ANTICANCER RESEARCH *28*: 1713-1718 (2008)

Induction of Apoptosis by Epigallocatechin Gallate and Autophagy Inhibitors in a Mouse Macrophage-like Cell Line

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Abstract. *We have previously reported that lignin carbohydrate complex and E. coli lipopolysaccharide (LPS), but not their precursors or (–)-epigallocatechin-3-gallate (EGCG), enhanced the nitric oxide (NO), citrulline and asparagines production by the mouse-macrophage-like cell line Raw264.7. Here the EGCG inhibition of NO production by LPS-stimulated Raw264.7 cells is reported. EGCG induced apoptotic cell death characterized by internucleosomal DNA fragmentation, whereas it inhibited the formation of secondary lysosomes and autophagosomes detected as granular dot-like distribution of acridine orange and microtubule-associated protein light chain 3 fused with green fluorescent protein (LC3-GFP). Autophagy inhibitors such as 3-methyladenine and bafilomycin A1 induced apoptotic cell death in the Raw264.7 cells. The inhibition of macrophage NO production by EGCG may be due to the apoptosis induction coupled with autophagy inhibition.*

The polyphenols present in green tea are flavonols, commonly known as catechins. Catechins show diverse biological activities such as antioxidant activity (1), inhibition of tumor growth (2), antibacterial activity (3, 4), and induction of apoptosis (5, 6). Most of the properties of green tea have been ascribed to the most abundant polyphenol, epigallocatechin-3-gallate (EGCG). We have recently reported that various flavones (7, 8), extract of Barbados cherry, a fruit of *Malpighia emarginata* DC (9), azulenes (10), tropolones (11), azulenequinones (12), trihaloacetylazulenes (13, 14), phenolcarboxylic acid monomers and polymers (15), Chinese medicines (16, 17) and Moxa smoke (18) inhibited nitric oxide (NO) production by mouse macrophage-like cells Raw264.7, whereas lignin carbohydrate complex (19) and some groups of Chinese medicines (16, 17) stimulated NO production. This suggested that most of the plant low molecular weight polyphenols inhibit NO production by macrophages. Based on these reports, the effect of EGCG on NO production and apoptosis in Raw264.7 cells was investigated

There are at least three types of cell death, apoptosis (type I programmed cell death characterized by blebbing, nuclear fragmentation, chromatin condensation, internucleosomal DNA fragmentation and loss of cell surface microvilli), autophagy (type II programmed cell death characterized by the formation of autophagosomes and autophagolysosomes engulfing the cytosol organelles) and necrosis (20, 21). Recently, cross-talk between apoptotic and autophagic pathways has been suggested (22, 23). Therefore, whether or not EGCG inhibits autophagy, and whether two popular autophagy inhibitors, 3-methyladenine (3MA) and bafilomycin A1 (BAF) induce apoptosis in Raw264.7 cells were also investigated.

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Key Words: EGCG, macrophage, apoptosis, autophagy.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), phenol red-free DMEM, fetal bovine serum (FBS) and Lipofectamine 2000 from Invitrogen, Carlsbad, CA, USA; 3-methyladenine (3MA), Hank's balanced salt solution (HBSS) and acridine orange from Sigma Chem. Ind., St. Louis, MO, USA; (–)-epigallocatechin-3-gallate (EGCG) and bafilomycin A1 (BAF) from Wako Pure Chem. Ind., Osaka, Japan and Caspase-Glo™ 3/7 Assay from Promega, Madison, WI, USA.

Cell culture. Mouse macrophage-like Raw264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere.

Assay for NO concentration. The Raw264.7 cells were initially inoculated at 0.5×10^6/mL in 24-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated for 20 hours before treatment. Near-confluent cells were treated for 24 hours with different concentrations of EGCG (0-500 μM) in phenol red-free DMEM supplemented with or without lipopolysaccharide

