(-)-Epigallocatechin-3-gallate inhibition of Epstein–Barr virus spontaneous lytic infection involves ERK1/2 and PI3-K/Akt signaling in EBV-positive cells

Sufang Liu1, Hongde Li1, Lin Chen1, Lifang Yang1, Lili Li1, Yongguan Tao1, Wei Li1, Zijian Li1, Haidan Liu1, Min Tang2, Ann M. Bode3, Zigang Dong3 and Ya Cao1,2,*

1Cancer Research Institute, Xiangya School of Medicine, Central South University, Key Laboratory for Cancer and Invasion of Ministry of Education, Changsha, Hunan 410078, China, 2Division of Hematology, Institute of Molecular Hematology, the Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China and 3The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912, USA

*To whom correspondence should be addressed. Tel: +86-731-84405448; Fax: +86-731-84470589; Email: ycao98@vip.sina.com

Correspondence may also be addressed to Zigang Dong. Tel: +507-437-9600; Fax: +507-437-9606; Email: zgdong@hi.umn.edu

Introduction

Epstein–Barr virus (EBV) reactivation into the lytic cycle plays certain roles in the development of EBV-associated diseases, including nasopharyngeal carcinoma and lymphoma. In this study, we investigated the effects of the tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) on EBV spontaneous lytic infection and the mechanism(s) involved in EBV-positive cells. We found that EGCG could effectively inhibit the constitutive lytic infection of EBV at the DNA, gene transcription and protein levels by decreasing the phosphorylation and activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt. By using cellular signaling pathway-specific inhibitors, we also explored the signaling mechanisms underlying the inhibitory effects of EGCG on EBV spontaneous lytic infection in cell models. Results show that specific inhibitors of Mitogen-Activated Protein Kinase Kinase (MEK) (PD98059) and phosphatidylinositol 3-kinase [PI3-K (LY294002)] markedly downregulated gene transcription and expression of BZLF1 and BMRF1 indicating that the MEK/ERK1/2 and PI3-K/Akt pathways are involved in the EBV spontaneous lytic cycle cascade. Therefore, one of the mechanisms by which EGCG inhibits EBV spontaneous lytic infection appears to involve the suppression of the activation of MEK/ERK1/2 and PI3-K/Akt signaling.

Abbreviations: AP-1, activator protein; DMSO, dimethylsulfoxide; EBV, Epstein–Barr virus; EGCG, (-)-epigallocatechin-3-gallate; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; LMP1, latent membrane protein 1; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; NPC, nasopharyngeal carcinoma; PBS, phosphate-buffered saline; PI3-K, phosphatidylinositol 3-kinase; PCR, quantitative PCR

Materials and methods

Cell lines and cell culture

B95.8, an EBV-positive marmoset B-cell line, and Raji, an EBV-positive lymphoblast-like cell line established from Burkitt’s lymphoma, were obtained from the American Type Culture Collection. CNE1-LMP1 is a stable LMP1-integrated NPC cell line (20,21). All cells were cultured in RPMI 1640 (Gibco

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Fig. 1. Detection of EBV-encoded proteins and EBV DNA in cells. (A) Cells were disrupted and immunoblot analysis was performed to determine the expression of EBNA1, LMP1, BZLF1 and BMRF1. (B) CNE1-LMP1 cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. BZLF1 or BMRF1 was visualized with mouse monoclonal anti-BZLF1 (1:100) or anti-BMRF1 (1:100) and a fluorescein isothiocyanate-conjugated secondary antibody (1:200). Incubation with PBS but no primary antibody and then with secondary antibody was used as a negative control. DNA was stained with...
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BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 10 μg/ml streptomycin and 10 IU/ml penicillin, and incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Chemicals and cell treatments

EGCG and LY294002 were purchased from Sigma Chemical (St Louis, MO), and PD98059 was purchased from Calbiochem (La Jolla, CA). EGCG and all inhibitors were prepared in autoclaved water and dimethylsulfoxide (DMSO; Sigma), respectively, as stock solutions for in vitro studies. Subconfluent cells were treated with the respective compounds at various concentrations for different times as indicated. Detailed treatment procedures are described in the Figure Legend 4. The final concentration of DMSO in the culture media was maintained at <0.1%, which had no significant effect on cell growth. Vehicle controls were included for all treatments. Preparation of cell lysates and immunoblot analysis

