Epigallocatechin gallate (EGCG) restores 25-hydroxy vitamin D levels in rheumatoid arthritis patients by attenuating ROS-mediated activation of NF-κB

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Abstract

Purpose: To investigate the mechanism by which epigallocatechin gallate (EGCG) attenuates ROS-induced pathogenesis in a rheumatoid arthritis (RA) patients.

Methods: Fifty patients (33 male and 17 female) aged between 25 and 60 years who satisfied ACR 1987 criteria for active RA, and thirty healthy individuals were recruited. Total peripheral blood mononuclear cells (PBMCs), neutrophils and monocytes were isolated from the blood samples of RA patients and healthy donors using commercial extraction kits. Levels of reactive oxygen species (ROS) and superoxide radical (O²⁻) in isolated cells were measured using fluorescence spectroscopy while the levels of the associated inflammatory cytokines (TNF-α and IL-6) were determined in PBMCs isolated from RA patients using enzyme-linked immunosorbent assay (ELISA). These parameters were monitored in EGCG-treated and untreated cells. Moreover, the status of 25(OH) vitamin D and NK-κB were investigated in lipopolysaccharide (LPS)-challenged PBMCs in the presence and absence of EGCG.

Results: Elevated levels of superoxide radical and ROS were observed in neutrophils from RA patients in the absence of EGCG, but in the presence of EGCG, the levels of the two parameters were significantly reduced (p < 0.05). Similarly, EGCG treatment downregulated the ROS-mediated increases in TNF-α and IL-6 in the PBMCs from RA patients (p < 0.05). Treatment of PBMCs with the ROS-inducing agent, LPS, resulted in the activation of NK-κB via phosphorylation, and also depletion of 25(OH) vitamin D levels (p < 0.05). However, pre-treatment of the LPS-challenged cells with EGCG inhibited NK-κB activation and 25(OH)-vitamin D depletion (p < 0.05). Moreover, 25(OH) vitamin D levels were restored in the presence of NK-κB inhibitor, flavopiridol, thereby confirming the direct regulatory role of NK-κB in 25(OH) vitamin D levels.

Conclusion: EGCG restores 25(OH) vitamin D levels by attenuating ROS-mediated NK-κB activation. Thus, EGCG may be a promising drug candidate for RA.

Keywords: Rheumatoid arthritis, Vitamin D, Epigallocatechin gallate, Oxidative stress, NK-κB
INTRODUCTION

Rheumatoid arthritis (RA) is a frequently-occurring autoimmune disorder associated with systemic complications, progressive disability, and mortality [1]. Recent reports have shown that oxidative stress is a major factor in the pathogenesis of the disease [2-4]. Oxidative stress is characterized by uncontrolled release of ROS. It is known to increase the release of numerous pro-inflammatory modulators such as cytokines and prostaglandins, which could further lead to tissue and joint lesions in RA patients.

To date, treatment regimens for RA include hormone-based drugs such as glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), anti-rheumatic agents (DMARDS), and anti-cytokine agents which are mainly intended to relieve the pain and minimize tissue damage [5]. Although these agents are known to improve the condition of the patient to a certain extent, they can also induce significant toxicity in patients [6]. Therefore, it is important to screen for less toxic and novel anti-RA drug candidates which can counter ROS generation.

Green tea (Camellia sinensis) is a widely consumed beverage all over the world, and it is highly beneficial to human health. Green tea possesses antibacterial, antioxidative, anti-inflammatory and anti-tumorigenic properties [5]. Catechins, which are the major components of green tea, comprise (-)-epigallocatechin gallate (EGCG), (-)-epicatechin-3-gallate, (-)-epigallocatechin, and (-)-epicatechin. (-)-Epigallocatechin gallate (EGCG; Figure 1A) is the most abundant catechin component of green tea, accounting for 50 to 80% of tea catechin: a cup of green tea contains 200 - 300 mg of EGCG [7].

In the present study, the antioxidant potential of EGCG was investigated in PBMCs and neutrophils derived from the blood of RA patients.