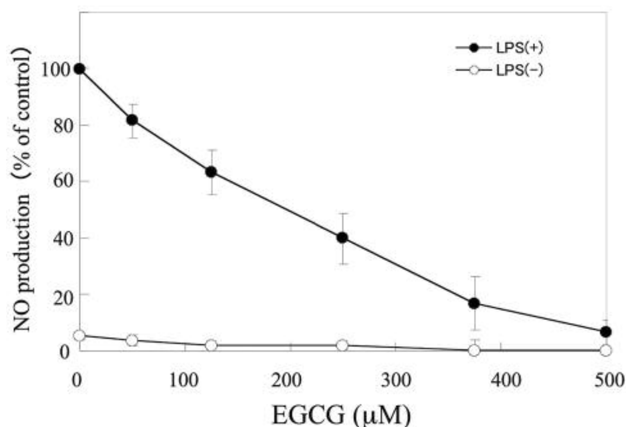

Figure 1. *Inhibition by EGCG of NO production by LPS-stimulated Raw264.7 cells. Raw264.7 cells were incubated for 24 hours without (–) or with (+) 100 ng/mL LPS in the presence of the indicated concentrations of EGCG, and the extracellular NO concentration was determined by Greiss reagent. The control represents the concentration of NO (approximately 20 μM) released into the culture medium by treatment with LPS (100 ng/mL) alone. Each value represents mean±S.D. of triplicate determinations.*

(LPS) (100 ng/mL). The NO production by the Raw264.7 cells was quantified by Greiss reagent, using the standard curve of NO_2^-.

Assay for DNA fragmentation. The cells were washed once with PBS (–) and lysed with 50 μL lysate buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate solution). The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 2 hours at 50˚C and then mixed with 50 μL NaI solution (7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0) and then 250 μL of ethanol. After centrifugation for 20 minutes at 20,000 xg, the precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA-2Na). Each sample (10 μL) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA-2Na). After staining with ethidium bromide, the DNA was visualized by UV irradiation, and photographed by charge coupled device (CCD) camera (Bio Doc-It, UVP, Inc., Upland, CA, USA).

Assay for caspase activation. The Raw264.7 cells were initially inoculated at 0.3×10^4/0.1 mL in 96-micro well plates (Becton, Dickinson and Company). Near-confluent cells were treated for 9-18 hours with EGCG (300 μM). The caspase activities were determined by the Caspase-Glo™ 3/7 Assay, according to the manufacturer's protocol. After treatment with EGCG, the cells were lysed with 100 μL of Caspase-Glo™ 3/7 Reagent in each well. After incubation for 30 minutes, the luminescence was measured by a luminescence reader (Micro Lumat, LB96P, EG & G Berthold, Germany).

Detection of acidic vesicular organelles with acridine orange staining. The acidic vesicular organelles were stained with acridine orange as described previously (24). The Raw264.7 cells were stained with 1 μg/mL acridine orange for 15 minutes. Samples were then examined under a laser scanning microscope LSM510 (Carl Zeiss Inc., Gottingen, Germany), using excitation filter 488 nm and emission filter >650 nm.

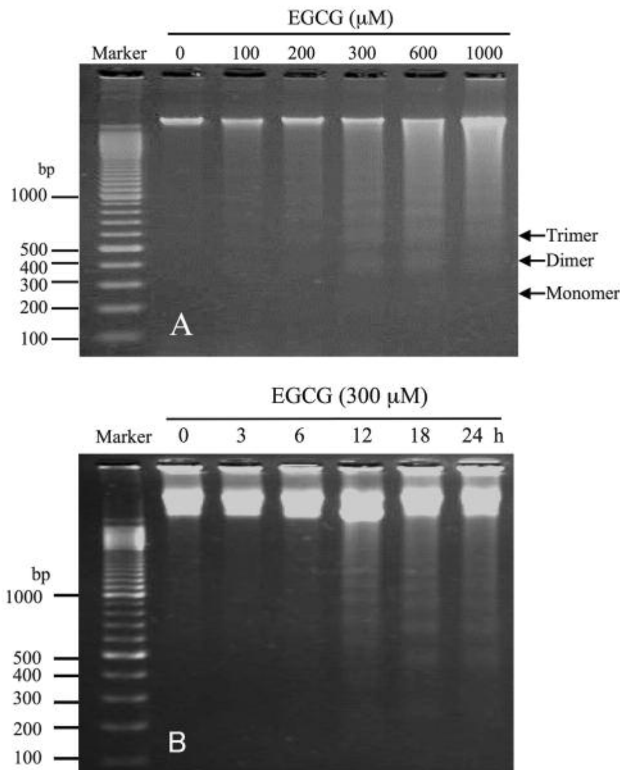

Figure 2. *Induction of DNA fragmentation by EGCG in Raw264.7 cells. (A) Dose-response: Raw264.7 cells were incubated for 24 hours with the indicated concentrations of EGCG. (B) Time course: Raw264.7 cells were incubated with 300 μM EGCG for the indicated times. DNA was then extracted and applied to agarose gel electrophoresis.*

