Int J Clin Exp Pathol 2016;9(5):5421-5428
www.ijcep.com /ISSN:1936-2625/IJCEP0024048

Original Article

EGCG suppresses NF-κB activation induced by gastroesophageal reflux contents in human esophageal epithelial cells

Chang Liu[1*], Jie Dai[2*], Heng Lu[1], Hui Shi[1], Youke Lu[1], Fangyu Wang[1]

[1]Department of Gastroenterology and Hepatology, Jinling Hospital, Nanjing Clinical School of Southern Medical University, Nanjing, China; [2]Department of Gastroenterology and Hepatology, The PLA Navy Anqing Hospital, Anqing, China. [*]Equal contributors.

Received January 14, 2016; Accepted March 26, 2016; Epub May 1, 2016; Published May 15, 2016

Abstract: Background and aims: Epigallocatechin-3-gallate (EGCG) is a natural component of green tea that has been shown to have inhibitory effects against the inflammation-induced onset and the development of carcinogen-induced tumors in animal models at different organ sites, including the esophagus. This study investigates the effect of mixed refluxate (acid, bile acids and trypsin) on the expression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway in normal human esophageal epithelial cells (HEECs) and the effect of EGCG pretreatment of cells on activation of NF-κB induced by the mixed refluxate. Methods: HEECs were cultured *in vitro* and treated with varying concentrations of EGCG in the absence or presence of GER contents. NF-κB DNA-binding activity was examined using an electrophoretic mobility shift assay (EMSA) and intracellular levels of NF-κB were evaluated using an ELISA. NF-κB reporter gene activity was measured using a luciferase reporter gene assay. The expression levels of NF-κB/p65, p-NFκB/p65, IκBα, p-IκBα, p-IKKα and proinflammatory cytokines, such as IL-6, IL-8, iNOS and COX-2 proteins, were examined using Western blot analysis. Results: Exposure of GER to HEECs results in a significant increase in NF-κB DNA-binding activity, intracellular levels of NF-κB and luciferase reporter activity compared to the control group. GER also induced the activation and nuclear translocation of NF-κB/p65, phosphorylation of IκBα and IKKα, and upregulated the expression of NF-κB-regulated proteins IL-6, IL-8, COX-2 and iNOS all of which were significantly downregulated by EGCG pretreatment. Conclusions: Our data suggest that EGCG can suppress GER-induced NF-κB activation and can downregulate the expression of NF-κB-regulated proteins in HEECs.

Keywords: EGCG, gastroesophageal refluxate(GER), HEECs, NF-κB

Introduction

Gastroesophageal reflux disease (GERD) is common in Western countries, and the incidence in United States alone is approximately 7-10% [1]. A mild form of this disease may cause no apparent mucosal damage, but chronic GERD can lead to severe lesions, erosion, ulceration, stricture formation and Barrett's esophagus (BE), a precancerous lesion that could potentially develop into esophageal adenocarcinoma [2]. Bile and acid are major constituents of the gastroesophagealrefluxate (GER) [3], and this mixture is closely associated with tumor progression [4]. Therefore, it is important to understand the molecular and cellular mechanisms of esophageal mucosal injury and the effects of the refluxate on the pathogenesis of GERD. There is a strong relationship between inflammation and cancer, and now, it is recognized that the tumor microenvironment is largely orchestrated by inflammatory cells and contributes to the neoplastic process by promoting cell proliferation, survival and migration [5]. Exposure to the carcinogens present in the GER contents can cause structural and functional changes in the esophageal squamous epithelium, including abnormal proliferation and differentiation of these cells, BE and Barrett's-related adenocarcinoma (BAA).

NF-κB is recognized as a key transcription factor involved in initiating and regulating inflammatory responses. NF-κB activity is regulated

by the cytoplasmic degradation of a related inhibitory protein known as IκBα. Once IκBα is inactivated and the Rel proteins are phosphorylated, the NF-κB dimers relocate to the nucleus [6]. The activated NF-κB binds to specific target sites in the nucleus and regulates the expression of genes related to inflammation, immune responses and cell survival [7]. Recently, both esophageal cell lines and biopsy cultures showed that GER contents can induce the expression of NF-κB dependent genes [8].

