of green tea. However, further research is needed before a full understanding of the mechanism of EGCG in tea is achieved in order to benefit the health of the general population.

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References