EXPERIMENTAL

Reagents

(-)-Epigallocatechin gallate (EGCG) and DCFH-DA were purchased from Sigma, USA. Hisep1077 and Granulosep 1119 were obtained from (Himedia, USA). Tumor Necrosis Factor –α (TNF-α) Elisa Kit [# ELH-TNFa-1] and IL-6 Human Elisa Kits (#ELH-IL6-CL-1) were purchased from Ray Biotech, USA. Anti-rabbit monoclonal p-NFκB p65 (D14E12) [8242] antibody, and anti-rabbit monoclonal β-Actin (13E5) (4970) [4970] antibodies were procured from Cell Signalling Technology, USA.

Subjects

A total of 50 RA patients (33 male and 17 female) between the ages of 25 and 60 years, were recruited for this study. The patients were selected from those who came for treatment in orthopaedics OPD of Department of Rheumatology and Immunology, Xi’an Fifth Hospital. The patients included in this study fulfilled American College of Rheumatology 1987 criteria [8]. Thirty healthy individuals (20 males and 10 females) were selected as a control population. Patients with any co-morbid conditions such as diabetes, cardiovascular disorder and hypertension, addiction to smoking or alcohol, and those undergoing any type of antioxidant therapy, were excluded from this study. All subjects submitted written consent for collection of blood. Blood samples from RA patients and healthy individuals were collected after an overnight fast. The study was conducted as per the approval of the ethical committee members of the Institutional Ethics committee and Research Advisory Committee (approval no. RA-05674), following the American College of Rheumatology 1987 criteria [8].

Isolation of neutrophils and monocytes from blood samples

Neutrophils and PBMCs from the blood of RA patients and healthy individuals were isolated using HiSep 1077 kit (an iso-osmotic solution containing polycose and diatrizoic acid dihydrate, with a density of 1.0770 g/ml), and Granulosep GSM 1119 kit (a mixture of polycose and sodium diatrizoate, with a density of 1.119 g/ml), in line with the manufacturer’s protocol. Granulosep GSM 1119 was used to isolate neutrophils, while HiSep1077 and Granulosep GSM 1119 were used to isolate the peripheral blood mononuclear cells (PBMCs) or monocytes.

Initially, a double gradient was created with equal volumes of HiSep 1077 overlaid on Granulosep 1119 layer (1.5 ml each). Then, 3 ml of whole blood was layered over the gradient and centrifugation was performed at 2000 g for 25 min at room temperature. Due to centrifugation, granulocytes got separated on the interphase of GranuloEl Sep 1119/HiSep 1077 layer. Neutrophils were then collected from the interphase layer, washed thrice in RPMI 1640 PR- media, and again centrifuged at 1200 g for 10 min. After
In the isolation of monocytes, HiSep 1077 was overlaid with an equal volume of whole blood (3 ml each), and centrifuged at 2000 g for 30 min at room temperature. The PBMCs or monocytes accumulated at the plasma/HiSep 1077 interphase, while the granulocytes and erythrocytes sedimented in the pellet. The supernatant was carefully removed and the monocytes were collected from the interphase RPMI 1640 PR media. The suspension was then centrifuged again at 1000 g for 10 min at room temperature. After centrifugation, the cell pellet was suspended in RPMI 1640 PR medium containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 % FBS, prior to use.

**Cell viability**

The effect of EGCG on the viability of PBMCs was determined using trypan blue exclusion assay. The cells were incubated with EGCG (0 - 50 μM) for 6 h, trypsinized and stained with 0.4 % trypan blue dye. Viable cells excluded the dye and showed clear cytoplasms when observed under the microscope in a hemocytometer. The percentage of viable cells (V) was determined as shown in Eq 1.

\[
V (\%) = \{1.00 - (N_b/N_t)\} \times 100 \quad \text{(1)}
\]

where \(N_b\) is the number of blue cells or dead cells, and \(N_t\) is the total number of cells.