Whole cell lysate preparation and immunoblot analysis were performed according to the method described previously (11). Protein concentration was determined using the BCA Assay Reagent (Pierce, Rockford, IL). The following antibodies were used for immunodetection with appropriate dilutions: mouse BZLF1 monoclonal antibody (AZ-69; Argene, Vailieres, France), EBNA1 antibody (ab25653; Abcam, Cambridge, UK), BMRF1 antibody (ab6524; Abcam), β-actin (Ac-15; Sigma), Akt antibody (9272; Cell Signaling Technology, Danvers, MA) and p-Akt (Ser473) (9271; Cell Signaling Technology). The antibodies against ERK1 (sc-93), p-ERK (Tyr-204) (sc-7383), goat anti-rabbit IgG-horseradish peroxidase (sc-2004) and goat anti-mouse IgG-horseradish peroxidase (sc-2005) were all from Santa Cruz (Santa Cruz, CA).

Immunofluorescence assay

After being washed with phosphate-buffered saline (PBS), cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. BZLF1 or BMRF1 was visualized with mouse monoclonal anti-BZLF1 (1:100) or anti-BMRF1 (1:100) and a fluorescein isothiocyanate-conjugated secondary antibody (1:200). Incubation with PBS but no primary antibody and then with secondary antibody was used as a negative control. DNA was stained with 4′,6-diamidino-2-phenylindole at room temperature for 30 s. The staining patterns were observed under a fluorescence microscope (Zeiss, Jena, Germany).

Whole cell lysate preparation and immunoblot analysis were performed according to the method described previously (11). Protein concentration was determined using the BCA Assay Reagent (Pierce, Rockford, IL). The following antibodies were used for immunodetection with appropriate dilutions: mouse BZLF1 monoclonal antibody (AZ-69; Argene, Vailieres, France), EBNA1 antibody (ab25653; Abcam, Cambridge, UK), BMRF1 antibody (ab6524; Abcam), β-actin (Ac-15; Sigma), Akt antibody (9272; Cell Signaling Technology, Danvers, MA) and p-Akt (Ser473) (9271; Cell Signaling Technology). The antibodies against ERK1 (sc-93), p-ERK (Tyr-204) (sc-7383), goat anti-rabbit IgG-horseradish peroxidase (sc-2004) and goat anti-mouse IgG-horseradish peroxidase (sc-2005) were all from Santa Cruz (Santa Cruz, CA).

Immunohistochemistry

Immunohistochemical staining for EBV lytic proteins in specimens from patients with NPC and lymphoma. Paraffin sections were screened for the expression of the EBV lytic proteins with the specific antibodies to detect BZLF1 and BMRF1. Positive signal implies that BZLF1 or BMRF1 expression occurs in tumor cells as shown by nuclear localization in different cases. Magnification, ×400.

Fig. 2. Immunohistochemical staining for EBV lytic proteins in specimens from patients with NPC and lymphoma. Paraffin sections were screened for the expression of the EBV lytic proteins with the specific antibodies to detect BZLF1 and BMRF1. Positive signal implies that BZLF1 or BMRF1 expression occurs in tumor cells as shown by nuclear localization in different cases. Magnification, ×400.

Thirty-three paraffin-embedded NPC tissues and 24 paraffin-embedded lymphoma tissues (Hodgkin’s lymphoma (19), Burkitt’s lymphoma (4) and NHL (1)) were obtained from the Department of Pathology at Hunan Tumor Hospital and Xiangya Hospital, Changsha, China. Immunohistochemistry was performed on 4.0 μm sections from formalin-fixed, paraffin-embedded tissues. The sections were deparaffinized and rehydrated. Antigen retrieval was achieved with diluted ethylenediaminetetraacetic acid (50:1) by microwaving for 10 min. Endogenous peroxidase activity was quenched with a 3% hydrogen peroxide block for 15 min at room temperature. The sections were treated with goat serum for 10 min and incubated at 4°C overnight with the primary antibody (BZLF1 diluted 1:100). The secondary antibody, biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted 1:200, was applied for 30 min at 37°C; followed by a 30 min incubation with Vectastain ABC Elite. The reaction was visualized by using diaminobenzidine chromogen (Dako) following the manufacturer’s instructions, and the slides were counterstained with Mayer’s hematoxylin. All slides were scored by two observers. The staining intensity and pattern were evaluated using a 0 to 3+ scale (0, completely negative; 1+, weak; 2+, intermediate; 3+, strong). A final score of ≥2+ or greater was required for the case to be considered positive.

