Green Tea Epigallocatechin-3-Gallate Mediates T Cellular NF-κB Inhibition and Exerts Neuroprotection in Autoimmune Encephalomyelitis

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Recent studies in multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), point to the fact that even in the early phase of inflammation, neuronal pathology plays a pivotal role in the sustained disability of affected individuals. We show that the major green tea constituent, (−)-epigallocatechin-3-gallate (EGCG), dramatically suppresses EAE induced by proteolipid protein 139–151. EGCG reduced clinical severity when given at initiation or after the onset of EAE by both limiting brain inflammation and reducing neuronal damage. In orally treated mice, we found abrogated proliferation and TNF-α production of encephalitogenic T cells. In human myelin-specific CD4+ T cells, cell cycle arrest was induced, down-regulating the cyclin-dependent kinase 4. Interference with both T cell growth and effector function was mediated by blockade of the catalytic activities of the 20S/26S proteasome complex, resulting in intracellular accumulation of IκB-α and subsequent inhibition of NF-κB activation. Because its structure implicates additional antioxidative properties, EGCG was capable of protecting against neuronal injury in living brain tissue induced by N-methyl-D-aspartate or TRAIL and of directly blocking the formation of neurotoxic reactive oxygen species in neurons. Thus, a natural green tea constituent may open up a new therapeutic avenue for young disabled adults with inflammatory brain disease by combining, on one hand, anti-inflammatory and, on the other hand, neuroprotective capacities. The Journal of Immunology, 2004, 173: 5794–5800.

Tea (Camellia sinensis) and its constituent polyphenols have been reported to possess anticarcinogenic properties in a wide variety of experimental systems in vitro and in vivo. Drinking tea, especially green tea, is associated with a lower incidence of human cancer (1, 2). Subsequent studies demonstrated that certain fractions of green tea, among them (−)-epigallocatechin-3-gallate (EGCG), promote apoptosis and cell cycle arrest of transformed cells (3–5). Moreover, immunomodulatory properties of green tea extracts such as inhibiting endotoxin-induced TNF-α production (6) and neutrophil-mediated effects have been recently described (7). In a mouse model of stroke, a neuroprotective role was observed (8) and linked in a Parkinson’s disease model to regulation of superoxide dismutase and catalase activity in the brain (9).

Multiple sclerosis (MS) is a multiphasic autoimmune disease of the CNS (10) in which myelin-specific CD4+ Th1 cells are thought to orchestrate the effector processes resulting in the destruction of the myelin sheath (11). Recent studies in MS and its animal model, experimental autoimmune encephalomyelitis (EAE) (12), suggest that already during the early phase of inflammation, neuronal pathology involving axonal transection and loss of parental cell bodies plays a pivotal role in disease severity (13, 14). It has become evident that long-term disability in MS correlates better with axonal damage than with the degree of demyelination (13, 14). We have very recently unraveled a process linking demyelination and neuronal damage (15). As a consequence of demyelination, the myelin breakdown product 7-ketocholesterol, which can be detected in the cerebrospinal fluid of MS patients, induces microglia-mediated apoptotic cell death of neurons in the brain stem motor region (15). However, currently approved therapies, which have to be administered parenterally in young disabled adults with neuroinflammatory disorder, primarily focus on the inflammatory aspect of the CNS disease (16), are only partly effective, and are often limited by side effects. In contrast, neuroprotective agents have not yet been established in this autoimmune disorder. In light of the pharmacological profile of EGCG, and its reported properties in immunity and neurodegeneration, we hypothesized a potential beneficial role of EGCG in the treatment of neuroinflammation and subsequently discovered the underlying mechanisms of action.

Materials and Methods

Induction, treatment, and evaluation of EAE

Female SJL/J mice (6–8 wk; ∼20 g body weight; Charles River Laboratories, Sulzfeld, Germany) were immunized s.c. with 75 μg of proteolipid
protein (PLP) peptide p139–151 (purity >95%; Pepceuticals, Leicester, UK) in a 0.2-ml emulsion consisting of equal volumes of PBS and CFA (Difco, Franklin Lakes, NJ) and containing 6 mg/ml Mycobacterium tuberculosis H37Rv (Difco). Bordetella pertussis toxin (20 ng; List Biological Laboratories, Campbell, CA) was administered i.p. at days 0 and 2. For treatment, EGGC (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% NaCl. EGGC or vehicle (0.9% NaCl) were administered by gavage (100 μl per mouse) twice daily. Mice were scored as follows: 0, no disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb weakness; and 5, moribund or dead animals (17). All procedures were conducted according to protocols approved by the local animal welfare committee.

