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Indoleamine 2,3-Dioxygenase, an Immunomodulatory Protein, Is Suppressed by (−)-Epigallocatechin-3-gallate via Blocking of γ-Interferon-Induced JAK-PKC-δ-STAT1 Signaling in Human Oral Cancer Cells

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Immune escape is a characteristic of cancer progression, but its underlying molecular mechanism is still poorly understood. An immunomodulatory protein, indoleamide 2,3-dioxygenase (IDO), is induced by γ-interferon (IFN-γ) in several immune cells; those cells are observed in cancer cell microenvironment and can enhance immune escape. Previous studies show that IDO is expressed in the process of tumor formation and associated with cancer cell immune tolerance. By locally degrading tryptophan, IDO inhibits the proliferation of T lymphocytes and induces T cell apoptosis, leading to suppression of T cell response. In this study, (−)-epigallocatechin-3-gallate (EGCG), the major constituent of green tea, is found to significantly inhibit the expression of IDO in human oral cancer cell lines. EGCG suppresses the induction of IDO at transcriptional level. Activation of STAT1 is discovered to play an important role in regulating IDO expression by IFN-γ. The study results demonstrate that EGCG can inhibit translocation of STAT1 into nucleus in IFN-γ-stimulated human oral cancer cells. In addition, both tyrosine and serine phosphorylation of STAT1 are revealed to be suppressed by EGCG. Moreover, phosphorylation of PKC-δ, JAK-1, and JAK-2, which are the upstream event for the activation of STAT1, are also inhibited by EGCG in IFN-γ-stimulated human oral cancer cells. These data show that EGCG inhibited IDO expression by blocking the IFN-γ-induced JAK-PKC-δ-STAT1 signaling pathway. This study indicates that EGCG is a potential drug for immune and target therapy to enhance cancer therapy by increasing antitumor immunity.

KEYWORDS: IDO; EGCG; IFN-γ; STAT1; human oral cancer

INTRODUCTION

Cancer cells create a microenvironment to promote tumor progression, survival, angiogenesis, and metastasis (*1*). In the human immune system, T cells play the major role against tumors (*2*). To survive, the tumor cell must develop defense strategies for immune recognition and response (*3*). The ability of tumor cells to escape destruction by the immune system is an important hallmark of tumor progression (*4*). An immunoregulatory enzyme, indoleamine 2,3-dioxygenase (IDO), is shown to mediate tumor immune escape (*5*).

IDO catalyzes the initial and rate-limiting step in catabolism of tryptophan, which is an amino acid essential for T cell proliferation (*6*). Basal level of IDO expression is found in epididymis, thymus, gut, lung, placenta, and dendritic cells and is upregulated when tissue is under infection and inflammation (*7*). By degrading tryptophan from the local microenvironment of a tumor, IDO can inhibit T cells, which are particularly sensitive to loss of tryptophan (*8*). IDO suppresses T cells by degrading

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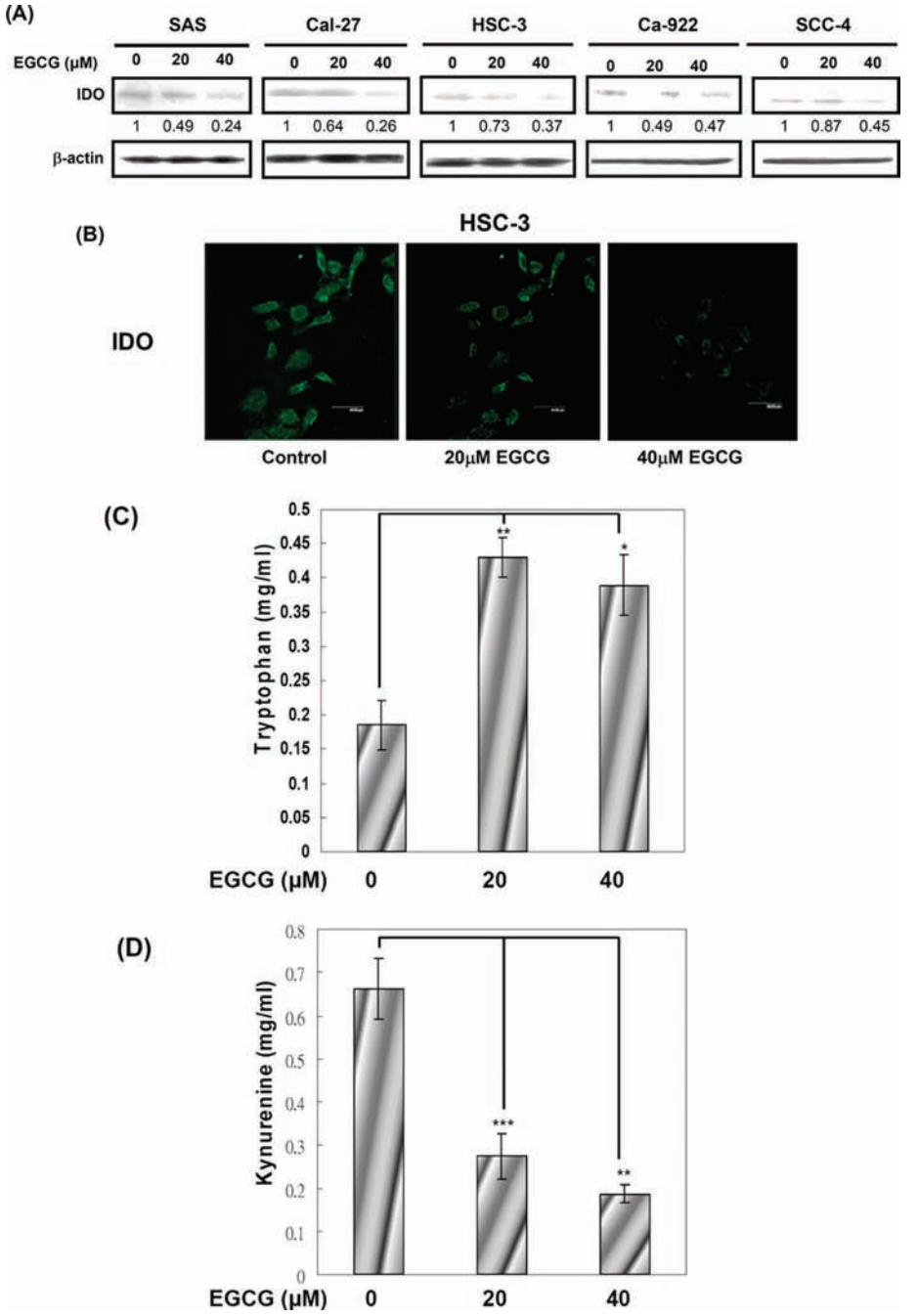