MTT assay

The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the effects of EGCG on cell viability as described previously (22). Briefly, cells were cultured in 96 well flat-bottomed plates and were starved in 0.1% FBS RPMI medium 1640 for 24 h. Then, serial dilutions of EGCG were added to the cells and incubated for the indicated time. Following the incubation period, 10 μl MTT (5 mg/ml) per well was added and then incubated for another 4 h at 37°C. After discarding the medium, 150 μl DMSO was added to each well and mixed thoroughly to dissolve the blue formazan crystals. The plates were subsequently read on a Bio-Rad 3350 microplate reader at a wavelength of 570 nm. Null control wells contained medium alone. Cell viability was calculated as the percentage of MTT inhibition using the following formula:

\[ \text{Viability (\%) = } \left( \frac{\text{OD}_{570 \text{blank}} - \text{OD}_{570 \text{treatment}}}{\text{OD}_{570 \text{blank}}} \right) \times 100\% \]

The experiments were repeated at least three times.
**Transient transfection and luciferase assay**

Cells were seeded in 24-well plates before transfection. pZip-Luc (kindly provided by Ichiro Saito, Tsurumi University School of Dental Medicine, Yokohama, Japan) (23) or pMP-Luc (kindly provided by Paul J. Farrell, Imperial College Faculty of Medicine, London, UK) (24) was cotransfected with an internal control pRL-SV40 using the Lipofectamine 2000 Transfection Reagent (Invitrogen) following the manufacturer’s instructions. At 4h after transfection, cells were treated with chemicals as designated. Cells were harvested at 24h after transfection and lysates were analyzed for luciferase activity using the Dual Luciferase Reporter Assay (Promega, Madison, WI) according to the manufacturer’s directions. The luciferase reporter plasmids were cotransfected with pRL-SV40 to correct for variations in transfection efficiency. The relative luciferase activity was normalized to the value of pRL-SV40 activity. The data are represented as means ± SD of three independent experiments performed in triplicate.

**RT–PCR and qPCR**

Total RNA from cultured cells was isolated using TRIzol following the manufacturer’s instructions (Invitrogen). Complementary DNA was synthesized using 1–5 μg of purified RNA, the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, EU) and Oligo(dT)12. Quantitative PCR (qPCR) was performed using a Rotor-Gene 6000 real-time (RT–PCR) instrument (Corbett Research, Sydney, Australia) and SYBR Premix Ex Taq™ II (TaKaRa, Dalian, China) and 2 μl complementary DNA with the following primers:

- **BZLF1:** forward (5′-CGG GGG TCT ATG G-3′) and reverse (5′-TCCAGGCTCTCCTCTGGG-3′) (antisense) and 5′-CTCCGACTGG-3′ (sense)

- **BMRF1:** forward (5′-CCA GGA TCT GAT GGG AG-3′) and reverse (5′-TCA AAA CTT TAG AGG CGA ATG G-3′) (antisense) and 5′-TCTCTGCTGAGTCTAAG-3′ (sense)

Relative mRNA abundance was calculated using β-actin as an internal control using the 2\(^{-ΔΔCt}\) method. For each experiment, mRNA levels from untreated cells were used as controls and set to 1. The mRNA changes are represented relative to untreated controls.

