Green tea polyphenol epigallocatechin gallate inhibits cell signaling by inducing SOCS1 gene expression

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Transmitting editor: T. Watanabe
Received 10 November 2009, accepted 27 January 2010

Abstract

Therapeutic effects of green tea involve an inhibitory function of its constituent polyphenol epigallocatechin gallate (EGCG) on cell signaling. The specificity and mechanism(s) by which EGCG inhibits cell signaling have remained unclear. Here, we demonstrate that green tea and EGCG induce suppressor of cytokine signaling 1 (SOCS1) gene expression, a negative regulator of specific cell signaling pathways. In mouse immune cells, EGCG induces SOCS1 expression via an oxidative (superoxide) pathway and activation of the signal transducer and activator of transcription 5 transcription factor. EGCG inhibited SOCS1-regulated cell signaling, but this inhibitory effect was abrogated in cells deficient in SOCS1. These findings identify a mechanism by which EGCG inhibits cell signaling with specificity, mediated by induction of the negative regulator SOCS1.

Keywords: immunotherapy, LPS signaling, STAT5

Introduction

Studies in experimental mice have demonstrated therapeutic effects of oral consumption of green tea in inflammatory/autoimmune disease (1) and both breast (2) and prostate cancers (3), which has led to increasing interest into the potential health benefits of green tea consumption in humans. Epigallocatechin gallate (EGCG) is the most abundant polyphenol found in green tea. Remarkably, treatment with EGCG alone (purified from green tea) is sufficient to inhibit various autoimmune diseases including Sjogren’s syndrome (4) and various cancers including breast and prostate (reviewed in 5) in experimental mice, indicating an important role for EGCG in mediating the therapeutic effects observed for green tea.

It has now been clearly established that EGCG has potent inhibitory effects on cell signaling pathways associated with inflammation and cancer cell growth. For example, EGCG inhibits the inflammatory IFN-γ/signal transducer and activator of transcription (STAT1) (6) and LPS (7) signaling pathways. EGCG also inhibits the EGF/MAP kinase pathway (8) (involved in cell growth and proliferation) leading to growth inhibition of cultured cancer cells and activation of genes with pro-apoptotic functions. Recent studies have demonstrated therapeutic effects of EGCG on the development of other diseases such as neurodegenerative disease (9) and diabetic nephropathy (10) in mice, which may involve inhibition of other signaling pathways.

The molecular mechanisms by which EGCG inhibits cell signaling pathways in cultured cells remain unclear. To date, these studies have focused particularly on EGCG inhibitory effects in cancer cell lines. Cancer chemoprotective effects of EGCG have been shown to involve binding of EGCG to the 67-kDa laminin receptor (11), a lipid raft protein that displays overexpression and abnormal surface localization in cancer cells (12), while EGCG-induced structural alteration of lipid raft composition in colon cancer cells inhibits EGF binding to its receptor (13). Conceivably, binding of EGCG to the 67-kDa laminin receptor mediates EGCG entry into lipid rafts and subsequent ‘interference’ in lipid raft-associated receptor signaling (including the EGF receptor). Such a mechanism, however, does not imply specificity in cell signaling inhibition by EGCG in cancer cells.

A clearer understanding of the specificity (if any) and mechanism(s) by which EGCG inhibits multiple pathways of disease (including non-cancer diseases) is important both for understanding the observed therapeutic effects of EGCG in vivo and also to rationalizing potential benefits of drinking...
green tea, the inclusion of EGCG in health products and food/drink and future research/development of tea-based therapies for specific human diseases.

Methods

Mice

Monocyte-specific suppressor of cytokine signaling 1 (SOCS1)-deficient C57BL/6 mice have been described previously (14). Wild-type (WT) and SOCS1-deficient C57BL/6 mice were maintained under standard conditions in a pathogen-free animal facility. Experiments were conducted in accordance with institutional ethics guidelines.