**Determination of superoxide radical (O\(_2^-\))**

Levels of superoxide radicals (O\(_2^-\)) in the neutrophils isolated from the blood of RA patients and healthy individuals were determined spectrophotometrically using a method reported in a previous study [7]. Neutrophils (1 x 10\(^6\) cells/mL) were preincubated with 10 μM EGCG for 1 h. The cells were then washed twice with PBS and incubated with cytochrome C (1 mg/mL) for 30 min at room temperature. In this assay, extracellular superoxide radicals (O\(_2^-\)) react with cytochrome C, resulting in its reduction which is reflected in reduction in absorbance at 550 nm. The cell suspension was then centrifuged at 6000 rpm for 5 min, and the absorbance (A\(_{550}\)) of the clear supernatant containing the reduced cytochrome C was measured at 550 nm using a plate reader. The number of superoxide radicals formed per ml of the reaction mixture was determined as shown in Eq 2 [9].

\[
O_2^- \text{ (nmol)} = 47.7 \times A_{550} \quad \text{(2)}
\]

**Determination of total ROS**

Total oxidative burden in the neutrophils isolated from RA patients’ blood were determined spectrofluorimetrically using the ROS sensitive fluorescence dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Neutrophils (1 x 10\(^6\) cells/mL) were treated with 10 μM EGCG for 1 h. Thereafter, the cells were washed with PBS and incubated with DCFH-DA (30 μM) for about 30 min at room temperature in the dark. Then, the cell extract was centrifuged at 6000 rpm for 5 min and the supernatant was discarded. The cell pellet obtained was resuspended in PBS buffer and fluorescence measurement was performed with a fluorimeter (Perkin-Elmer LS50B fluorimeter (2H2 INC, Ontario, Canada) (Ex485/Em530 nm).

**Evaluation of pro-inflammatory cytokines**

The major pro-inflammatory cytokines TNF-α and IL-6 were estimated using ELISA assay with commercially available kits (Ray Bio® Human TNF-alpha ELISA Kit and Human IL-6 ELISA kit), following the manufacturers’ protocols.

**Assay of 25-hydroxy vitamin D**

Levels of 25(OH)-D were measured independently in serum samples isolated from three sets of samples: blood samples from healthy individuals, blood samples from RA patients, and RA patient blood samples incubated with EGCG for 1 h. Blood samples from all sets were then allowed to clot and sera were separated. Levels of 25(OH)-D were determined using ELISA kit (Abcam, ab213966), following the manufacturer’s protocol.

**Western blotting**

Total proteins were extracted from the cells in the following groups: untreated PBMCs (control), cultured PBMCs (1x 10\(^6\) cells/mL) treated with LPS (1 μg/mL) for 30 min to induce inflammatory response, and cultured PBMCs (1x 10\(^6\) cells/mL) preincubated with 10 μM EGCG for 6 h prior to LPS treatment. Cells from each set were lysed using RIPA lysis buffer (Sigma, USA, # R0278) and protein contents of the lysates were estimated using the Bradford reagent (Sigma, USA, # B6916-500M). The cellular proteins were then separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then electroblotted onto nitrocellulose membranes (Bio-Rad, USA) according to the manufacturer’s protocol, using
TransBlot Turbo Transfer System (Biorad, USA). Thereafter, the membranes were incubated with the appropriate primary antibodies (1:1000 dilution) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody (1:5000 dilution). Finally, protein bands were visualized using the HRP-substrate (Clarity and Clarity Max ECL Western Blotting Substrate, Biorad, USA).

**Statistical analysis**

All results are presented as mean ± SD. Comparisons between two groups were performed using independent t-test and Mann-Whitney U test. Comparisons amongst multiple groups were performed using one-way ANOVA and post hoc tests. All statistical analyses were done using Graph Pad Prism software, version 5.0 (Graph Pad Software Inc, San Diego, CA). Values of $p < 0.05$ indicated significant difference.

**RESULTS**

**Effect of EGCG on human PBMC**

Cytotoxicity of EGCG (Figure 1A) on PBMC was monitored using trypan blue exclusion assay. The PBMCs from healthy donors were treated with different concentrations of EGCG for 6 h and cell viability was monitored using MTT assay. The results showed that EGCG did not exhibit any significant cytotoxicity at doses of 1 - 10 µM. However, slight cell death was observed at doses of 25 and 50 µM (Figure 1 B).