**DNA extraction and quantification of EBV copy number**

To measure EBV genomic copy number, qPCR targeting the BamHI-W segment was performed with DNA extracted from cells using the universal genomic extraction kit (TaKaRa) according to the kit handbook. To detect EBV DNA copies, the BamHI-W fragment was used as an index with a standard of 10 ng DNA as 50 copies of EBV in one Raji cell (26). The Raji DNA samples were diluted 10-fold from 107 to 102 copies for a standard curve. DNA (500 ng) was used in the first round of PCR for EBV copies using the following primers: forward (5′-TCA AAA CTT TAG AGG CGA ATG G-3′) and reverse (5′-CGG GGG TCT ATG G-3′). The second round of PCR was performed using another pair of primers: forward (5′-CTGA AGG TGG TGT CCT AAC-3′) and reverse (5′-GGC TTA TTC CTC TTT TCC CCT CTA-3′). The second round of PCR was performed using another pair of primers: forward (5′-CCG GGC GGG TCT ATG G-3′) and reverse (5′-GGC TTA TTC CTC TTT TCC CCT CTA-3′) and the special TaqMan probe (FAM-TGG TCG TGG TGC TGC TGC TAT C-TAMRA). The RT–qPCR assay with a fluorogenic probe was performed using the TaqMan PCR kit and a Model 7700 Sequence Detector (Applied Biosystems, Foster City, CA).

**Flow cytometer analysis**

To detect EBV lytic proteins, cells were washed with PBS, followed by fixing with 4% paraformaldehyde for 30 min and finally treated for 5 min with PBS containing 0.1% Triton X-100. Cells were then washed with PBS, treated with 1% bovine serum albumin in PBS for 1 h and incubated with 1:200-diluted monoclonal anti-BZLF1 or monoclonal anti-BMRF1 at 4°C overnight. Next, the cells were washed with PBS and incubated with 1:200-diluted fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Santa Cruz) for 1 h at 37°C. Incubation with PBS, but no primary antibody, and then with secondary antibody was used as a negative control. Finally, cells were resuspended in 1% paraformaldehyde and analyzed on a FACSCalibur flow cytometer using the CellQuest software (BD, San Diego, CA).

**Assay of ERKs and Akt kinase activities**

ERKs or Akt kinase activity was estimated using an ERKs kinase assay kit or an Akt kinase assay kit according to the manufacturer’s instructions (Cell Signalling Technology). In brief, cells were washed once with ice-cold PBS and disrupted with lysis buffer. ERK or Akt was immunoprecipitated using a specific ERKs or Akt-immobilized antibody bead slurry with gentle rocking overnight at 4°C. The cell lysate/immmobilized antibody complex was centrifuged at 13 000 r.p.m. for 30 s at 4°C and the pellet washed two times with 500 μl of 1× cell lysis buffer, and then twice with 500 μl of 1× kinase buffer. The kinase reactions were carried out in the presence of 200 μM adenosine triphosphate at 30°C for 30 min using 2 μg of fusion protein Elk-1 or GSK-3 as a specific substrate for ERKs or Akt, respectively. The phosphorylated proteins were detected by immunoblotting using a specific phospho-Elk-1 or phospho-GSK-3α/β (Ser21/9) antibody.

**Statistical analysis**

All statistical calculations were performed with the statistical software program SPSS (v. 12.0). Differences between various groups were evaluated by the Student’s t-test, and a P value < 0.05 was considered statistically significant.

**Results**

**Detection of EBV lytic proteins and EBV DNA copies in cell lines and biopsies**

EBV latency-associated gene expression is consistently detected in NPC and lymphoma cells and is, therefore, regarded as one of the factors in the oncogenesis of these malignancies. However, various lines of evidence indicate that lytic infection of EBV can also occur in NPC tumor cells and lymphomas (27,28). B95.8 cell line is a well-known EBV-positive cell line, which is permissive for virus lytic replication. In this study, B95.8 cells were used as the cell model and also as a positive control. To determine the EBV presence and lytic infection in NPC cell model, we used RT–PCR to quantify EBV DNA load and immunoblotting and immunofluorescence to analyze the EBV-associated lytic proteins.