Epigallocatechin-3-gallate (EGCG), the major polyphenol found in green tea, is reported to have antioxidant, anti-inflammatory, anti-mutagenic, anti-angiogenic and chemopreventive effects [9-13]. The mechanisms used to describe EGCG's anti-tumor activity include the induction of detoxifying enzymes, inhibition of the activation of carcinogens and inhibition of signaling pathways that control cell proliferation and tumor growth [14]. Although there is extensive literature describing the inhibitory effects of EGCG, most of these studies were conducted in models in which the esophageal carcinogenesis was already well established. It is well known that esophageal tumors result in irreversible structural and functional damage to several tissues and organs. Therefore, it is necessary to investigate the effects of EGCG on healthy esophageal cells, which are exposed to GER contents, and assess the resulting effects that are involved in esophageal carcinogenesis.

In this study, we investigated the relationships between the activated NF-κB induced by GER contents and the anti-inflammatory effect of EGCG in human esophageal epithelial cells (HEECs). Additionally, the non-malignant HEECs used in this study provide clear evidence of specific molecular mechanisms involved in GER -induced esophageal carcinogenesis.

Materials and methods

Antibodies and reagents

Chenodeoxycholic acid (CDCA) and EGCG (>98% pure) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Trypsin (1:250) was obtained from Gibco (Rockville, MD, USA). IκBα, p-IκBα (Ser32/36), IKKα, NF-κB/p65, p-NFκB/p65 (Ser^{536}) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). IL-6, IL-8, COX-2 and iNOS antibodies were purchased from KeyGENBioTECH Co. Ltd (Nanjing, China).

Cell culture

HEECs were purchased from American Type Culture Collection (Manassas, VA, USA). HEECs were cultured in RPMI-1640 medium obtained from Gibco (Rockville, MD, USA) and supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin G, which were purchased from Invitrogen (Carlsbad, CA, USA). Cells were harvested with 0.05% trypsin and maintained at 37°C in an environment containing 5% CO_2.

Cell treatments

A solution of EGCG dissolved in 1X PBS was used for this study. Cells (80-90% confluent) were treated with various concentrations of EGCG (5, 10, 20 μM) at 37°C for 4 h in RPMI-1640 medium following which, the media was removed, the cells were washed with PBS and treated with GER contents (including acid, CDCA, and trypsin) for 12 h and harvested. The acidic culture conditions (pH 6.5), the CDCA concentration (200 μmol/L), and the trypsin concentration (10 U/mL) were recommended by Kawabe et al. [15].

Preparation of total cell lysate

After the treatment of HEECs with EGCG, GER or both, the culture medium was aspirated, and cells were washed thrice with PBS. The cells were incubated in 0.4 ml ice-cold lysis buffer (150 mM NaCl, 100 mM Na_3VO_4, 50 mM Tris-HCl, 20 mM NaF, 1 mM PMSF (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and 0.5% NP-40) containing freshly added protease inhibitor cocktail (Cell Signaling Technology, Beverly, MA, USA). The cells were centrifuged for 20 min (12,000 *g*) at 4°C, and the supernatant (total cell lysate) was collected and stored at -80°C. The protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Preparation of cytosolic and nuclear lysates

Following the treatment of HEECs with EGCG, GER contents or both, the culture medium was