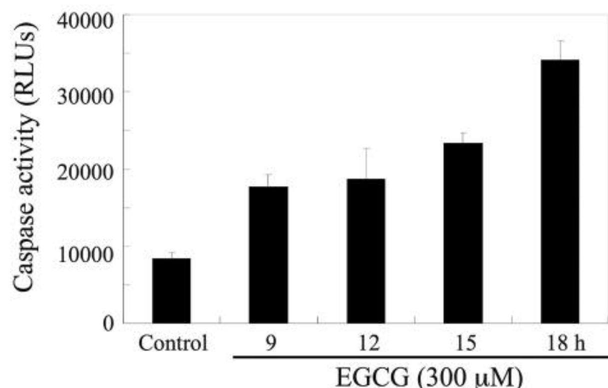

Figure 3. *Activation of caspase-3 by EGCG. Raw264.7 cells were incubated without (control) or with 300 μM EGCG for the indicated times, and the caspases-3 activity was quantified by the release of 7-amino-4-trifluor-methyl coumarin (AFC) from the fluorescent caspase substrate (DEVD-AFC). RLU: relative light unit. Each value represents mean±S.D. from triplicate determinations.*

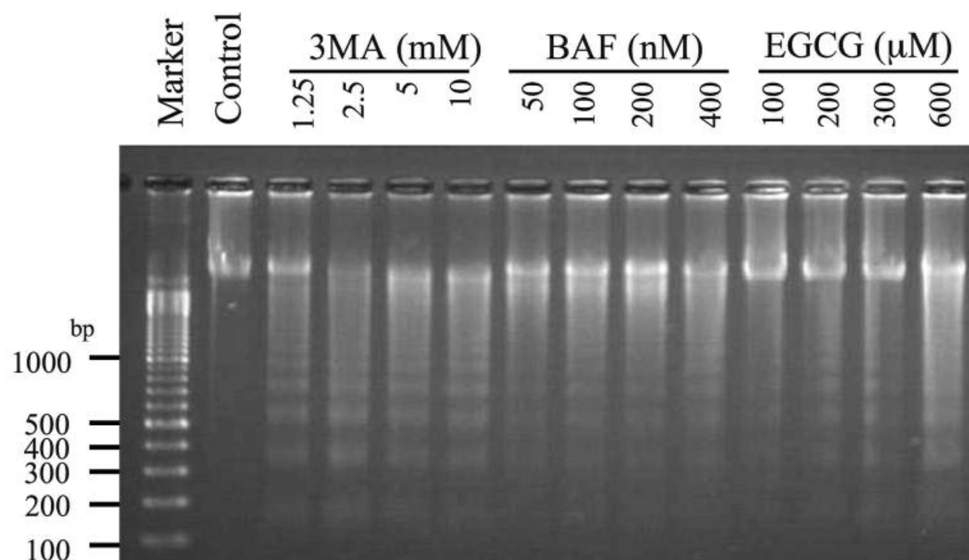

Figure 4. *Induction of internucleosomal DNA fragmentation by autophage inhibitors. Raw264.7 cells were incubated for 24 hours with the indicated concentrations of 3-methyladenine (3MA), bafilomycin A1 (BAF) or EGCG. DNA was then extracted and applied to agarose gel electrophoresis.*

Transfection. To investigate the possibility that EGCG induced autophagy by another method, microtubule-associated protein 1 light chain 3 (LC3)-green fluorescent protein (GFP) fusion protein was constructed (25, 26). The Raw264.7 cells were inoculated at 0.3×10^6/well in a 24 well plates (Becton, Dickinson and Company), and the next day, the cells were transfected with a mixture of 0.7 μL of Lipofectamine 2000 and 1.5 ng of plasmid DNA (pAcGFP1-LC3, a GFP-LC3 fusion protein expression vector) (24). After transfection for 18 hours, the cells cultured for 4 hours in DMEM or HBSS [to induce autophagy (27)] with different concentrations of EGCG.

Assay for LC3 accumulation in autophagosomes. The GFP-LC3 transfected Raw264.7 cells were observed by a laser scanning microscope LSM510, using excitation filter 488 nm and emission filter 505-530 nm (24).

Results

Inhibition of NO production by EGCG. LPS (100 ng/mL) significantly stimulated the NO production by the Raw264.7 cells, and the NO released into the culture medium was elevated from the background level to approximately 20 μM (Figure 1). EGCG alone did not significantly induce the NO production by unstimulated Raw264.7 cells, whereas it effectively inhibited the NO production by LPS-stimulated Raw264.7 cells in a concentration-dependent manner (Figure 1).