**Figure 1:** A. Structure of EGCG. B. Trypan blue exclusion assay indicating the viability of PBMCs from ten healthy individuals in the presence of different concentrations of EGCG (0-50 µM) for 6 h; *p < 0.05, vs untreated control cells

**Neutrophils from RA patients had enhanced levels of superoxide anion and ROS**

The involvement of ROS in the pathogenesis of RA was confirmed by measuring levels of superoxide radical and total ROS in neutrophils isolated from healthy donors ($n = 30$) and RA patients ($n = 50$). Superoxide radical was estimated spectrophotometrically using the superoxide-sensitive cytochrome C reduction. The results indicated that the superoxide levels were highly elevated in neutrophils from RA patients, when compared to the healthy group, by about 6 folds (Figure 2 A). Similarly, total ROS as determined using the fluorescence probe DCF-DA was highly elevated by 11 folds in neutrophils from RA patients, relative to the healthy controls (Figure 2 B). Thus, these results confirm the involvement of ROS in RA pathogenesis.

**Figure 2:** A. Levels of superoxide radical in neutrophils isolated from the blood samples of RA patients ($n = 50$) and healthy individuals ($n = 30$); $p < 0.05$, vs control. B. Levels of ROS in neutrophils isolated from the blood samples of RA patients ($n = 50$) and healthy individuals ($n = 30$); $p < 0.05$ vs control

**EGCG in neutrophils quenched ROS from RA patients**

Pre-treatment of neutrophils isolated from RA patients with EGCG significantly reduced the ROS burden, as was confirmed by determination of superoxide and total ROS levels (Figure 3). There was a significant 3-fold reduction in superoxide burden in EGCG-treated neutrophils, and under similar conditions, total ROS was also reduced by 2 folds (Figure 3). Thus, the oxidative burden in RA samples was significantly reduced in the presence of EGCG.

**Figure 3:** Levels of superoxide radical and total ROS in neutrophils isolated from blood samples of healthy individuals, blood samples of RA patients and the isolated neutrophils from the blood samples of RA patients treated with EGCG. A. Superoxide radical $p < 0.05$, vs control; $p < 0.05$, vs RA. B. Total ROS, $p < 0.05$, vs control; $p < 0.05$ vs RA
EGCG reduced levels of pro-inflammatory cytokines in PBMCs derived from RA patients

The pro-inflammatory cytokines TNF-α and IL-6 were measured in the PBMCs isolated from healthy donors and RA patients in the presence and absence of EGCG. Increased levels of TNF-α and IL-6 were found in RA patient samples, when compared to their healthy counterparts. There was a 6-fold increase in TNF-α level in RA patient-derived PBMCs, whereas level of IL-6 was increased by 8 folds, when compared to the healthy PBMCs. Interestingly, treatment of RA patient-derived PBMCs with EGCG resulted in significant decreases in the levels of TNF-α and IL-6, relative to the untreated PBMCs from RA patients (Figure 4 A and B).

EGCG inhibited ROS-mediated down-regulation of vitamin D in PBMC cells

To find out the mechanism by which EGCG modulated vitamin D levels, PBMCs isolated from healthy donors were challenged with lipopolysaccharide (LPS). Treatment of PBMCs with LPS (1 µg/mL) induced ROS generation in a time-dependent fashion, with the highest ROS obtained after 15 min of incubation (Figure 5 A). As expected, preincubation of LPS-challenged cells with EGCG significantly reduced the levels of ROS (Figure 5 B). Very interestingly, it was observed that 25-(OH) vitamin D levels were restored in LPS-challenged cells pre-treated with EGCG. Thus, these results indicate that 25-(OH) vitamin D levels were markedly reduced by ROS and significantly restored in the presence of EGCG (Figure 5 C).

EGCG inhibited ROS-mediated activation of NF-κB in LPS-challenged cells

To investigate the role of NF-kB in ROS-mediated inflammatory responses, the status of activated NF-κB was investigated by monitoring the levels of its phosphorylated P65 subunit. It was observed that the expression of p-P65 was increased by 2.7 folds in LPS-challenged PBMC cells, when compared to the untreated control cells. Interestingly, the elevated expression of p-P65 was reduced significantly in the presence of EGCG (Figure 6 A and B).
Inhibition of NF-κB-activation restored 25(OH) vitamin D levels in LPS-challenged cells

To investigate whether the activation of NF-κB had any effect on vitamin D levels, the LPS-challenged cells were treated with flavopiridol (FP), a pharmacological inhibitor of NF-κB, and levels of vitamin D were monitored. Treatment of PBMCs with LPS resulted in the reduction of 25(OH) vitamin D level by 4.2-folds. However, when the LPS-challenged PBMCs were treated with FP, significant restoration of vitamin D level was observed (Figure 6 C). Thus, blocking NF-κB activation restored the vitamin D levels in oxidatively-challenged cells.