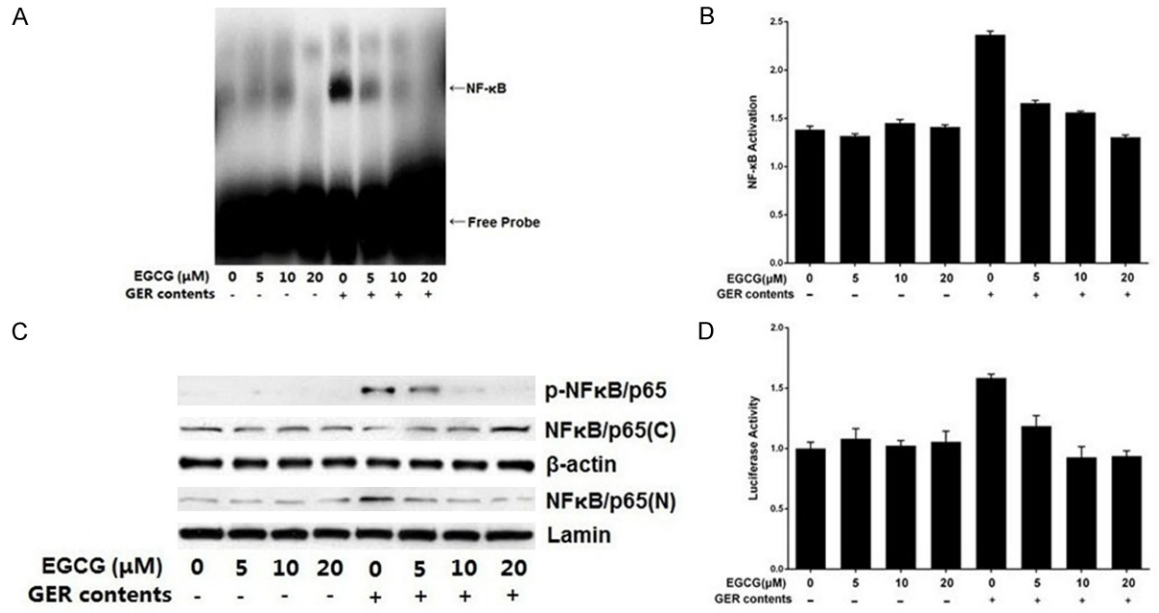

Figure 1. EGCG inhibited GER content-induced activation of NF-κB in HEECs. A. EGCG treatment of HEECs inhibits GER content-induced NF-κB DNA-binding activity. Cells were pretreated with various concentrations of EGCG (5-20 μM) for 4 h and then exposed to GER contents; nuclear extracts were prepared and assayed for NF-κB transcriptional activity using EMSA. The gel is representative of three experiments with similar results. B. EGCG inhibited GER content-induced NF-κB/p65 activity. The effect of EGCG on NF-κB/p65 was evaluated using an ELISA. The values are presented as the mean ± SEM. C. EGCG inhibited GER content-induced NF-κB/p65 phosphorylation, as shown by a decrease in nuclear NF-κB/p65 levels with a concomitant increase in the total cytosolic NF-κB/p65 levels in EGCG treated samples. Cytosolic and nuclear extracts were prepared for Western blot analysis. Equal protein loading was achieved using β-actin and laminin as loading controls. D. EGCG inhibited GER content-induced NF-κB-dependent reporter gene expression. HEECs were seeded at a concentration of 1.0×10^5 cells per well in 6-well plates and co-transfected with an NF-κB-driven luciferase reporter construct and pSV40-β-gal plasmid. After 48 h, cells were treated with GER contents with or without EGCG and Luciferase activity was measured and normalized with respect to β-galactosidase activity. Values are presented as the mean ± SEM. GER contents: acidified media (pH 6.5) treated with CDCA (200 μmol/L) for a total of 12 h along with trypsin (10 U/mL) during the final hour.

aspirated, and the cells were washed thrice with PBS. The cells were incubated in 0.4 ml ice-cold lysis buffer (10 mM KCl, 10 mM HEPES (pH 7.9), 1 mM PMSF, 1 mM DTT, 0.1 mM EDTA and 0.1 mM EGTA) containing freshly added protease inhibitor cocktail for 20 min, following which 12.5 μl of 10% NP-40 was added and the contents were mixed. The cells were centrifuged at 13,000 *g* for 2 min at 4°C. The supernatant (cytosolic lysate) was collected and stored at -80°C. The pellet (nuclear lysate) was resuspended in 50 μl of ice-cold nuclear extraction buffer (20 mM HEPES (pH 7.9), 1 mM PMSF, 1 mM DTT, 1 mM EDTA, 1 mM EGTA and 0.4 M NaCl) containing freshly added protease inhibitor cocktail for 40 min with intermittent mixing. The tubes were centrifuged at 13,000 *g* for 6 min at 4°C. The supernatant (nuclear extract) was collected and stored at -80°C. The protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Electrophoretic mobility shift assay

The nuclear protein extracts from HEECs were prepared as described earlier. For the DNA binding assay, an end-labeled biotinylated double-stranded NF-κB oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was used. Binding reactions were conducted using the nonradioactive Lightshift™ chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) according to the manufacturer's directions. Reaction products were separated through a 6% DNA retardation gel (Invitrogen, Carlsbad, CA, USA) and transferred to a Biodyne B membrane (Pierce, Rockford, IL, USA). The membrane was exposed to X-ray film and developed using the Chemiluminescent Nucleic Acid Detection Module (Pierce, Rockford, IL, USA).