Cell preparations

Human PBMCs were purified from whole blood samples obtained from healthy donors by density centrifugation on Ficol-Paque (GE Healthcare, Buckinghamshire, UK). Fractionated human PBMCs (monocyte, B- and T-cell-enriched populations) were purified, respectively, by positive selection using MACs™ CD14, CD19 and CD3e MicroBeads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer’s protocol. Splenic monocytes from 8-week-old C57/BL6 WT or SOCS1 knockout mice (13) were prepared by adherence to tissue culture plastics for 2 h, followed by vigorous washing with room temperature PBS to remove the non-adherent fraction, followed by removal of adherent monocytes by vigorous pipetting with ice-cold PBS. Monocytes were further purified by B- and T-cell depletion using MACs CD3 and CD19 Microbeads (Miltenyi Biotec), according to the manufacturer’s protocol. The purity of cell-enriched populations was routinely >90% as determined by flow cytometric analysis.

Cell cultures and in vitro stimulation

Human PBMCs were cultured in RPMI medium (Wako Pure Chemicals Industries, Osaka, Japan), supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA), 1% penicillin G and streptomycin (Sigma Ltd, St Louis, MO, USA). Mouse splenic monocytes were cultured in DMEM medium (Wako Pure Chemical Industries), supplemented with 10% fetal calf serum (Hyclone Laboratories), 1% penicillin G and streptomycin (Sigma Ltd). Green tea extract (GTE), a standardized preparation (60.0% weight = (−)-epigallocatechin-3-O-gallate by HPLC, ≤0.1% of caffeine by HPLC), was a kind gift of Indena Inc. (Milan, Italy). Polyphenols (purified from green tea): (−)-EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epicatechin (EC)—were purchased from Sigma Ltd. GTE and individual green tea polyphenols were dissolved fresh in sterile water and treated to cells at the concentrations indicated in figure legends. IL-6, IFN-γ and granulocyte/monocyte colony stimulating factor (GMCSF) (Sigma Ltd) were prepared in sterile water and treated to cells at the concentrations indicated in figure legends.

Antibodies

Western blot analysis was performed using polyclonal antibodies to mouse SOCS1 (International Reagents, Tokyo, Japan), a monoclonal antibody to human SOCS1 (Abcam, Cambridge, UK) and monoclonal antibodies to total or phosphorylated STAT1, STAT3, STAT5 and STAT6 (Cell Signaling, Danvers, MA, USA) and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA interference

RNA interference (RNAi) was performed using STAT1, STAT3 and STAT5 short inhibitory RNA (siRNA) oligonucleotides and siRNA control oligonucleotide (Qiagen, Stanford, CA, USA) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. STAT protein knockdown was confirmed by western blot analysis of STAT protein expression and required a minimum of 4-day siRNA oligonucleotide transfection. By this method, we typically achieved >80% STAT-specific protein knockdown.

Reverse transcription–polymerase chain reaction

Total RNA from PBMC samples and mouse splenocytes was prepared using an RNaseasy kit (Qiagen) according to the manufacturer’s instructions. cDNA was prepared using the Quantitect Reverse Transcription System (Qiagen). Real-time reverse transcription (RT)–PCR was performed on cDNA samples using the QuantiTect SYBR Green PCR kit (Qiagen) and an iCycler (Bio-Rad, Hercules, CA, USA) to quantify levels of expression of SOCS1, IL6/12, tumor necrosis factor (TNF)-α and control actin levels. PCR primer sequences are available on request.

Results

EGCG induces SOCS1 gene expression in immune cells

We previously reported the cloning and characterization of SOCS1, a negative regulator of cell signaling pathways (15). The specificity of SOCS1 inhibitory function on cell signaling is mediated via an SH2 homology 2 (SH2) and kinase inhibitory region (KIR), which together inhibit JAK activation, and via the SOCS box, which mediates specific protein degradation (such as NFkappaB) by ubiquitylation (reviewed in 16). Since SOCS1 is involved in the negative regulation of LPS/JAK (17, 18) and IFN-γ/JAK signaling (19) pathways also inhibited by EGCG (6, 7), we explore here the hypothesis that SOCS1 mediates the inhibitory effect of EGCG on cell signaling.