DISCUSSION

The direct association between ROS and RA has been well documented [2-5]. Enhanced ROS generation facilitates RA pathogenicity and triggers the secretion of pro-inflammatory cytokines. Recent clinical and pre-clinical studies have shown that vitamin D plays a pivotal role in the onset and pathogenesis of RA [10-14]. Active vitamin D, also known as 25-hydroxy vitamin D is known to affect the immune cells via its immunomodulatory activities [11]. It has also been reported that vitamin D is involved in maintaining ROS homeostasis in vivo [12]. Moreover, a study has shown that vitamin D inhibits the release of pro-inflammatory cytokines such as interferon-γ, TNF-α, and IL-2 [13]. In the present study, elevated levels of superoxide radical and total ROS were observed in the neutrophils isolated from the peripheral blood of RA patients, when compared to the healthy counterparts. These results are consistent with previous findings.

Although there have been a few reports on the effect of EGCG on RA [15,16], the immunomodulatory role of EGCG has not been investigated. The present study has demonstrated a very promising role of EGCG in controlling the pathogenicity of RA at the cellular level. The most important result obtained is the negligible toxicity of EGCG in PBMCs isolated from healthy donors. Furthermore, pre-treatment of neutrophils/PBMCs from RA patients reduced their oxidative burden and also the levels of pro-inflammatory cytokines.

Since vitamin D plays an important role in ROS haemostasis [13], and also modulates pro-inflammatory cytokines, the levels of 25-(OH) vitamin D in EGCG-treated and untreated groups were investigated. Very interestingly, it was observed that 25-(OH) vitamin D levels in the plasma from blood samples of RA patients were restored on pre-treatment with EGCG. To delineate the exact mechanism involved in EGCG-induced modulation of vitamin D levels in RA patients, PBMCs were isolated from healthy donors and challenged with LPS in the presence and absence of EGCG. Extant literature indicates that EGCG suppresses inflammatory responses triggered by LPS in several cell lines and animal models [15,16]. As expected, LPS treatment generated significant ROS in PBMCs, but EGCG effectively suppressed the production of ROS. Moreover, pre-incubation with EGCG restored 25-(OH) vitamin D levels in LPS-challenged cells, which is consistent with previous results from clinical samples.

Nuclear factor-κB (NF-κB) family of transcription factors is composed of homo- or heterodimers of any of the five proteins NF-κB1, NF-κB2, RelA (p65), RelB and cRel, which collectively play crucial roles in inflammatory responses of the immune system [17]. Several reports suggest that the generation of ROS leads to the activation of NF-κB via phosphorylation of P-65 subunit by several stress-responsive kinases [18,19]. Interestingly, it was also observed in this study that levels of phosphorylated P-65 were increased significantly in LPS-challenged PBMCs, but pre-incubation with EGCG reversed that effect. These results indicate that ROS-mediated activation of NF-κB is a crucial event in RA, as was confirmed by in vitro studies. Further investigations were done to find out whether depletion of 25-(OH) vitamin D was a direct effect of ROS-mediated NF-κB-activation. To confirm the effect of NFκB on vitamin D, levels of vitamin D were monitored in the
presence of flavopiridol (FP), a well-known inhibitor of NF-κB [20, 21]. In the presence of FP, the level of 25-(OH) vitamin D was significantly restored in LPS-challenged PBMCs, thereby confirming the involvement of NF-κB in ROS-mediated pathogenesis of RA.

There is no previous report on the regulation of 25-(OH) vitamin D by EGCG in RA and hence this study demonstrates that EGCG by its antioxidant property can restore the levels of 25-(OH) vitamin D by suppressing ROS-induced activation of NF-κB (Figure 7).

**Figure 7:** A schematic representation of the inhibitory action of EGCG on NF-κB activation

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