Enzyme-linked immunosorbent assay

Quantitative analysis of NF-κB/p65 activity in the culture media of treated cells was conducted using the NF-κBTrans^{AM} ELISA kit from Active Motif (Carlsbad, CA, USA). All procedures were performed according to the manufacturer's protocol.

Western blot analysis

70 μg of protein was resolved using PAGE (5-10% gels), transferred to PVDF membranes and blocked with 5% non-fat milk in TBS (150 mM NaCl and 20 mM Tris, pH 7.6) containing 0.05% Tween-20. The membranes were incubated overnight at 4°C with the appropriate monoclonal/polyclonal primary antibodies, and then incubated with corresponding HRP-conjugated secondary antibody for 2 h. The protein bands were visualized using the ECL kit (Pierce, Rockford, IL, USA) and imaged using X-ray films.

Luciferase activity

HEECs (1.0×10^5) were plated onto 6-well plates and co-transfected with 6 μg NF-κB-driven luciferase reporter construct and 8 μg pSV40-β-gal plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of exposure to the transfection mixture, cells were incubated in medium containing EGCG for 4 h and subsequently exposed to GER contents for 12 h. The wells were washed with PBS, incubated with Passive Lysis Buffer (Pierce, Rockford, IL, USA) for 20 min on a shaker, and the lysates were collected. Luciferase activity was determined using the Reporter Luciferase Assay System (Promega, Madison, WI, USA) according to the methods provided by the manufacturer and the values obtained were normalized to the β-galactosidase activity (Clontech, Mountain View, CA, USA).

Results

EGCG suppresses GER content-induced NF-κB DNA-binding activity

To exclude the possibility of DNA damage caused due to high concentrations of EGCG, the effects of EGCG on cell viability were assessed using the MTT assay. We found that EGCG pretreatment at concentrations of 5-20 μM for 4 h had an insignificant effect on the viability of HEECs. The results indicate that the NF-κB DNA-binding activity was significantly enhanced after HEECs were exposed to GER contents (**Figure 1A**: *lane* 5). EGCG pretreatment significantly inhibited GER content-induced NF-κB DNA-binding activity of HEECs in a dose-dependent manner (**Figure 1A**: *lane* 6, 7, 8). In addition, the levels of NF-κB DNA-binding activity of cells treated with EGCG alone (**Figure 1A**: *lane* 2, 3, 4) were not significantly different from those of the untreated group (**Figure 1A**: *lane* 1). This indicates that the physiological concentrations of EGCG may not substantially affect the NF-κB DNA-binding activity.

EGCG suppresses GER content-induced phosphorylation and nuclear translocation of NF-κB/p65

Western blot analysis shows that GER contents induce NF-κB/p65 (Ser^{536}) phosphorylation leading to the nuclear translocation of NF-κB/p65. EGCG pretreatment of HEECs inhibited this GER content-induced NF-κB/p65 phosphorylation in a dose-dependent manner (**Figure 1C**). In addition, there is an increase in total cytosolic NF-κB/p65 levels with a concomitant decrease in nuclear NF-κB/p65 levels in EGCG pretreated samples, suggesting that EGCG suppresses GER content-mediated NF-κB/p65 migration from the cytosol to the nucleus (**Figure 1C**). ELISA analysis further confirmed this effect. The activity of nuclear NF-κB/p65 that was induced by the GER contents was effectively suppressed by EGCG (**Figure 1B**).