Figure 1. EGCG reduces IDO expression and activity in human oral cancer cell lines. (**A**) Five human oral cancer cell lines (SAS, Cal-27, HSC-3, Ca-922, and SCC-4) were treated with 20 or 40 μM EGCG and incubated for 24 h. IDO proteins were detected by Western blotting using IDO monoclonal antibody. These data were representative of those obtained in at least three separate experiments. The values below the figures represent change in protein expression of the bands normalized against β-actin. (**B**) HSC-3 cells were treated with 20 or 40 μM EGCG, stained with anti-IDO antibody (green), and then examined under a confocal microscope (Leica SP2). (**C**) HPLC was used to measure IDO enzyme activity. HSC-3 cells were treated with 20 or 40 μM EGCG, and then tryptophan levels in cell culture medium were measured. (**D**) To detect the levels of kynurenine, culture medium of HSC-3 cells alone and HSC-3 cells treated with 20 or 40 μM EGCG were assayed by HPLC. Values are represented as mean $\pm$ SD. Asterisks indicate values significantly different from the control: $*$, $P < 0.05$; $***$, $P < 0.001$.

tryptophan and increasing the level of its degradation product, kynurenine. These activities result in suppression of T cell response by inducing T cell apoptosis (*5*). Recently, IDO has been discovered in human tumor cells and is involved in escaping from T cell recognition and destruction. Expression of IDO is important in the process of tumor formation and is implicated in tumor immune resistance (*9*). Inhibition of IDO can potentiate the antitumor activity and increase sensitivity of cytotoxic chemotherapeutic drugs (*10*).

A number of pathways are related to IDO expression, such as NF-κB and MAPK signaling pathways (*11*). γ-Interferon (IFN-γ) is the major inducer of IDO expression. It is well-known that Janus-activated kinase-signal transducer, an activator of the transcription (JAK-STAT) pathway, is mediated by IFN-γ for inducing IDO expression (*11*). IFN-γ binding to IFN-γ receptor results in tyrosine phosphorylation of two receptor-associated tyrosine kinases of JAK1 and JAK2, which phosphorylate the downstream protein STAT1 at tyrosine 701, and protein kinase C

δ (PKC-δ) phosphorylates STAT1 at serine 727 (*12*). Both tyrosine and serine phosphorylations of STAT1 lead to homodimerization. The activated STAT1 translocates into the nucleus and promotes transcription of IFN-γ-stimulated genes (*8*).

(−)-Epigallocatechin-3-gallate (EGCG) is the major polyphenol component of green tea (*13*). It is known to have antioxidant (*14*), anti-inflammatory, and anticarcinogenesis activities (*15*). Various anticancer activities have been investigated, such as the induction of cell apoptosis, inhibition of cell proliferation (*16*), inhibition of angiogenesis (*17*), and blocking the PI3-kinase/Akt and MAPK signaling pathways (*18*). In breast cancer, EGCG has been studied extensively by using nude mouse xenograft models (*19*). Previously, epidemiological studies showed that green tea could improve the clinical outcome of human breast cancer (*20*). In the presence of copper iron, EGCG inhibited the growth of prostate cancer cells (*21*). EGCG could also induce apoptosis of hepatoma cells via mitochondrial pathways (*22*). Therefore, EGCG is an advisible anticancer drug that might be used for cancer therapy.