First, the EBV latent and lytic proteins were examined using monoclonal antibodies. Immunoblot analysis revealed a constitutive expression of EBV latent proteins, EBNA1 and LMP1, and lytic infection gene products, BZLF1 and BMRF1 (Figure 1A). The expression level of EBV proteins, LMP1, EBNA1, BZLF1 and BMRF1, in B95.8 cells was more notable than in CNE1-LMP1 cells. Immunofluorescence staining
EGCG inhibits EBV lytic infection

**A**

CNE1-LMP1 cells

B95.8 cells

Relative luciferase activity (Fold induction)

<table>
<thead>
<tr>
<th>EGC (μM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
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<td>BZLF1-reporter</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>BMRF1-reporter</td>
<td>*</td>
<td>*</td>
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**B**

EPLF1 mRNA level

BMRF1 mRNA level

<table>
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<tr>
<th>EGC (μM)</th>
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<th>20</th>
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<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>B95.8</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

**C**

CNE1-LMP1

B95.8

EGCG (μM) 0 5 10 20

BMRF1

BZLF1

β-Acin

**D**

CNE1-LMP1 cells

B95.8 cells

Counts

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<td>8.54%</td>
<td></td>
</tr>
<tr>
<td>EGCG 0 μM</td>
<td>10.54%</td>
<td>8.54%</td>
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Counts

<table>
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<th>40.27%</th>
<th>68.99%</th>
</tr>
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<tbody>
<tr>
<td>EGCG 20 μM</td>
<td>0.47%</td>
<td>40.27%</td>
<td></td>
</tr>
<tr>
<td>EGCG 0 μM</td>
<td>18.57%</td>
<td>68.99%</td>
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</table>
revealed that the CNE1-LMP1 cells constitutively expressed the BZLF1 and BMRF1 proteins (Figure 1B). For quantification of EBV copy number, a RT–PCR assay targeting the BamHI-W segment of the EBV genome was developed. The BamHI-W assay has been reported to be more sensitive and suitable than EBER for applications in EBV detection, presumably because it targets a reiterated sequence that is present at approximately 10 copies per EBV genome (29). We used the DNA from Raji cells that contain 50 copies per cell (30). A 10-fold serial dilution of the reference was used as a standard of the viral DNA number. The estimation of viral genomes in each sample was expressed as the number of a RT–PCR assay targeting the BamHI-W segment of the EBV genome was developed. The BamHI-W assay has been reported to be more sensitive and suitable than EBER for applications in EBV detection, presumably because it targets a reiterated sequence that is present at approximately 10 copies per EBV genome (29). We used the DNA from Raji cells that contain 50 copies per cell (30). A 10-fold serial dilution of the reference was used as a standard of the viral DNA number. The estimation of viral genomes in each sample was expressed as the number of viral genomes copy per 50 ng of purified DNA. Experiments were performed at least twice to improve reliability of the results. We observed that EBV DNA was present in both CNE1-LMP1 cells and B95.8 cells, and the copies of EBV DNA in CNE1-LMP1 cells (1.35 × 10^3 copies/50 ng DNA) were significantly lower than in B95.8 cells (4.82 × 10^6 copies/50 ng DNA) (P < 0.01) (Figure 1C).

To determine whether EBV lytic protein expression also existed in vivo, immunohistochemistry to detect BZLF1 and BMRF1 was performed on formalin-fixed, paraffin-embedded tissue from NPC and lymphomas. Positive signal implies that BZLF1 or BMRF1 expression occurs in tumor cells as shown by nuclear localization in different cases. We observed BZLF1- and BMRF1-positive cells within the NPC and lymphoma tissues. We detected BZLF1 expression in 9 (27.3%) of the 33 NPC patients and in 7 (29.17%) of 24 lymphoma patients and observed BMRF1 expression in 2 (6.06%) of the 33 NPC patients and in 1 (4.16%) of the 24 lymphoma patients studied (Figure 2).

The inhibitory effect of EGCG on EBV spontaneous lytic infection

The viability of CNE1-LMP1 cells and B95.8 cells after treatment with EGCG was assessed by MTT assay. EGCG exhibited dose- and time-dependent inhibitory effects on these cells with an IC_{50} of 20 µM (Figure 3). Thus, the concentrations of EGCG (5–20 µM) were the doses chosen for subsequent experiments.