EGCG suppresses GER content-induced NF-κB reporter activity

GER contents can activate NF-κB, which can lead to the transcriptional activation of NF-κB-inducible genes. To determine whether EGCG could inhibit GER content-induced NF-κB promoter activity, HEECs were transiently transfected with the NF-κB promoter-luciferase reporter plasmid and were exposed to GER contents in the absence and presence of EGCG. An increase in luciferase reporter expression was observed in GER content-exposed cells compared to the control group (**Figure 1D**), suggesting that NF-κB was activated. We found that EGCG pretreatment of HEECs not only suppressed NF-κB DNA-binding activity but also

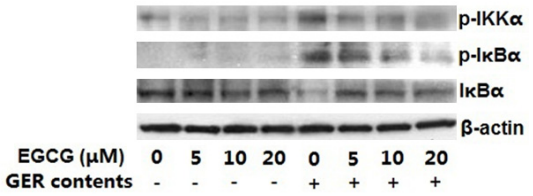

Figure 2. EGCG inhibited GER content-induced phosphorylation of IκBα. HEECs were pretreated with various concentrations of EGCG (5-20 μM) for 4 h and then exposed to GER contents. To examine the effect of EGCG on the expression levels of IKKα and IκBα proteins, cytosolic lysates were prepared for Western blot analysis. The gel is representative of three experiments with similar results. GER contents: acidified media (pH 6.5) treated with CDCA (200 μmol/L) for a total of 12 h along with trypsin (10 U/mL) during the final hour.

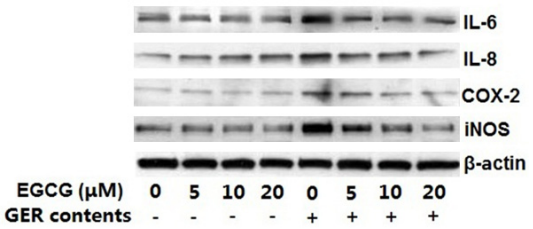

Figure 3. EGCG inhibited GER content-induced activation of inflammatory markers. HEECs were pretreated with various concentrations of EGCG (5-20 μM) for 4 h and then exposed to GER contents, after which the cells were harvested and total cell lysates were prepared for Western blot analysis. The gel is representative of three experiments with similar results. GER contents: acidified media (pH 6.5) treated with CDCA (200 μmol/L) for a total of 12 h along with trypsin (10 U/mL) during the final hour.

led to a significant decrease in GER content-induced NF-κB promoter activity.

EGCG suppresses GER content-induced phosphorylation of IKKα and IκBα

A key step in NF-κB activation is the phosphorylation of IκBα by the IκB kinase (IKK) complex (IKKα, IKKβ and IKKγ/NEMO). To determine whether EGCG's inhibition of NF-κB activation was because of its impact on IκBα degradation, the cytosolic levels of IKKα and IκBα were measured using immunoblotting. The results showed that GER content exposure led to an increased phosphorylation of IKKα and IκBα, and EGCG pretreatment of HEECs suppressed these phosphorylations in a dose-dependent manner (**Figure 2**). Our data indicate that pretreatment of HEECs with EGCG led to the inhibition of GER content-induced IKKα activation, phosphorylation and degradation of IκBα, and subsequent NF-κB activation.

EGCG suppresses GER content-induced activation of inflammatory markers

To determine whether EGCG could decrease the production of proinflammatory cytokines, we used Western blotting to analyze the whole cell extracts. The results showed that GER contents exposure to HEECs led to an increased expression of inflammatory markers such as IL-6, IL-8, COX-2 and iNOS proteins. Pretreatment of HEECs with EGCG led to a significant inhibition of GER content-induced increase in the expression of these proteins (**Figure 3**) indicating that EGCG potentially has anti-inflammatory properties.

Discussion

Many studies have been conducted on esophageal cancer cell lines to evaluate whether EGCG could reduce their viability and invasiveness. In our study, normal esophageal cells pretreated with EGCG showed downregulation of GER content-activated NF-κB. The development of esophageal inflammation and cancer is associated with oxidative stress induced by reflux contents such as gastric acid, bile acids and trypsin [16]. Dvorak et al. showed that exposing immortalized human esophageal epithelial cells HET-1A to pH 2 or 4 for 1 minute led to the production of reactive oxygen species (ROS) and low pH in combination with bile acids induced mitochondrial oxidative stress and DNA damage [17, 18]. These changes may cause cancer. Indeed, previous studies have shown that mitochondrial DNA mutations frequently occur in dysplastic BE and esophageal adenocarcinoma, probably due to oxidative damage [19].