Induction of apoptosis by EGCG. EGCG induced internucleosomal DNA fragmentation in both concentration- (Figure 2A) and time- (Figure 2B) dependent manners. EGCG induced caspase-3 activation in the Raw264.7 cells (Figure 3).

Induction of apoptosis by autophagy inhibitors. The class III phosphoinositide-3-kinase (PI3K) inhibitor, 3MA, and vacuolar-type proton ATPase (V-ATPase) inhibitor, BAF, clearly induced internucleosomal DNA fragmentation in the Raw264.7 cells (Figure 4).

Effect of EGCG autophagy. Treatment of the Raw264.7 cells with EGCG considerably reduced the incidence of acidic organelles, that was detected by acridine orange staining (Figure 5A and B). Similarly, treatment of the Raw264.7 cells with 3MA (Figure 5C) or BAF (Figure 5D) reduced the incidence of acidic organelles slightly and completely respectively.

After culturing for 4 hours in Hank's balanced salt solution (HBSS), the cells became autophagic displaying the granular dot-like distribution of LC3-GFP fluorescence in autophagosomes (Figure 6B). EGCG dose-dependently reduced the translocation of the LC3-GFP fusion protein into the autophagosomes in the starvation-inducted Raw264.7 cells (Figure 6C-F).

Discussion

In this study, EGCG, at the concentration which induced the apoptosis, inhibited NO production by LPS-stimulated Raw264.7 cells. Additionally the activation of caspase-3 and DNA fragmentation occurred 9 hours after EGCG treatment. The mechanism by which EGCG induces apoptosis has so far been poorly understood. Apoptosis is a genetically regulated program for cell death that can be inhibited by Bcl-2 and

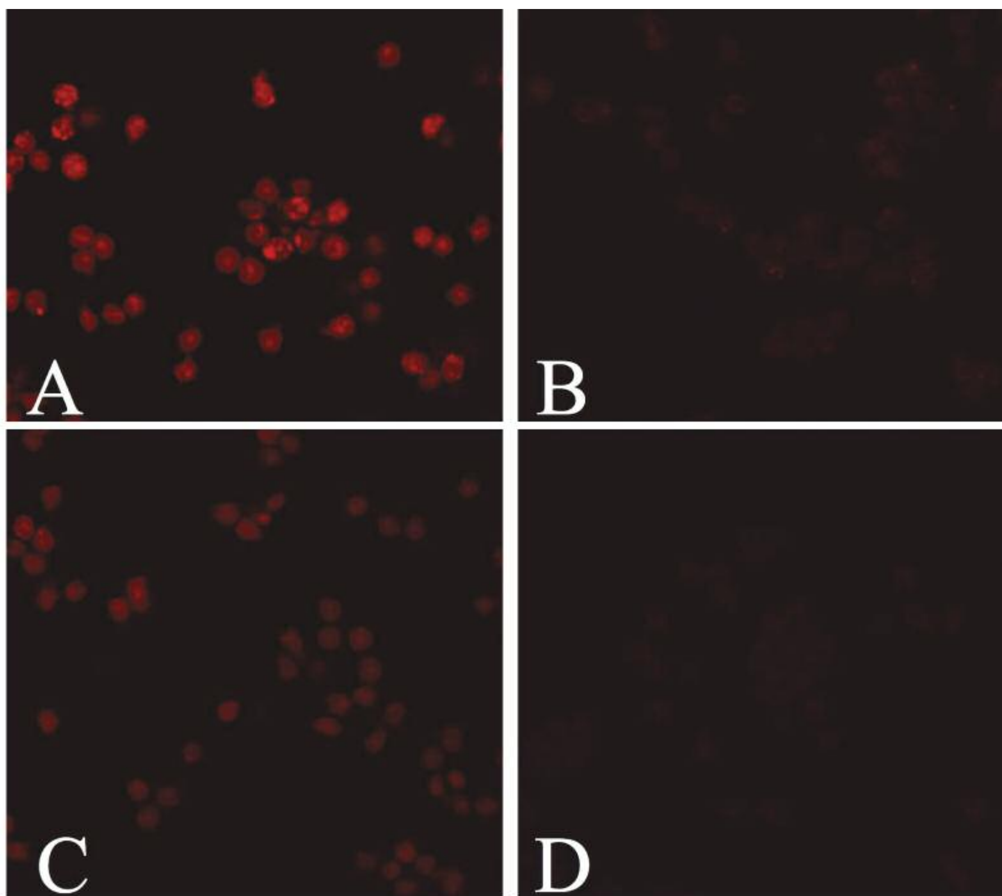

Figure 5. *Inhibition of acidic organelle production by EGCG and autophagy inhibitors. Raw264.7 cells were incubated for 2 hours without (control) (A) or with ECGC (300 μM) (B), 3-methyladenine (3MA) (2.5 mM) (C) or bafilomycin A1 (BAF) (100 ng/mL) (D), and stained with 1 μg/mL acridine orange for 15 minutes. Samples were then examined under a laser scanning microscope LSM510, using excitation filter 488 nm and emission filter >650 nm.*