In human oral cancer, EGCG suppresses oral cancer cell invasion by demethylation of MMP inhibitors (*23*). However, there have been few studies about the effects of EGCG on immune escape, especially in human oral cancer. In IFN-γ-activated human macrophages, IDO was inhibited by antioxidants (*24*) and EGCG suppressed IDO expression in IFN-γ-stimulated bone marrow-derived dendritic cells (BMDCs) (*25*). In this study, we investigate whether EGCG could reduce IDO expression in human oral cancer cells. We demonstrated that IDO expression and activity were significantly inhibited by EGCG and led to inhibition of IDO-mediated tumor immune escape. Moreover, we also proved that EGCG suppressed transcription of IDO gene by blocking the IFN-γ-triggered JAK-PKC-δ-STAT1 signaling pathway.

MATERIALS AND METHODS

Reagents and Antibodies. EGCG was purchased from Sigma Chemical Co. (St. Louis, MO). JAK inhibitor and rottlerin were purchased from Calbiochem (San Diego, CA). Lipofectamin 2000 reagent was purchased from Invitrogen (Carlsbad, CA). Antibodies for JAK-1, JAK-2, STAT1, phospho-STAT1 (Ser727), phospho-STAT1 (Tyr701), phospho-JAK1 (Tyr1022/1023), and phospho-JAK2 (Tyr1007/1008) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for IDO and mouse and rabbit conjugated with HRP were purchased from Chemicon (Temecula, CA).

Cell Culture. Human oral squamous carcinoma cell lines HSC-3, SAS, and Ca-922 were obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan). SCC-4 and Cal-27 oral cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). HSC-3 and SCC-4 were grown in DMEM/F-12 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco BRL). SAS, Ca-922, and Cal-27 were cultured in DMEM (Invitrogen) containing 10% FBS. Cells were grown in a humidified incubator at 37 °C under 5% CO_2.

HPLC. To precipitate proteins, 100 μL of 15% perchloric acid was added to 200 μL of culture medium supernatant. After centrifugation for 5 min at 12000 rpm, 200 μL of supernatant was added to 500 μL of $NaHCO_3$ solution. Three hundred microliters of sample was added to 300 μL of dabsyl chloride solution and centrifuged at 12000 rpm for 5 min. Ten microliters of sample was loaded into a HPLC column (TSK ODS 100S). Tryptophan and kynurenine were detected by using UV light with excitation and emission wavelengths of 425 and 360 nm, respectively. The area of peak was determined and quantitated.

Western Blotting. Cells (1×10^6) were washed with PBS before lysing with the global lysis buffer (10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/mL leupeptin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 20 mM Tris-HCl, 137 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate, and 10 μg/mL aprotinin, adjust pH to 7.9). The cell lysates were centrifuged, and the supernatant was used to determine the protein content by the Bio-Rad protein assay kit (Bio-Red Laboratories). Proteins of 100 μg were used and resolved by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane (BioTrace, U.K.). The membrane was blocked by blocking buffer (5% nonfat milk and 0.2% v/v Tween 20 in TBST). The PVDF membrane was then incubated with primary antibodies, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5000 dilution, Roche Applied Science, Indianapolis, IN). Reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL). The intensity of the bands was scanned and quantified with Photoshop software.

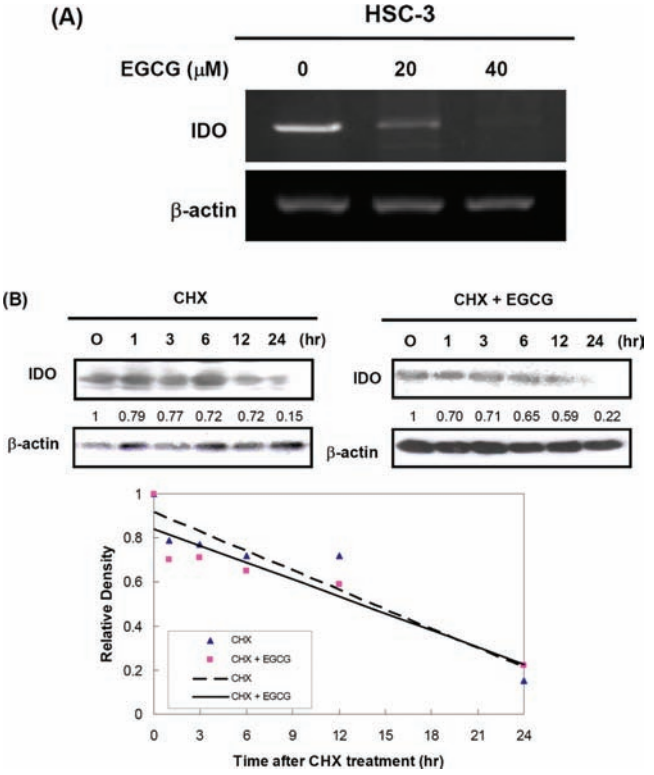

Figure 2. EGCG suppresses IDO expression at transcriptional level. (**A**) HSC-3 cells were treated with 20 or 40 μM EGCG, and then mRNA levels of IDO and β-actin were analyzed by RT-PCR. (**B**) HSC-3 cells were cultured with 20 μg/mL CHX in the presence or absence of 40 μM EGCG for the indicated times. (Top) IDO and β-actin protein levels from three independent experiments. (Bottom) Quantification of IDO expression normalized against the level of β-actin control. IDO expression at the 0 h time point was set as 1.