Oxidative stress is closely related to the activation of several signaling pathways, including the NF-κB, Janus kinase/Signal Transducer and Activator of Transcription signal transducer and activator of transcription (JAK/STAT) and MAPK pathways, that can upregulate the expression of pro-survival, anti-apoptotic and angiogenic proteins [20, 21]. Activation of these pathways and expression of anti-apoptotic proteins was shown to be increased in BE [22-24].

We speculate that the oxidative stress induced by the mixed refluxate (acid and bile acids) may lead to the activation of anti-apoptotic and pro-survival pathways in BE and BAA. However, DNA damage in anti-apoptosis cells is dangerous and can result in cell mutations due to replication errors, clonal expansion of mutated cells and tumor progression. Acquired somatic mutations may be an important determining factor in esophageal carcinogenesis and may alter the signal transduction in the esophagus. The squamous epithelium of distal esophagus is frequently exposed to acid and bile acids during a reflux episode. An analysis of the esophageal aspirates of GERD patients suggests that bile acids are found in the esophageal aspirates of 86% of the patients [25]. Therefore, it is necessary to develop chemopreventive strategies for high-risk patients with GERD or for those suffering from chronic reflux.

Pathways activated by EGCG in healthy cells, compared to tumor cells, create a different oxidizing environment, which is conducive to the survival of normal cells and the destruction of tumor cells. Studies have shown that epithelial cells in skin, oral mucosa and gastrointestinal regions, which are in frequent contact with plant-derived polyphenols, are able to develop mechanisms to reduce the toxicity from these compounds [26]. However, high doses of green tea polyphenols in other healthy human cells (which lack these antitoxic mechanisms) or tumor cells (which may have lost these protective mechanisms) may have cytotoxic effects. Therefore, it is important to find an optimal dose of EGCG that can selectively promote the death of tumor cells while protecting the normal cells.

The activation of NF-κB can be induced by microenvironmental signals, such as acid, bile, hypoxia, cytokines, and genetic factors [27]. NF-κB activation is closely associated with an increase in the anti-apoptotic responses and the growth-promoting potential of cells leading to a broad spectrum of stresses, which may lead to a malignant transformation of the cells. In this study, GER content-induced NF-κB activation resulted in the NF-κB/p65 nuclear translocation, which was suppressed by EGCG pretreatment, suggesting that EGCG has the potential to suppress carcinogenesis induced by GER contents.

Cytokine-induced neutrophil accumulation inesophageal inflammation induced by the reflux of gastroduodenal contents has been previously demonstrated [28]. Cytokines, such as IL-1, IL-6, IL-8 and MCP-1, may be secreted during this inflammatory reaction. Rafiee et al. [1] showed that an acidic environment results in increased IL-6 and IL-8 release by normal esophageal epithelial cells. Transcriptional factors, especially those regulated by NF-κB activation, play an important role in the induction of cytokines, COX-2, iNOS, acute-phase proteins, cell adhesion molecules and growth factors [29]. These inflammatory mediators attract inflammatory cells to the tissues damaged by GER contents and can activate other enzymes. The inducible isoform of nitric oxide synthase (iNOS) is considered more important during tumor progression than the other isoforms (eNOS and nNOS) because it is involved in maintaining the nitric oxide levels during inflammatory responses. Nitric oxide can directly damage DNA, inhibit the repair of damaged DNA, regulate transcriptional factors, enhance oncogene expression, block cell apoptosis and promote angiogenesis and is therefore associated with the processes of tumor initiation and development [30]. Moreover, studies have found that a selective nitric oxide synthase inhibitor prevents the progression of rat esophageal tumors induced by nitrosomethylbenzylamine [31]. Soma et al. [32] showed that GER contents induced the expression of COX-2 in both normal and cancerous esophageal cells. Abdel-Latif et al. [33] speculated that the COX-2 expression in the esophagus may be directly related to the signal transduction pathways involved in tumor progression. Our results show that EGCG suppresses the activation of inflammatory markers (IL-6, IL-8, COX-2 and iNOS) induced by GER contents, suggesting that EGCG can prevent the formation of an inflammatory environment and thus, inhibit pathogenesis of GER contents-induced esophageal diseases.