To characterize the effect of EGCG on EBV spontaneous lytic infection in cells, a transient transfection assay was performed using a BZLF1 and BMRF1 promoter/luciferase reporter system. Results showed that EGCG effectively suppressed the transcription activity of the BZLF1 and BMRF1 genes in both CNE1-LMP1 and B95.8 cells in a dose-dependent manner (Figure 4A). The effect of EGCG on the mRNA level of EBV spontaneous lytic genes was measured using RT–qPCR. In accordance with the results from transfection assay, the mRNA expression levels of the lytic genes, BZLF1 and BMRF1, were also strongly reduced after EGCG treatment (Figure 4B).

To confirm the mRNA results, the inhibitory effect of EGCG on EBV lytic protein expression was assessed by immunoblot analysis, which was performed using monoclonal anti-BZLF1 and anti-BMRF1. Upon treatment with different concentrations of EGCG, expression of both BZLF1 and BMRF1 was substantially decreased in a dose-dependent manner (Figure 4C). Moreover, flow cytometry analysis of EGCG-treated cells revealed that following treatment with EGCG (20 µM), the percentage of the cell population expressing BZLF1 and BMRF1 decreased from 73.69% and 15.57% to 27.39% and 2.89%, respectively, in CNE1-LMP1 cells, and from 98.54% and 68.99% to 78.98% and 40.27% in B95.8 cells (Figure 4D).

Further, we examined whether EGCG suppresses lytic replication of the viral load. EBV DNA copies were quantified using a RT–PCR method. Results indicated that the copy number of EBV DNA in treated CNE1-LMP1 cells was significantly reduced by EGCG in a dose-dependent manner. However, our data showed that EGCG had no statistically significant effect on the copy number of EBV DNA in treated B95.8 cells (Figure 4E). These results indicate that EGCG effectively inhibits the constitutive lytic infection of EBV in CNE1-LMP1 and B95.8 cells.

Involvement of the MEK/ERK1/2 and PI3-K/Akt pathways in EGCG’s inhibition of EBV lytic infection

The MEK/ERK1/2 and PI3-K/Akt signaling pathways were reported to play certain roles in EBV lytic replication, and EGCG was shown to suppress the activities of these pathways (18). Thus, we hypothesized that EGCG inhibits EBV spontaneous lytic infection by suppressing these signaling pathways. First, using phospho-specific...
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Fig. 5. Inhibitory effects of EGCG on activation of ERK1/2 and Akt in CNE1-LMP1 and B95.8 cells. (A) Effect of EGCG on phosphorylation of ERK1/2 and Akt. The cells were starved in 0.1% FBS RPMI medium 1640 for 24 h and then cultured with different concentrations of EGCG for 24 h. Cells were then disrupted and immunoblot analysis was performed. (B) In vitro kinase assays of ERK1/2 and Akt were used to evaluate the effects of EGCG. Cells were starved in 0.1% FBS RPMI medium 1640 for 24 h and then cultured with different concentrations of PD98059 and LY294002 for 24 h. Cells were then disrupted and subjected to immunoblot analysis as described in Materials and methods. To ensure that equal amounts of ERK or Akt were immunoprecipitated, we also immunoblotted the precipitate for total ERK or Akt.

antibodies against ERK1/2 and Akt, we found that the phosphorylation of these kinases was suppressed by EGCG (Figure 5A). ERKs or Akt kinase activity was estimated using an ERK kinase assay kit or an Akt kinase assay kit. ERK or Akt was first immunoprecipitated from cell extracts and then incubated with their specific substrate, Elk-1 or GSK-3 fusion protein, respectively, in the presence of adenosine triphosphate and kinase buffer. ERKs- or Akt-dependent Elk-1 or GSK-3 phosphorylation was then measured by immunoblotting using a phospho-antibody that recognizes Elk-1 or GSK-3 when phosphorylated. The kinase assays further confirmed that the activation of ERK1/2 and Akt was inhibited by EGCG in a dose-dependent manner (Figure 5B). These data indicate that EGCG suppresses the activation of the ERK1/2 and Akt signaling pathways.