Transient Tranfection and Luciferase Assay. For reporter assay, 1 day before transfection, 1×10^5 HSC-3 cells were plated onto 24-well plates, grown to 90% confluence, and then cotransfected with pRL-CMV (containing the Renilla luciferase reporter gene) and pIDO-Luc (the IDO promoter-directed firefly luciferase reporter) by using Lipofectamine 2000 (Invitrogen). After 6 h of incubation, the medium was replaced with 10% FBS. After transfection, the HSC-3 cells were treated with EGCG at various concentrations with or without IFN-γ stimulation. For luciferase assays, cells were lysed in lysis buffer (Promega, Madison, WI), and the firefly luciferase and Renilla luciferase activities were dectected with dual luciferase assay kits (Promega).

Reverse Transcriptase (RT)-PCR. Total RNA was isolated from 1×10^6 cells by TRIzol (Invitrogen). Ten micrograms of total RNA was used for the first-strand cDNA synthesis. The oligonucleotide primers used for IDO amplification were 5′-GGCAAAGGTCATGGAGATGT-3′ (forward) and 5′-GCTTGCAGGAATCAGGATGT-3′ (reverse). PCR was performed for 30 cycles under the condition of 94 °C for 60 s, 56 °C for 90 s, and 72 °C for 60 s. PCR products were visualized under UV light after 2% agarose gel electrophoresis.

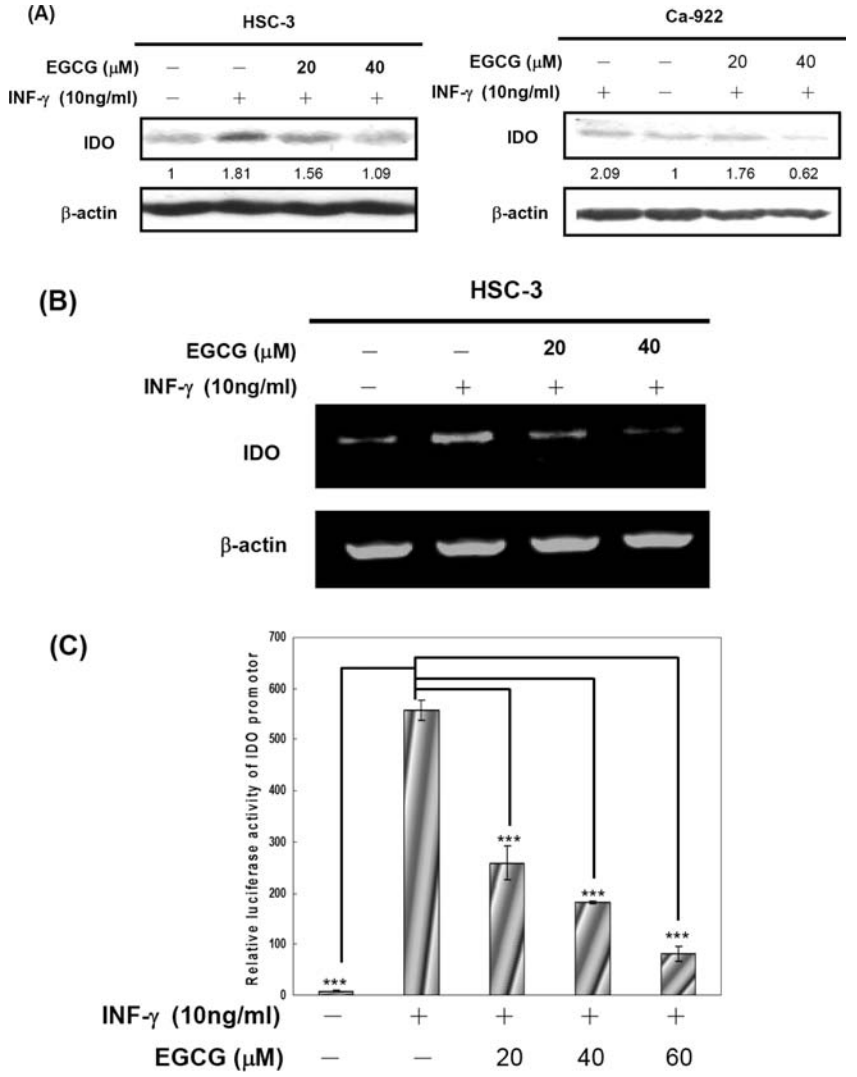

Figure 3. EGCG inhibits the expression and activity of IDO in IFN-γ-stimulated oral cancer cell lines. HSC-3 and Ca-922 cells were preincubated in the absence or presence of various concentrations (20 or 40 μM) of EGCG for 1 h and then treated with IFN-γ (10 ng/mL) for 24 h. (**A**) IDO proteins were assayed by Western blot analysis. These data are representative of those obtained in at least three independent experiments. The values below the figures represent change in protein expression of the bands normalized against β-actin control. (**B**) By using RT-PCR, mRNAs of IDO and β-actin were measured. (**C**) IDO promoter activity of HSC-3 cells analyzed by luciferase reporter assays. Values are represented as mean $\pm$ SD. Asterisks indicate values significantly different from the control: ***, $P < 0.001$.