Proliferation and apoptosis

Human CD4+ T cell lines (TCLs) specific for human myelin basic protein (MBP; FN8 and MA14) or birch pollen (SW21) were established with a modified “split-well” protocol as previously described (17). For proliferation, 0.7 × 10^6 cells were stimulated with 2 × 10^5 autologous-irradiated (3000 rad) PBMC as APCs and Ag, or with anti-CD3 (OKT3; coated at 1 μg/ml; American Type Culture Collection, Manassas, VA) and 2.5 μg/ml anti-CD28 (R&D Systems, Minneapolis, MN), respectively. For murine bulk cell cultures, cells suspensions from draining lymph nodes were stimulated in 96-well microtiter plates (2 × 10^5 cells/well) with peptide.

1[H]Thymidine incorporation (0.5 μCi/well; Amersham Biosciences, Piscataway, NJ) was measured 72 h after stimulation with Ag and 48 h with anti-CD3/CD28. Radioactivity was detected using a MicroBeta beta counter (PerkinElmer, Wellesley, MA) after 18 h. For apoptosis, DNA fragmentation of susceptible Jurkat T cells was determined by flow cytometry of hypodiploid DNA as described previously (17).

Immunohistochemistry

Spinal cords and brain stems from mice transcardially perfused with 4% paraformaldehyde were postfixed overnight. Vibratome sections (Thermo Electron, Waltham, MA) were immunolabeled and developed using the avidin-biotinylated enzyme complex technique (ABC-Elite; Vector Laboratories, Burlingame, CA). Neurons positive for active caspase 3 were counted in brain stem sections (four ocular fields per section and six mice per group) (15).

Flow cytometry

Single-cell suspensions were prepared from draining lymph nodes, and a portion was stained with anti-CD25-APC (BD Pharmingen, San Jose, CA). Before staining, cells were incubated with anti-CD14/CD28 (BD Pharmingen) to prevent unspecific Ab binding. For intracellular staining, cells were treated with anti-CD14/CD32 (BD Pharmingen, San Jose, CA), anti-CD19-PE (BD Pharmingen), and anti-Cd11c-PerCP (Serotec, Raleigh, NC) to prevent unspecific Ab binding. For intracellular staining, cells were activated for 4 h (5 ng/ml PMA, 1 μg/ml ionomycin), and brefeldin A (5 μg/ml) was added for the last 2 h. Cells were fixed, permeabilized with FACS buffer containing saponin (PBS, 0.5% saponin, 0.5% BSA, 0.1% NaN3), stained with anti-IFN-γ-PE/TNF-α-APC/Cy7/4-APC Abs (BD Pharmingen), and analyzed on a FACS Calibur using CellQuest software (BD Pharmingen).

Western blot

Cells were washed with PBS and resuspended in lysis buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin). Total protein (30 μg/ml per lane) was separated by 10% SDS-PAGE. After transfer, blots were incubated with anti-IκB-α (Chemicon International, Temecula, CA), anti-cyclin-dependent kinase (CDK4; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p21WAF1 (Santa Cruz Biotechnology), or anti-ubiquitin (Sigma-Aldrich) following HRP-conjugated secondary Ab (DakoCytomation, Carpinteria, CA) and ECL system (Amersham Biosciences) as described (18).

Brain slice cultures

Organotypic entorhinal-hippocampal slice (350 μm thick) cultures from 10-day-old SJL mice were prepared on a tissue chopper (Bachofner) and cultured as described (19). Neuronal cell death was induced with recombinant TRAIL and N-methyl-D-aspartate (NMDA; Sigma-Aldrich) after 24 h. For TRAIL production, N-terminal truncated human TRAIL (residues 95–281) containing an N-terminal 6-His tag was expressed in Escherichia coli BL21 cells. After purification on Ni2+-nitrilotriacetate-agarose (Clontech Laboratories, Palo Alto, CA), untagged proteins were dialyzed against PBS. Damage in brain slices was detected by addition of 5 μg/ml propidium iodide (30 min at 36°C). Slices were examined using a rhodamine filter (488 nm/515 nm) with a dark-field inverse fluorescence microscope (Olympus, Melville, NY). For resectioning, slices were immersed before the preparation of 18-μm thick horizontal cryostat sections, and quantification of PI+ cells was conducted as previously described (15).