In Fig. 1, we demonstrate that green tea and its constituent polyphenol EGCG potently induce SOCS1 gene expression in immune cells. Cultured human PBMCs were treated with either GTE or polyphenols purified from green tea—EGCG, EGC, ECG and EC. SOCS1 expression (mRNA) was induced in PBMCs in response to GTE or EGCG treatment compared with untreated cells and with control IL6-treated cells (Fig. 1A). Minimal SOCS1 induction by EGC was observed and SOCS1 induction could not be detected following either ECG or EC treatment. In Fig. 1C, we confirm induction of SOCS1 protein by EGCG (at 25–100 μM EGCG), in agreement with our analysis by RT-PCR (Fig. 1A).

We next determined the relative induction of expression of SOCS1 by EGCG in individual mononuclear cell populations.
As shown in Fig. 1D, EGCG induced SOCS1 expression (mRNA) in monocytes and B cells, with induction occurring at 10 min of 100 μM EGCG treatment. At 30 min of EGCG treatment (100 μM), SOCS1 mRNA levels were equivalent to levels induced by IL6 treatment for 30 min. In T cells, EGCG (100 μM) induced minimal SOCS1 expression (mRNA) above basal levels, at 30 min of treatment.

EGCG induces activation of the STAT5 transcription factor

We previously described SOCS1 as a STAT-induced STAT inhibitor (15). Thus, having established that EGCG is an inducer of SOCS1 expression, we investigated if EGCG is an inducer of STAT activation. For this analysis, we utilized mouse splenic monocytes, which we confirmed induce SOCS1 mRNA (Fig. 2A) and protein (Fig. 2B) in response to
EGCG treatment, in agreement with our analysis in human monocytes (Fig. 1). As shown in Fig. 2C, EGCG treatment induced the phosphorylation (activation) of the STAT5 transcription factor, with levels equivalent to that achieved by treatment with GMCSF alone. We were unable to detect marked activation of the STAT1, STAT3 or STAT6 transcription factors in response to EGCG treatment compared with untreated cells. The induction of STAT5 activation in monocytes following EGCG treatment occurs from 5 min of EGCG treatment (Fig. 2D), which parallels the induction of SOCS1 mRNA that we show occurs at 5 min of EGCG treatment (compare Fig. 2D and E).

We next investigated if endogenous STAT5 is required for induction of SOCS1 expression by EGCG. For this analysis, we employed STAT siRNA transfection in cultured mouse splenic monocytes and measured the induction of SOCS1 expression following EGCG treatment by real-time RT–PCR. As shown in Fig. 3A, EGCG was unable to induce SOCS1 expression (mRNA) in cells deficient in STAT5. Conversely, EGCG induced SOCS1 expression in cells deficient in STAT1 or STAT3. STAT5 deficiency did not inhibit the induction of SOCS1 by IFN-γ.

**EGCG induces STAT5 activation and SOCS1 expression via an oxidative pathway**

A previous study demonstrated that EGCG exerts oxidant activity in cell culture via the generation of hydrogen peroxide and superoxide molecules (20). In another study, inhibitory effects of EGCG on EGF signaling were shown to be mediated via EGCG auto-oxidation and generation of superoxide molecules (21). Thus, we investigated if activation of the STAT5 transcription factor and induction of SOCS1 expression by EGCG is also mediated via an oxidative (superoxide) pathway. As shown in Fig. 3, EGCG induced STAT5 activation (Fig. 3B) and SOCS1 expression (Fig. 3C); however, in cells pre-treated with superoxide dismutase (SOD), which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, clear abrogation of EGCG STAT5...
activation/SOCS1 induction was observed. Pre-treatment with catalase (that catalyzes the decomposition of hydrogen peroxide into hydrogen and water) had no effect on EGCG STAT5 activation/SOCS1 induction. Similarly, pre-treatment with SOD had no effect on GMCSF STAT5 activation or IFN-γ induction of SOCS1 expression.