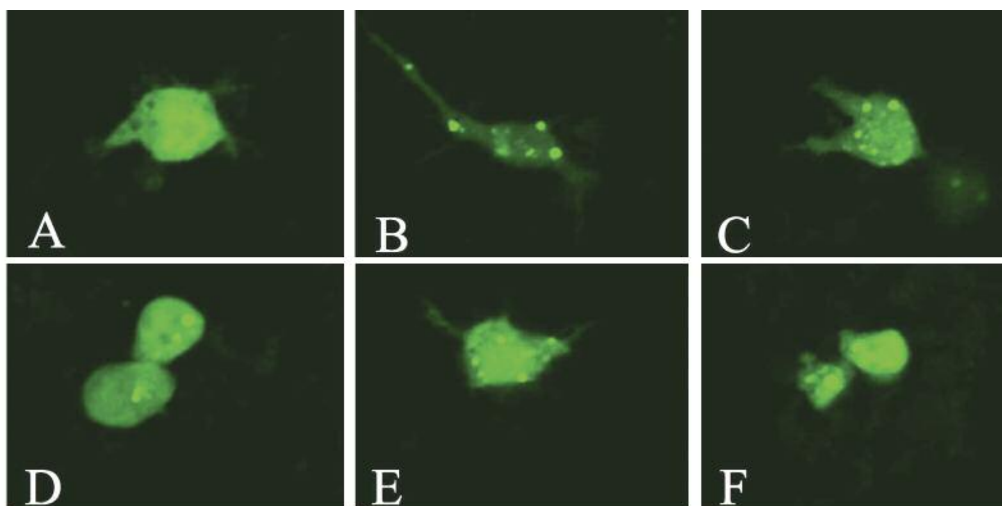

Figure 6. *Inhibition of GFP-LC3 accumulation by EGCG in the autophagosomes under starvation conditions. Raw264.7 cells transiently transfected with GFP-LC3 were cultured for 4 hours in DMEM (A) or Hank's balanced salt solution (HBSS) with 0 (B), 100 (C), 250 (D), 500 (E) or 750 (F) μM EGCG. Small dots represent autophagosomes. Samples were examined under a laser scanning microscope LSM510, using excitation filter 488 nm and emission filter 505-530 nm.*

Bcl-xL, and promoted by Bax protein (28, 29). The balance between the expression of pro-apoptotic and anti-apoptotic proteins determines the fate of the cells in many systems. Although there are diverse types of cell death, both apoptosis and autophagy tightly regulate cell survival and death (22, 23). The antiapoptotic protein, Bcl-2, is known to interact with the autophagy protein, Beclin 1 (30). Bcl-2 not only functions as an antiapoptotic protein, but also as an anti-autophagy protein *via* its inhibitory interaction with Beclin 1, suggesting the possible inter-conversion between apoptosis and autophagy. Autophagy inhibitors such as 3MA and BAF were therefore investigated for their possible apoptosis-inducing activity in the Raw264.7 cells. As expected, this was the case (Figures 4 and 5), further confirming that autophagy plays an important role in the survival of Raw264.7 cells. In addition, EGCG reduced the granular distribution of acridine orange into the autolysosomes of the Raw264.7 cells. These results indicated that EGCG inhibited physiological levels of autophagy in the Raw264.7 cells. When GFP-LC3, a highly specific fluorescent autophagy marker in mammalian cells was used to measure autophagosome production (25, 26), EGCG was shown to inhibit the starvation-induced autophagy in the Raw264.7 cells.

The present study suggests that EGCG may modify the function and survival of macrophages. Further studies are required to demonstrate the relationship between apoptosis and autophagy in this cell line.

Acknowledgements

We thank Drs. Jiang Y and Prof. Ohmori for their excellent advice. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (Sakagami, No.19592156).

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Received December 14, 2007
Revised February 20, 2008
Accepted March 11, 2008