Immunofluorescent Staining. HSC-3 cells were seeded onto a 6-well plate. All samples were fixed for 20 min with 4% formaldehyde and then incubated in 0.2% Triton X-100 for 15 min at 4 °C. After three washings with PBS, all slides were incubated 1 h with blocking buffer containing 4% BSA and then treated with primary antibody overnight at 4 °C. After three washings with PBS, all slides were incubated with fluorescence-labeled secondary antibody at 37 °C in the dark and then stained with DAPI (Sigma). The above experimental results were analyzed by confocal imaging microscopy.

Statistical Analysis. All values were expressed as mean $\pm$ SD. Each value was the mean of at least three individual experiments in each group. Student's *t* test was used for statistical comparison. Asterisks indicate that the values were significantly different from the control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

RESULTS AND DISCUSSION

EGCG Promotes Down-regulation of IDO in Human Oral Cancer Cell Lines. To investigate whether the inhibition of EGCG on the IDO protein level is a general phenomenon for human oral cancer cell lines, Western blotting was performed to determine the IDO protein level in human oral cancer cells SAS, Cal-27, HSC-3, Ca-922, and SCC-4 treated with various concentrations of EGCG at 37 °C for 24 h (**Figure 1A**). The results showed that the expression of IDO in human oral cancer cell lines was suppressed by EGCG in a dose-dependent manner. Moreover, suppression of IDO by EGCG was detected by immunofluorescence staining. The IDO protein in HSC-3 was stained by using an anti-IDO antibody (green). Down-regulated IDO in HSC-3 by EGCG was observed by confocal microscopy (**Figure 1B**). To characterize the effects of EGCG treatment on IDO enzyme activity, we assayed the levels of tryptophan and its degradation products, kynurenine, in culture medium. HPLC data showed that culture medium from HSC-3 cells with EGCG treatment exhibited higher levels of tryptophan (**Figure 1C**) and lower levels of kynurenine (**Figure 1D**) by comparison with control. Our results demonstrate that treatment with EGCG significantly reduced IDO activity in HSC-3 cells. Cancer cells may induce immunosuppression and evasion through several different mechanisms. Some of these mechanisms are well-known and demonstrated. Recently, a novel mechanism of IDO-induced tumor-derived immune escape was discovered. IDO was expressed by a variety of cells, including

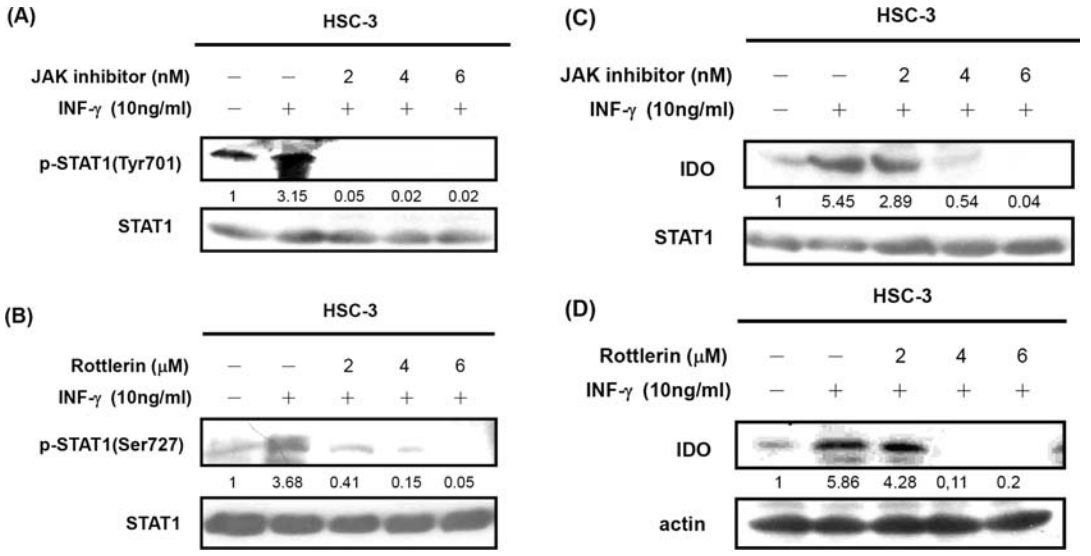

Figure 4. IFN-γ induces IDO expression via JAK-STAT signaling pathway. (**A**, **B**) HSC-3 cells were pretreated with the JAK inhibitor or rottlerin for 1 h and then treated with IFN-γ (10 ng/mL) for 30 min. STAT1 and STAT1 tyrosine701 or serine727 phosphorylation was detected by Western blot analysis. (**C**, **D** Before stimulation with IFN-γ, HSC-3 cells were incubated in the presence or absence of JAK inhibitor or rottlerin for 1 h at the indicated concentration. IDO proteins were detected by Western blotting with IDO monoclonal antibody. These data are representative of those obtained in at least three independent experiments. The values below the figures represent change in protein expression of the bands normalized against β-actin control.