EGCG inhibits cell signaling via SOCS1

Next we investigated a physiological role for SOCS1 in EGCG inhibition of cell signaling. Since SOCS1 participates, but is not indispensable for negative regulation of LPS responses (17), we tested the known inhibitory effect of EGCG treatment on LPS signaling (7) in splenic monocytes of control compared with monocyte-conditional SOCS1 knockout mice. As shown in Fig. 4, EGCG inhibited LPS-induced IL-6 (Fig. 4A) and IL-12 (Fig. 4B) expression (mRNA); however, this inhibitory effect was abrogated in cells deficient in SOCS1. Interestingly, EGCG did not inhibit LPS induction of TNF-α expression (Fig. 4C), a pathway that we have previously demonstrated is not directly regulated by SOCS1 in monocytes (18). EGCG at the doses used in this analysis was not observed to induce IL-6, IL-12 or TNF-α gene expression (data not shown).

Discussion

Although animal studies have clearly demonstrated therapeutic effects of treatment with green tea in various diseases, the molecular mechanisms and specificity by which its constituent polyphenol EGCG inhibits multiple pathogenic pathways of disease have remained unclear. Conceivably, these inhibitory effects may involve the induction of expression of a negative regulator mutually involved in the inhibition of multiple signaling pathways. Since EGCG inhibits signaling pathways that are negatively regulated by SOCS1 protein (6, 7), we explored in this study a role for SOCS1 in mediating the inhibitory effect of EGCG on cell signaling.

In cultured human PBMCs, we demonstrated that treatment with green tea or EGCG induces SOCS1 gene expression with expression levels (mRNA and protein) equivalent to that observed following treatment with IL6 alone. Similar levels of SOCS1 induction to GTE treatment were achieved by treating cells with EGCG at an equivalent concentration to that constituent in GTE treatment. This indicates that the SOCS1 inducing activity of green tea in PBMCs occurs mainly via the bioactivity of constituent EGCG. In agreement with this, minimal or no SOCS1 induction was observed following treatment of cultured PBMCs with other green tea polyphenols (EGC, ECG or EC). Further analysis indicated that EGCG induction of SOCS1 expression was strongest in monocyte and B-cell populations, with minimal induction of SOCS1 occurring in T cells. However, we note that levels of SOCS1 are constitutively high in T cells purified from PBMCs compared with both purified monocytes and B cells and that treatment of T cells with IL6 also induced minimal SOCS1 induction above basal levels.

Studies on the bioavailability of EGCG in humans following oral EGCG consumption have shown that salivary EGCG concentrations can reach ~50 μM EGCG (total conjugated and unconjugated EGCG) (22) while serum EGCG
concentrations can reach \( \sim 7 \mu M \) EGCG (total conjugated and unconjugated EGCG) (23). These reported EGCG concentrations in vivo differ to the concentrations of EGCG required in this study to induce SOCS1 expression in cultured cells. An extensive literature review of the chemistry, bioactivity and health benefits of green tea and its constituent polyphenols (principally EGCG) has been published (24). As the authors highlight in their review, it is clear that in vitro, EGCG inhibits the cellular activity of biomedically relevant molecular targets at concentrations (micromolar range) that exceed physiological serum levels of EGCG in humans (nanomolar range). However, it is also clear that the consumption of green tea or EGCG has significant protective effects in murine disease models, while epidemiological studies in humans suggest potential health benefits of green tea and EGCG consumption.

To explain the apparent discrepancy in physiologically relevant EGCG concentrations reported in in vitro studies compared with in vivo studies, the review authors suggest several possible explanations. Thus, for example, studies indicate that the effects of EGCG are enhanced in the presence of other green tea polyphenols. Also studies indicate that in vivo, EGCG and other green tea polyphenols are metabolized to form more potent and bioactive compounds. It has also been speculated that EGCG may accumulate in tissues over time to concentrations that exceed those measured in serum samples. Furthermore, in this study, we later discuss a potential role for inflammatory microenvironments in enhancing EGCG function and the production of SOCS1 protein.