Conclusions

In this study, we have shown that EGCG inhibits the GER content-mediated activation of NF-κB and reduces the production of inflammatory markers such as IL-6, IL-8, COX-2 and iNOS in HEECs. According to our results, long-term exposure to low levels of green tea derived

EGCG could play a chemopreventive role in patients with chronic GERD and in those suffering from chronic reflux. Further studies to test the efficacy of EGCG in animal models with inflammation-associated esophagus injury and to elucidate the mechanisms by which this natural product modulates proinflammatory signal transduction pathways will be conducted.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 812-70453) to Fang-Yu Wang.

Disclosure of conflict of interest

None.

Address correspondence to: Fangyu Wang, Department of Gastroenterology and Hepatology, Jinling Hospital, Nanjing Clinical School of Southern Medical University, 305 East Zhongshan Road, Nanjing 210002, Jiangsu Province, China. Tel: (0086) 25-80861051; Fax: (0086) 25-80861051; E-mail: wangfangyu1965@163.com

References

[1] Rafiee P, Nelson VM, Manley S, Wellner M, Floer M, Binion DG and Shaker R. Effect of curcumin on acidic pH-induced expression of IL-6 and IL-8 in human esophageal epithelial cells (HET-1A): role of PKC, MAPKs, and NF-kappaB. Am J PhysiolGastrointest Liver Physiol 2009; 296: G388-398.

[2] Thrift AP, Kramer JR, Qureshi Z, Richardson PA and El-Serag HB. Age at onset of GERD symptoms predicts risk of Barrett's esophagus. Am J Gastroenterol 2013; 108: 915-922.

[3] Champion G, Richter JE, Vaezi MF, Singh S and Alexander R. Duodenogastroesophageal reflux: relationship to pH and importance in Barrett's esophagus. Gastroenterology 1994; 107: 747-754.

[4] Erichsen R, Robertson D, Farkas DK, Pedersen L, Pohl H, Baron JA and Sorensen HT. Erosive reflux disease increases risk for esophageal adenocarcinoma, compared with nonerosive reflux. Clin Gastroenterol Hepatol 2012; 10: 475-480, e471.

[5] Coussens LM and Werb Z. Inflammation and cancer. Nature 2002; 420: 860-867.

[6] Sizemore N, Lerner N, Dombrowski N, Sakurai H and Stark GR. Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. J Biol Chem 2002; 277: 3863-3869.

[7] Lin A and Karin M. NF-kappaB in cancer: a marked target. Semin Cancer Biol 2003; 13: 107-114.

[8] Cronin J, Alhamdani A, Griffiths AP, Baxter JN, Brown T and Jenkins GJ. In vitro and ex vivo models of extended reflux exposure demonstrate that weakly acidic mixed reflux heightens NF-kB-mediated gene expression. Dis Esophagus 2011; 24: 360-370.

[9] Higdon JV and Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Crit Rev Food Sci Nutr 2003; 43: 89-143.

[10] Kada T, Kaneko K, Matsuzaki S, Matsuzaki T and Hara Y. Detection and chemical identification of natural bio-antimutagens. A case of the green tea factor. Mutat Res 1985; 150: 127-132.

[11] Nechuta S, Shu XO, Li HL, Yang G, Ji BT, Xiang YB, Cai H, Chow WH, Gao YT and Zheng W. Prospective cohort study of tea consumption and risk of digestive system cancers: results from the Shanghai Women's Health Study. Am J ClinNutr 2012; 96: 1056-1063.

[12] Dryden GW, Lam A, Beatty K, Qazzaz HH and McClain CJ. A pilot study to evaluate the safety and efficacy of an oral dose of (-)-epigallocatechin-3-gallate-rich polyphenon E in patients with mild to moderate ulcerative colitis. Inflamm Bowel Dis 2013; 19: 1904-1912.

[13] Jung YD and Ellis LM. Inhibition of tumour invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea. Int J Exp Pathol 2001; 82: 309-316.

[14] Yang CS, Liao J, Yang GY and Lu G. Inhibition of lung tumorigenesis by tea. Exp Lung Res 2005; 31: 135-144.

[15] Kawabe A, Shimada Y, Soma T, Maeda M, Itami A, Kaganoi J, Kiyono T and Imamura M. Production of prostaglandin E2 via bile acid is enhanced by trypsin and acid in normal human esophageal epithelial cells. Life Sci 2004; 75: 21-34.