To determine if the ability of EGCG to inhibit the ERK1/2 and PI3-K/Akt pathways plays a role in the attenuation of EBV lytic infection, a pharmacological approach was adopted using each kinase-specific inhibitor. PD98059, a specific inhibitor of MEK1 that acts by inhibiting activation of ERK1/2, and LY294002, a specific inhibitor of PI3-K that acts by inhibiting activation of Akt, were tested for their effects on EBV lytic infection. Our results showed that treatment of cells with each of these inhibitors dramatically decreased the phosphorylation levels of ERK1/2 and Akt. In addition, these inhibitors suppressed the expression of the EBV lytic proteins in a dose-dependent manner (Figure 6A and B). A transient transfection assay performed using a BZLF1 and BMRF1 promoter/luciferase reporter system showed that the inhibitors each effectively and significantly suppressed the transcription activity of the BZLF1 and BMRF1 genes in our cell models in a dose-dependent manner (Figure 6C and D, P < 0.05).

Next, to determine the inhibitory effect of the inhibitors on EBV lytic gene expression due to the suppressive effects on the genes at the mRNA level, we performed qPCR to estimate BZLF1 and BMRF1 mRNA expression after PD98059 or LY294002 treatment. Consistent with the data from the transfection assay and immunoblot analysis, the qPCR results showed that the inhibitors each effectively and significantly suppressed the mRNA expression of the BZLF1 and BMRF1 genes in a dose-dependent manner (Figure 6E and F, P < 0.05).

Overall, these results indicated that the MEK/ERK1/2 and PI3-K/Akt signaling pathways are involved in EGCG inhibition of EBV spontaneous lytic infection in both CNE1-LMP1 and B95.8 cells.

Discussion

The life cycle of EBV includes latent and lytic stages. In most asymptomatic carriers of EBV, the cells containing latent viral genomes are periodically reactivated to the lytic cycle. Previous studies focused on the effects of EBV latent infection and revealed that viral LMP1 is an oncoprotein (19). But much of the literature points to the fact that EBV...
Figure A: Western blot analysis of CNE1-LMP1 and B95.8 cells treated with PD98059 at different concentrations (Con, DMSO, 25, 50 μM). The blots show changes in P-ERK1/2 (Tyr-204), total ERK1, BMRF1, and BZLF1 proteins.

Figure B: Western blot analysis of CNE1-LMP1 and B95.8 cells treated with LY294002 at different concentrations (Con, DMSO, 5, 10 μM). The blots show changes in p-Akt (Ser473), total Akt, BMRF1, and BZLF1 proteins.

Figure C: Bar graph showing relative luciferase activity for BZLF1 and BMRF1 reporters in CNE1-LMP1 and B95.8 cells treated with PD98059 at different concentrations (DMSO, 25, 50 μM). Significant differences are indicated by asterisks.

Figure D: Bar graph showing relative luciferase activity for BZLF1 and BMRF1 reporters in CNE1-LMP1 and B95.8 cells treated with LY294002 at different concentrations (DMSO, 5, 10 μM). Significant differences are indicated by asterisks.
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spontaneous lytic replication takes place in various EBV-associated tumors (8–10). Our results also indicated that the EBV spontaneous lytic infection exists in NPC and lymphoma cells (Figure 1).

Although the lytic protein BZLF1 can induce the viral replication cycle and subsequent cell death, accumulating evidence indicates that expression of BZLF1 does not always result in virus production and cell death. The abortive lytic infection occurs not only in NPC but also in Hodgkin’s disease, non-Hodgkin lymphoma in an immunocompromised host and EBV-associated gastric cancer (28,31–33). Our results also showed that the basal level of early gene BMRF1 in gene transcription and protein expression was much lower than the immediate-early gene BZLF1 (Figures 1A and 4). In addition, the results from immunohistochemistry showed that the percentage of BZLF1-positive cells in NPC and lymphoma biopsies was much larger than the percentage of BMRF1-positive cells (Figure 2). The findings of our study imply that abortive lytic infection may occur in NPC and lymphoma.

Viral lytic genes, such as BZLF1, BMRF1, or cellular genes induced by viral lytic proteins could potentially encode paracrine factors that promote tumor growth. BZLF1 has been reported as having various malignancy-promoting actions (34). BZLF1 has the potential to create the best cellular environment for EBV replication by suppressing the cell-mediated immunity of the host (35,36), regulating the expression of the cellular proteins and the activities of signaling pathways (34,37,38), manipulating the cell cycle (38,39) and interacting with host proteins physically (40,41).