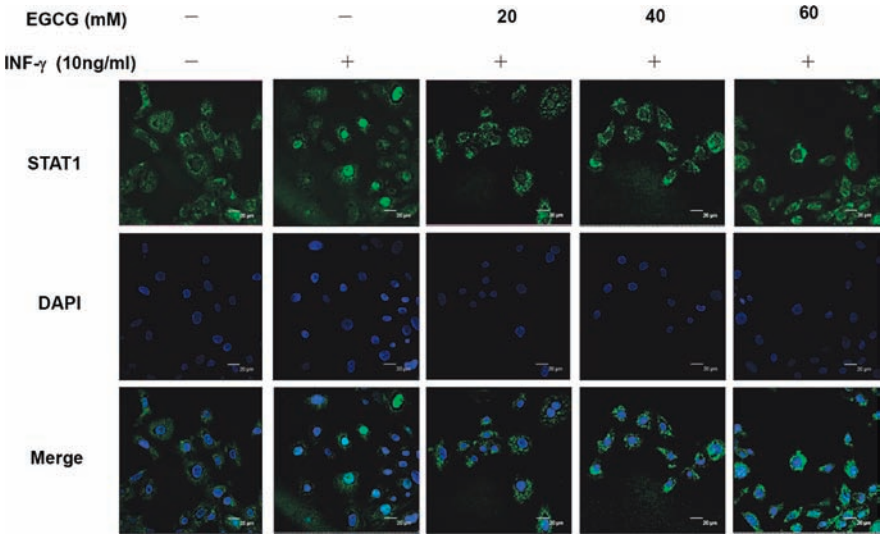

Figure 5. EGCG inhibits STAT1 translocation into nucleus. HSC-3 cells were pretreated with various concentrations of EGCG for 1 h and then stimulated with 10 ng/mL IFN-γ for 12 h. The translocation of STAT1 was demonstrated by immunofluorescent staining with STAT1 antibody.

different subsets of both murine and human tumor cells (*9*). Our results indicate that EGCG could decrease the expression and activity of the immunomodulatory protein IDO, an enzyme-induced immune tolerance, and suppression of T cells in human oral cancer cell lines.

EGCG Treatment Suppresses IDO Expression at the Transcriptional Level. To investigate the molecular mechanisms of suppression of IDO by EGCG, a semiquantitative RT-PCR analysis was performed. The amplification of cDNA with primers specific for IDO and β-actin (as internal control gene) were used for PCR. The RT-PCR results showed that the IDO mRNA expression level was significantly repressed by EGCG in a dose-dependent manner (**Figure 2A**). The results indicate that IDO mRNA expression was significantly reduced by EGCG at the transcriptional level in HSC-3 cells. To further determine whether IDO protein stability was accelerated by EGCG, we treated HSC-3 cells with the translation inhibitor cycloheximide (CHX) or with the inhibitor plus EGCG and then measured the relative IDO level in these cells. As shown in **Figure 2B**, the IDO protein level was not significantly decreased in HSC-3 cells treated with CHX plus EGCG compared with CHX alone. This result demonstrates that the expression and activity of IDO in human cancer cell lines is regulated by EGCG. IDO enzyme activity not only induces tumor immune escape but is also involved in tumor growth. EGCG inhibits IDO mRNA expression in a dose-dependent manner. These results suggest that EGCG induces IDO depletion at the transcriptional level.

EGCG Inhibits IFN-γ-Induced IDO Expression in HSC-3 Cells. IFN-γ is the major inducer of IDO expression. To investigate whether EGCG suppresses IFN-γ-induced IDO expression, we analyzed IFN-γ-stimulated IDO expression in HSC-3 and Ca-922 cells with EGCG treatment by Western blotting. We found that IFN-γ-induced expression of IDO was repressed in a dose-dependent manner by EGCG in HSC-3 and Ca-922 cells (**Figure 3A**).

To address the mechanism by which EGCG decreased the protein level of IDO in IFN-γ-stimulated HSC-3 cells, we examined the mRNA level of IDO. Using semiquantitative RT-PCR, the results showed a significant increase of the IDO mRNA in IFN-γ-induced HSC-3, whereas EGCG inhibited the increase of IDO mRNA in a dose-dependent manner (**Figure 3B**). To further determine whether IDO promoter was affected by EGCG, HSC-3 cells were transfected with the plasmid containing a luciferase reporter gene driven by IDO promoter. By using luciferase assay, the effect of EGCG on the IDO promoter activity was measured. As shown in **Figure 3C**, EGCG exerted its inhibitory effect on IFN-γ-induced IDO promoter activation via a dose-dependent manner when HSC-3 cells were incubated with EGCG for 24 h. These results indicate that EGCG suppresses IFN-γ-induced IDO protein synthesis at the transcriptional level. Previous studies reported that antioxidants inhibit IDO in IFN-γ-activated human macrophages (*24*) and that curcumin significantly inhibited the expression and enzymatic functions of IDO in IFN-γ-stimulated murine BMDCs (*9*). Among the antioxidants that were reported to have inhibiting effects on IDO expression and enzyme activity, EGCG, an antioxidant that could eliminate superoxide and free radicals, was used in this study. We have previously demonstrated that EGCG inhibits protein synthesis, lipogenesis, and cell cycle progression through activation of AMPK, which is associated with cancer (*26*). Cancer cells create a microenvironment with cytokines that promotes cancer cell survival (*1*). The most important purpose of this study is to investigate the inhibiting mechanism of EGCG on IDO-mediated tumor immune escape. Here, we found IFN-γ could strongly induce IDO expression in human oral cancer cells. Furthermore, we showed that IFN-γ-induced IDO, an enzyme associated with tumor immune escape and T cell suppression, was down-regulated by EGCG treatment. These results indicate that EGCG inhibits IFN-γ-induced IDO expression and leads to recovery of the IDO-mediated cancer immune escape. Therefore, we provide a potential tactic of cancer immunotherapy in human oral cancers via suppression of IDO by EGCG.