HT22 neuronal cell culture assays

For assessment of viability, hippocampal HT22 cells (20) were seeded at 5000 neurons per well in 96-well plates and cultured for 16 h. On the following day, cells received fresh medium and were incubated with EGGC 2 h before incubation with TRAIL with and without cycloheximide (CHX; 0.5 μg/ml). Crystal violet assay was performed 16 h thereafter as previously described (21). Briefly, cells were stained for 30 min with 0.5% crystal violet in 20% methanol. After washing and drying, crystal violet was dissolved in 50 ml/well of 0.1 M sodium citrate solution diluted in 50% ethanol and subsequently quantified photometrically by absorbance at 550 nm (Dynatech Laboratories, Chantilly, VA). Values are expressed as the percentage of survival compared with untreated controls. Total protein extracts for immunoblots were prepared similarly. For detection of intracellular peroxides, intracellular accumulation of reactive oxygen species (ROS) was induced by 100 μM buthionine sulfoximine (BSO) in HT22 cells (20). Cells were incubated with 10 μM 5-carboxy-2′,7′-dichloro-l-hydrofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR) for 30 min at 37°C. Wells without cells were loaded with DCFDA serving as negative controls. Fluorescence measurements were made on a Fluorostar plate reader (SLT Labinstruments) at 488 nm and Ex_{250} A 530 nm.

Statistics

For group comparisons, the Mann-Whitney U test was applied. For EAE courses, ANOVA with Bonferroni adjustment was performed as previously described (17).

Results

Green tea polyphenol EGGC prevents and reverses disability in autoimmune encephalomyelitis

As a first step, we explored the therapeutic effect of the green tea polyphenol EGGC in EAE. This polyphenol was chosen because it is easily absorbed from the digestive tract and penetrates the brain, reaching levels similar to those found in lung, liver, kidney, and other organs (22). SJL/J mice were started on oral therapy with EGGC (300 μg per mouse in 0.9% NaCl) or vehicle (0.9% NaCl) twice daily at the time of immunization with PLP p139–151, resulting in relapsing-remitting EAE (17). EGGC treatment enhanced animals’ recovery from disease manifestation and significantly protected them from long-term neurological deficits, as indicated by the mean clinical scores over an observation period up to 131 days (Fig. 1A). EGGC administration started at the onset of acute disease prevented mice from relapses and prolonged neurological sequelae during subsequent relapse with a maximum disease score of 1.4 ± 0.3 in the EGGC group vs 2.7 ± 0.5 in the vehicle group (p < 0.05) and significantly lower mean clinical disease scores (Fig. 1B). The therapeutic effect of oral EGGC was found to be dose-dependent: a dose of 60 μg per mouse (administered twice daily) was sufficient to attenuate the course of EAE, whereas a lower dosage (12 μg per mouse twice daily) was not (Fig. 1C). Histological examination revealed large inflammatory lesions throughout the brain stem and spinal cord in vehicle-treated mice, while EGGC-treated animals showed significantly reduced inflammation (Fig. 2A).

Analysis of the T cell response

To unravel the underlying mechanisms, we next examined T cell response ex vivo. Draining lymph node cells from immunized animals treated with EGGC for 10 days exhibited a markedly lower proliferative response to the encephalitogenic PLP p139–151 peptide than control animals (Fig. 2B). Further, oral EGGC led to a
FIGURE 1. Oral EGCG therapy inhibits and reverses relapsing-remitting EAE. A, For the preventive treatment paradigm, EGCG (●, 300 μg per mouse twice daily) or vehicle (○, NaCl 0.9%) was orally administered from the day of immunization on. Disease severity was significantly reduced in the EGCG group (μ, F_{31-131} (1,101) = 6.7, p < 0.05; ANOVA; n = 6 per group). B, For treatment of established disease, mice were randomized into 2 groups after disease manifestation on day 12 and subsequently received either oral EGCG or vehicle. Clinical disease was significantly reduced in the EGCG group (μ, F_{50-75} (1,26) = 4.9, p < 0.05; ANOVA; n = 9 per group). C, Dose titration experiments showed that 60 μg of EGCG per mouse administered twice daily attenuated EAE (●), as compared with the vehicle control group (○), F_{10-16} (1,7) = 5.0, p < 0.05; ANOVA, n = 8 per group). In contrast, a lower dose (12 μg of EGCG per mouse administered twice daily; ▲) had no significant effect.

FIGURE 2. EGCG inhibits myelin-specific inflammatory responses. A, Representative H&E staining of spinal cord longitudinal sections from EAE in Fig. 1A. Number of inflammatory foci in the brain stem and spinal cord was assessed. B and C, Four mice per group received EGCG (filled bars) or vehicle (open bars) from day 1 after immunization. Ten days later, lymph node cells were assessed for proliferation in response to PLP (B) or TNF-α synthesis (C). The upper graph in C is a representative flow cytometry histogram. The thick bold line represents the staining of cells from vehicle-treated animals; the thin line with gray shading below represents the staining of cells from EGCG-treated animals; the thin line without shading represents the staining of cells from isotype controls (see labels). The lower graph shows the percentage of TNF-α-positive cells (open bars, vehicle-treated group; filled bars, EGCG-treated group). Data show mean ± SEM; * p < 0.05.
reduction in TNF-α synthesis, demonstrated by intracellular FACS staining ex vivo (Fig. 2C). In contrast, expression of IL-4 or IFN-γ was unaffected. There was no link between successful EAE therapy and expansion of regulatory T cells, determined by comparable fractions of CD4<sup>+</sup>CD25<sup>+</sup> cells in EGCG- and vehicle-treated animals (data not shown). Similarly, EGCG treatment did not affect the proportion of CD4/CD8 cells.