NPC is known to be closely associated with EBV infection, several retrospective and prospective investigations suggest that EBV lytic infection might also play a substantial role in pathogenesis. Elevated antibody titers against EBV lytic antigens, representing EBV lytic infection in vivo, correspond with advanced cancer stages, poor prognosis or tumor recurrence of NPC (6,7). The serologic marker of EBV reactivation into lytic infection also serves as a risk factor of NPC (42). In addition, the presence of lytic viral genome and lytic gene products, such as BZLF1, further supports the notion that EBV reactivations do occur in some cases (43). The presence of lytic viral genome and lytic gene products, such as BZLF1, also occurs in lymphomas (27). EBV reactivation into lytic EBV gene expression contributes to EBV-associated lymphoproliferative disease, potentially through induction of paracrine B-cell growth factors. BZLF1-induced IL-13 production facilitates B-cell proliferation and may contribute to the pathogenesis of EBV-associated lymphoproliferative disorders, such as post-transplantation lymphoproliferative disease and Hodgkin’s lymphoma (44). A recent study showed the ability of methotrexate (MTX), which is used in patients with rheumatoid arthritis and polymyositis, to increase the level of lytic infectious EBV in patients. This, combined with its potent immunosuppressive effects, may collaborate to induce EBV-positive lymphomas (45). Our data confirm that EBV lytic infection proteins are present not only in NPC and lymphoma cell lines but also in patient biopsies, consistent with the previous studies.

The above evidence has challenged the traditional concept that EBV only transforms cells in its latent infection and has also broadened our knowledge regarding the mechanism of EBV-associated carcinogenesis. By increasing the horizontal transmission of the virus.
from cell to cell, EBV lytic infection might increase the total number of latently infected cells. In addition, important implications of EBV lytic infection might include the idea that the infected host cells or nearby cells are transformed, or that the biologic characteristics of these transformed cells are changed and the degree of malignancy might increase along with the ease with which metastasis can occur. Thus, if EBV lytic infection is inhibited, the potential of the malignancy in the infected cells might be suppressed or reversed.

Cancer chemoprevention by the natural compound, EGCG, has been studied by many investigators and has received much attention in recent years. The inhibitory effects of EGCG against carcinogenesis at different organ sites have been demonstrated in many animal models (16,46). Several studies have shown that EGCG can inhibit EBV lytic infection, but the mechanism involved in the control of EBV gene expression by EGCG is not well understood. Choi et al. (47) found that EBV-induced B-cell outgrowth and B lymphocyte transformation was dramatically inhibited by treatment with EGCG (50 μM), supporting the important role of acetylation in cancer initiation and progression. Taniguchi et al. (48) and Chang et al. (49) demonstrated that EGCG blocks EBV lytic cycle induced by chemicals in Burkitt's lymphoma cell lines. However, in recent years, considerable progress has been made in the mechanisms of cancer chemoprevention by EGCG. In our studies, we used the NPC cell line (CNE1-LMP) and lymphoblastoid cell line (B95.8) with EBV spontaneous lytic replication that mimics the natural state of the infected cells. We found that EGCG inhibits EBV spontaneous lytic replication without any inducer and involving the inhibition of the activation of MEK/ERK1/2 and PI3-K/Akt signaling.

EGCG exerts its anticancer effect by modulating a wide spectrum of molecular targets, thus inhibiting tumor development and progression. Located at the cell membrane, EGCG suppresses the activation of multiple receptor tyrosine kinases, including epidermal growth factor receptor, insulin-like growth factor-I receptor, G3BP1, ZAP-70 and vimentin (56). With EGCG have been identified and include laminin, GRP-78, insulin-like growth factor-I receptor, G3BP1, ZAP-70 and vimentin (56).

In summary, this study presented novel experimental evidence describing the mechanisms underlying the inhibition of EBV spontaneous lytic replication by EGCG in both epithelial NPC and B-cell lymphoma models. Our findings support the future investigation of EGCG as an anticancer and chemoprevention agent for EBV-associated malignancies. Further study regarding the inhibitory effect of EGCG on EBV lytic infection in vivo is needed.

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References


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