Thus, it is conceivable that although physiologically relevant concentrations of EGCG differ from in vitro and in vivo studies, the physiologically relevant effects of EGCG may be equivalent.

Clearly, further analysis is necessary to confirm a physiological role for EGCG in the induction of SOCS1 in vivo. However, a potential role for EGCG in targeting SOCS1-regulated pathways in vivo is indicated by our previous study (25), in which we demonstrated a protective effect of EGCG on ischemia-reperfusion-induced apoptosis in mouse cardiac myocytes, involving inhibition of the STAT1 transcription factor, which is negatively regulated by SOCS1.

We next explored the molecular mechanisms of SOCS1 induction by EGCG. Since we previously demonstrated that SOCS1 is a STAT-induced protein (15), we therefore determined if EGCG is an inducer of STAT protein activation. Using mouse splenic monocytes (that we demonstrated induce SOCS1 expression in response to EGCG), we observed that EGCG strongly induced STAT5 activation (phosphorylation), with levels equivalent to that achieved by treatment with GMCSF alone, but had minimal effect on the activation of other STATs (STAT1, STAT3 and STAT6). The induction of STAT5 activation in response to EGCG paralleled that of SOCS1 induction, indicating a possible role for STAT5 in inducing SOCS1 expression by EGCG. To explore this hypothesis, we utilized mouse monocytes deficient in specific STAT proteins (including STAT5) by STAT RNAi analysis. We showed that the induction of SOCS1 by EGCG critically required endogenous STAT5, but occurred independently of other STAT proteins (STAT1, STAT3 and STAT6). This requirement of STAT5...
for induction of SOCS1 expression by EGCG parallels the known role of STAT5 in signal transduction via the IL2 receptor common gamma chain, which induces SOCS1 expression (26). The relevant contribution of specific STAT5 isoforms (5a and 5b) to SOCS1 induction by EGCG was difficult to determine. In our analyses (data not shown), deficiency in STAT5a alone or STAT5b alone by RNAi analysis in mouse splenic monocytes had no effect on EGCG SOCS1 induction. However, by this method, STAT5 isoform-specific knockdown by RNAi leads to ‘compensatory’ induction of expression (protein level) of the counterpart STAT5 isoform. Thus, while these observations indicated that neither isoform by itself is critically required for SOCS1 induction by EGCG, the precise contribution of individual STAT5 isoforms to SOCS1 induction by EGCG was not possible to clearly determine.

Since EGCG has been shown to inhibit cell signaling via its oxidant activity in cultured cells (20, 21), we investigated an involvement of an oxidative pathway in mediating STAT5 activation/SOCS1 induction by EGCG. These analyses, using enzyme inhibitors of superoxide (SOD) and hydrogen peroxide (catalase), demonstrated the requirement of superoxide but not hydrogen peroxide, in mediating activation of the STAT5/SOCS1 pathway by EGCG. Previous studies have demonstrated that reactive oxygen species (such as hydrogen peroxide) can induce STAT activation (27, 28), which parallels the observed role of superoxide in STAT5 activation/SOCS1 induction by EGCG in the present study. In the study of Simon et al. (27), treatment of cultured Rat-1 fibroblast cells or A-431 carcinoma cells with hydrogen peroxide led to the induction of STAT1 and STAT3 activation in minutes, independent of new protein synthesis. A role for superoxide in STAT activation has previously been reported (29, 30). In the study of Madamanchi et al. (29), SOD deficient mice (SOD2 knockout mice) displayed constitutive STAT3 activation in aortic smooth muscle cells. In the study of Schieffer et al. (30), angiotensin II-induced activation of STAT1 and STAT3 was shown to be critically dependent on superoxide generation by NAD(P)H oxidase in rat aortic smooth muscle cells.

Taken together, these studies (including the present study) highlight a role for reactive oxygen species (including superoxide) in the regulation of STAT transcription factor activation. The precise mechanisms linking cellular redox status and STAT activation and the differences in the specificity of STAT activation by different reactive oxygen species, however, presently remain unclear.