STAT1 Phosphorylation via JAK-STAT Signaling Is Essential for IFN-γ-Induced IDO Expression in HSC-3 Cells. Because IFN-γ-stimulated IDO expression was inhibited by EGCG in HSC-3 cells, we next examined whether IFN-γ-stimulated IDO protein synthesis was involved in the JAK-STAT signaling pathway in HSC-3 cells. We also examined whether phosphorylation of tyrosine 701 and serine 727 of STAT1 was triggered in IFN-γ-stimulated HSC-3 cells and dependent on activation of JAK1/2 and PKC-δ. After pretreatment with JAK inhibitor for 1 h before stimulation with IFN-γ for 30 min, Western blot data showed phosphorylation of STAT1 at tyrosine 701 was abrogated by JAK inhibitor (**Figure 4A**) and PKC-δ inhibitor; rottlerin suppressed phosphorylation of STAT1 at serine 727 in IFN-γ-stimulated HSC-3 cells (**Figure 4B**). Moreover, we also found that the IDO expression induced by IFN-γ was reduced by the JAK inhibitor in HSC-3 cells (**Figure 4C**). Rottlerin treatment also suppressed the IFN-γ-induced IDO expression in a dose-dependent manner (**Figure 4D**).

It has been known previously that IDO induction was through a number of pathways, such as PI3K- and JNK-dependent pathways (*27*), noncanonical NF-κB pathway (*28*), and the IFN-γ-induced JAK-STAT signaling pathway (*11*). In this study, we found that blocking JAK and PKC-δ activation in IFN-γ-stimulated human oral cancer cells may inhibit STAT1 phosphorylation and results in down-regulation of IDO expression. The results as shown in **Figure 4** demonstrate that IFN-γ-induced IDO expression in HSC-3 cells mediated the activation of STAT1

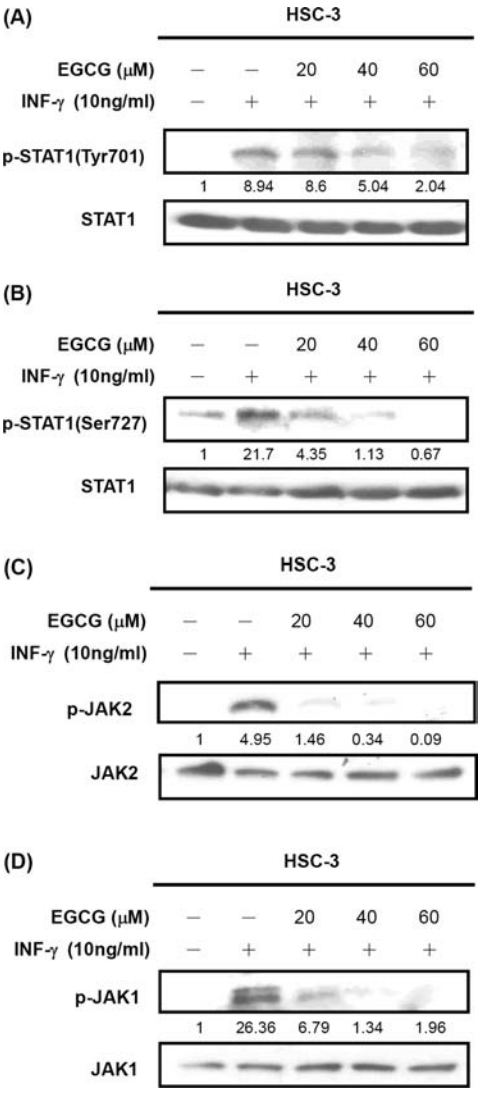

Figure 6. EGCG suppresses IFN-γ-induced phosphorylation of JAK1/2 and STAT1 (tyrosein 701 and serine 727) in HSC-3 cells. HSC-3 cells were pretreated with various concentrations of EGCG for 1 h and then stimulated with 10 ng/mL of IFN-γ for 30 min. Cell lysates were used for immunoblotting analysis. (**A**, **B**) STAT1 and phospho-STAT1 (tyrosine 701 and serine 727) were detected by Western blot analysis. (**C**) Immunoblotting was used to measure levels of JAK1 and phospho-JAK1. (**D**) Immunoblotting was used to measure levels of JAK2 and phospho-JAK2. These data are representative of those obtained in at least three independent experiments. The values below the figures represent change in protein expression of the bands normalized against β-actin control.

via JAK and PKC-δ signaling pathways. Therefore, these results indicate that activation of STAT1, which is phosphorylated by JAK at tyrosine 701 and by PKC-δ at serine 727, plays an important role in IFN-γ-induced IDO expression in human oral cancer cells.