[16] Yoshida N. Inflammation and oxidative stress in gastroesophageal reflux disease. J Clin Biochem Nutr 2007; 40: 13-23.

[17] Dvorak K, Fass R, Dekel R, Payne CM, Chavarria M, Dvorakova B, Bernstein H, Bernstein C and Garewal H. Esophageal acid exposure at pH < or = 2 is more common in Barrett's esophagus patients and is associated with oxidative stress. Dis Esophagus 2006; 19: 366-372.

[18] Dvorak K, Payne CM, Chavarria M, Ramsey L, Dvorakova B, Bernstein H, Holubec H, Sampliner RE, Guy N, Condon A, Bernstein C, Green SB, Prasad A and Garewal HS. Bile acids

in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's esophagus. Gut 2007; 56: 763-771.
[19] Miyazono F, Schneider PM, Metzger R, Warnecke-Eberz U, Baldus SE, Dienes HP, Aikou T and Hoelscher AH. Mutations in the mitochondrial DNA D-Loop region occur frequently in adenocarcinoma in Barrett's esophagus. Oncogene 2002; 21: 3780-3783.
[20] Yu H and Jove R. The STATs of cancer--new molecular targets come of age. Nat Rev Cancer 2004; 4: 97-105.
[21] Angelo LS, Talpaz M and Kurzrock R. Autocrine interleukin-6 production in renal cell carcinoma: evidence for the involvement of p53. Cancer Res 2002; 62: 932-940.
[22] Souza RF, Shewmake K, Terada LS and Spechler SJ. Acid exposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus. Gastroenterology 2002; 122: 299-307.
[23] Abdel-Latif MM, O'Riordan J, Windle HJ, Carton E, Ravi N, Kelleher D and Reynolds JV. NF-kappaB activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. Ann Surg 2004; 239: 491-500.
[24] Dvorakova K, Payne CM, Ramsey L, Holubec H, Sampliner R, Dominguez J, Dvorak B, Bernstein H, Bernstein C, Prasad A, Fass R, Cui H and Garewal H. Increased expression and secretion of interleukin-6 in patients with Barrett's esophagus. Clin Cancer Res 2004; 10: 2020-2028.
[25] Kauer WK, Peters JH, DeMeester TR, Feussner H, Ireland AP, Stein HJ and Siewert RJ. Composition and concentration of bile acid reflux into the esophagus of patients with gastroesophageal reflux disease. Surgery 1997; 122: 874-881.
[26] Yamamoto T, Hsu S, Lewis J, Wataha J, Dickinson D, Singh B, Bollag WB, Lockwood P, Ueta E, Osaki T and Schuster G. Green tea polyphenol causes differential oxidative environments in tumor versus normal epithelial cells. J Pharmacol Exp Ther 2003; 307: 230-236.
[27] Karin M, Cao Y, Greten FR and Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2002; 2: 301-310.
[28] Yamaguchi T, Yoshida N, Tomatsuri N, Takayama R, Katada K, Takagi T, Ichikawa H, Naito Y, Okanoue T and Yoshikawa T. Cytokine-induced neutrophil accumulation in the pathogenesis of acute reflux esophagitis in rats. Int J Mol Med 2005; 16: 71-77.
[29] Sahnoun Z, Jamoussi K and Zeghal KM. [Free radicals and antioxidants: physiology, human pathology and therapeutic aspects (part II)]. Therapie 1998; 53: 315-339.
[30] Rao CV. Nitric oxide signaling in colon cancer chemoprevention. Mutat Res 2004; 555: 107-119.
[31] Chen T, Nines RG, Peschke SM, Kresty LA and Stoner GD. Chemopreventive effects of a selective nitric oxide synthase inhibitor on carcinogen-induced rat esophageal tumorigenesis. Cancer Res 2004; 64: 3714-3717.
[32] Soma T, Shimada Y, Kawabe A, Kaganoi J, Kondo K, Imamura M and Uemoto S. Induction of prostaglandin E synthase by gastroesophageal reflux contents in normal esophageal epithelial cells and esophageal cancer cells. Dis Esophagus 2007; 20: 123-129.
[33] Abdel-Latif MM, Duggan S, Reynolds JV and Kelleher D. Inflammation and esophageal carcinogenesis. Curr Opin Pharmacol 2009; 9: 396-404.