In vivo, urinary oxidative products of EGCG have been identified in mice following high-dose intraperitoneal administration of EGCG (31). Interestingly, nitric oxide (that accumulates in inflammatory and tumor tissue) displays an increased potency in production of reactive oxygen species at low oxygen partial pressures, which are present in most normal tissues (32). Conceivably, therefore, inflammatory microenvironments may enhance EGCG auto-oxidation, the production of superoxide and in turn SOCS1 induction. It will be of particular interest therefore to assess the physiological role of EGCG oxidation and SOCS1 induction in vivo using various inflammatory/autoimmune and cancer murine disease models.

Finally, we investigated a role for SOCS1 in EGCG inhibition of cell signaling using splenic monocytes of control and SOCS1 monocyte-conditional knockout mice. In this analysis, we observed that EGCG inhibited LPS induction of IL-6 and IL-12 expression, in agreement with a previous study (7). However, clear abrogation of this inhibitory effect was observed in SOCS1 deficient monocytes. Thus, using the LPS-IL6/12 signaling pathways, we confirmed a physiological role for the negative regulator SOCS1 in EGCG inhibition of cell signaling. Such a mechanism may involve inhibition of LPS/JAK2 activation, which we have previously reported to be negatively regulated by SOCS1 (18), additionally SOCS1 inhibits LPS-induced NF-kappaB-mediated pro-inflammatory cytokine production (that is discussed below). The absence of an inhibitory effect on LPS-TNFα production in either WT or SOCS1 deficient monocytes indicates specificity in the inhibitory effect of EGCG on LPS responses. Conceivably, this specificity is dependent on SOCS1, which we previously demonstrated to display selectivity in inhibition of LPS signaling and is not directly involved in the negative regulation of the LPS-TNFα pathway (18).

However, while the induction of SOCS1 represents a potential anti-inflammatory therapeutic mechanism of EGCG, previous studies have demonstrated that EGCG can inhibit IFNγ-induced STAT1 activation (6) and LPS-induced NF-kappaB-mediated pro-inflammatory gene expression (33). Conceivably, these reported inhibitory effects of EGCG may in part be mediated by SOCS1 protein, which blocks STAT1 and NF-kappa B activation, reviewed extensively in 16. Thus, SOCS1 inhibits the catalytic activity of IFN-γ receptor-associated JAK to activate STAT1, by binding to the activation loop of the catalytic domain through its KIR and SH2 domains. The observation that SOCS1 knockout mice are hyper-responsive to LPS stimulation, leading to enhanced production of pro-inflammatory cytokines such as IL-6, indicates an important negative regulatory role for SOCS1 in LPS signaling pathways. The mechanisms by which SOCS1 inhibits LPS-induced pro-inflammatory cytokine production may in part be mediated by SOCS1 directly interacting with the p65 subunit of NF-kappa B, facilitating ubiquitylation and degradation of p65. In addition, SOCS1 binds tyrosine-phosphorylated MyD88-adaptor-like (MAL) protein inducing ubiquitylation and degradation of MAL, which in turn suppresses MAL-dependent p65 phosphorylation and transactivation of NF-kappa B. Thus, it is conceivable that although the reported anti-inflammatory effects of EGCG have been suggested to occur via different mechanisms, the induction of SOCS1 gene expression by EGCG may play a central role in the anti-inflammatory function of EGCG.

In summary, our study details for the first time a regulatory mechanism with specificity, by which EGCG inhibits cell signaling in immune cells. Our findings may also help to explain how green tea/EGCG can inhibit multiple pathways of disease, via induction of the negative regulator SOCS1. Deficiency in SOCS1 is associated with the development of various diseases including inflammatory autoimmune diseases, various cancers and specific allergic diseases (16). Thus, future studies to determine the therapeutic potential of EGCG in specific diseases, in which SOCS1-regulated pathways of immune cells represent therapeutic targets, will be of particular interest.
**References**