IFN-γ-Induced Nuclear Accumulation of STAT1 Is Inhibited by EGCG in HSC-3 Cells. Recent papers have shown that activation of STAT1 can be inhibited by several natural products (*29*). To detect the potential effect of EGCG on the translocation of STAT proteins in IFN-γ-stimulated HSC-3 cells, we performed an immunofluorescence assay by using monoclonal antibody, which specifically recognizes STAT1 viewed under confocol microscope. HSC-3 cells were pretreated with various concentrations of EGCG for 1 h before IFN-γ stimulation. IFN-γ treatment could induce STAT1 translocation into nucleus. However, the

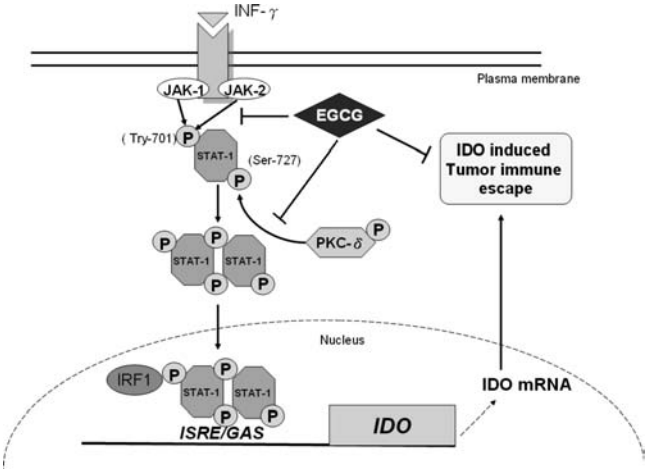

Figure 7. IFN-γ-induced IDO expression is regulated by EGCG in human oral cancer cell line. Binding of IFN-γ with its receptors induces activation of JAK1/2 and PKC-δ through tyrosine phosphorylation. Phosphorylation of STAT1 at tyrosine and serine is from the activation of JAK1/2 and PKC-δ. The dimerization of STAT1 results in nuclear translocation and binding to the GAS site of the IDO promoter. Phosphorylation of JAK1/2, PKC-δ, and STAT1 is inhibited by EGCG. Blocking of the IFN-γ-induced JAK-PKC-δ-STAT1 signaling pathway by EGCG suppresses IDO expression and reduces IDO-mediated tumor immune escape.

IFN-γ-triggered translocation of STAT1 was impaired with EGCG treatment in a dose-dependent manner (**Figure 5**). STAT1 is an important transcriptional factor for IFN-γ-stimulated gene transcription (*30*). The activated STAT1 dimerized and translocated into the nucleus was associated with transcription initiation of IDO gene (*11*). STAT1 binds two sequence elements of IDO promoter, interferon activation sequences (GAS) and interferon-stimulated response element (ISRE). Both of the two sequence elements of IDO promoter are related to IFN-γ response and promote IDO gene transcription. In the present study, we used immunofluorescent staining to confirm that EGCG was able to inhibit STAT1 translocation into nucleus and resulted in down-regulation of IDO gene transcription.

Phosphorylation of JAK1/2 and PKC-δ Is Suppressed by EGCG in IFN-γ-Stimulated HSC-3 Cells, Leading to Inhibition of IFN-γ-Triggered STAT1 Phosphorylation. We have shown that EGCG may block IFN-γ-induced translocation of STAT1, leading to inhibition of IDO expression. To investigate whether the decrease of IDO expression by EGCG resulted from blocking of the IFN-γ-induced JAK-STAT signaling pathway, HSC-3 cells were treated with various concentrations of EGCG for 1 h before stimulation with IFN-γ for 30 min. The phosphorylation of STAT1 was analyzed by Western blotting. As shown in **Figure 6A**, EGCG dramatically reduced the levels of phosphorylation of STAT1 (tyrosine 701) in IFN-γ-stimulated HSC-3 cells in a dose-dependent manner. Similarly, the phosphorylation of STAT1 at serine 727, which was activated by PKC-δ, was reduced by EGCG (**Figure 6B**). To further trace the upstream event, other proteins related to the JAK-STAT signaling pathway were examined in addition to STAT1. Before stimulation with IFN-γ for 30 min, HSC-3 cells was pretreated with various doses of EGCG for 1 h. Western blotting data showed that EGCG treatment significantly reduced the phosphorylation of JAK1 in IFN-γ-stimulated HSC-3 cells (**Figure 6C**). As shown in **Figure 6D**, in IFN-γ-stimulated HSC-3 cells, treatment with EGCG also reduced the level of JAK2 phosphorylation in a dose-dependent manner. In the present study, we discover a different mechanism for EGCG inhibiting immune escape of oral cancer cells. We demonstrated that IFN-γ was able to induce an immune tolerance protein, IDO expression via JAK-PKC-δ-STAT1 signaling pathway. Moreover, EGCG treatment may suppress IDO gene transcription by blocking the JAK-PKC-δ-STAT1 signaling pathway (**Figure 7**). Our results suggest a new mechanism showing EGCG can inhibit IFN-γ-induced IDO expression in human oral cancer cells and provide a potential drug for clinical cancer immunotherapy in the future.

ABBREVIATIONS USED

IDO, indoleamine 2,3-dioxygenase; IFN-γ, gamma-interferon; EGCG, (−)-epigallocatechin-3-gallate; JAK, Janus-activated kinase; STAT, signal transducer and activator of transcription; PKC, protein kinase C; RT, reverse transcriptase.

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