**EGCG inhibits T cell proliferation by suppressing CDK4 and up-regulating IκB-α**

In human CD4<sup>+</sup> Th1 MBP-specific T cells, EGCG, in a dose-dependent manner, blocked expansion of cells stimulated with peptide-loaded autologous APCs (Fig. 3A) and cells proliferating in response to direct TCR engagement by anti-CD3/CD28 stimulation (Fig. 3B). The micromolar EGCG concentrations used in this and the following in vitro assays are likely to be achieved in humans, as indicated by previous investigations (23). Interestingly, no indication of EGCG-mediated T cell death was observed with trypan blue exclusion assay (data not shown). Even in apoptosis-susceptible Jurkat T cells, no DNA fragmentation was induced (Fig. 3C), excluding a direct death-promoting role for EGCG in T cells. Western blot analysis showed that inhibition of the T cell proliferation by EGCG can be explained by interference on the cell cycle level, down-regulating expression of the CDK4. This effect was not mediated by p27<sup>kip1</sup>, a CDK inhibitor and negative cell cycle regulator (Fig. 4A). In contrast, we observed that EGCG interfered with NF-κB activation. While in untreated human T cells the crucial NF-κB-inhibitor IκB-α (24) was down-regulated upon stimulation, EGCG inhibited this pathway in a dose-dependent manner, resulting in an intracellular accumulation of IκB-α (Fig. 4B). Further, TNF-α stimulation of T cells resulted in a strong degradation of IκB-α within a few hours (Fig. 4C), which was completely reversed in the presence of EGCG. Since degradation of IκB-α, a prerequisite of NF-κB activity, is mediated by the cytosolic proteasome complex (24), intracellular accumulation of IκB-α by EGCG may originate from inhibition of proteasome activity. This assumption was supported by a similar IκB-α accumulation pattern in T cells in the presence of the synthetic 26S proteasome inhibitor MG132 (Fig. 4D).

**FIGURE 3.** EGCG inhibits human Ag-specific CD4<sup>+</sup> T cell proliferation. A, Proliferation of the human MBP-specific TCL FN8 stimulated with (+) or without (−) Ag presented by irradiated autologous APCs. B, The same TCL was stimulated with (+) or without (−) anti-CD3/CD28 Abs. C, Specific DNA fragmentation was assessed in Jurkat T cells incubated with murine CD95 ligand (10 U/ml) as positive control or EGCG, respectively.

**FIGURE 4.** EGCG interferes with cell cycle, NF-κB activation, and proteasome in CD4<sup>+</sup> T cells. A, Human TCL MA14 was assessed for CDK4 and p27<sup>kip1</sup> protein levels 24 h after anti-CD3/CD28 stimulation. B, The same TCL stimulated with anti-CD3/CD28 was used to analyze the NF-κB inhibitor IκB-α protein regulation by EGCG. C, Human TCL SW21 was cotreated with TNF-α (20 ng/ml) without (upper panel) or with EGCG (40 μg/ml; middle panel) or with proteasome inhibitor MG132 (10 μM; bottom panel) for indicated time periods. D, Accumulation of ubiquitinated proteins (arrows) in human T CL after stimulation with TNF-α (20 ng/ml) in the presence of EGCG (40 μg/ml).
proteasome inhibitor MG132 (Fig. 4C). Indeed, the rapid increase in ubiquitinated proteins, the substrates of the proteasome complex, confirmed the involvement of proteasome inhibition in T cells by EGCG (Fig. 4D).

**EGCG directly inhibits neuronal cell death by interference with ROS formation**

Considering the reported effects of green tea compounds in neurodegenerative models, we analyzed whether the therapeutic efficacy of EGCG in EAE involves protection against neuronal damage. Indeed, we found reduced axonal pathology in EGCG-treated animals in the chronic phase (Fig. 5A). Quantitative analysis of caspase 3-positive neurons, shown to be present in the brain stem motor area of mice suffering from EAE (15), revealed significantly fewer apoptotic neurons in EGCG-treated as compared with vehicle-treated animals (Fig. 5B). Accordingly, we observed similar effects of EGCG on neurons in living organotypic hippocampal brain slice cultures from SJL/J mice, a system in which neurons can be maintained in an in vivo-like fashion (19). Damage was induced by exposing slices to neurotoxic NMDA and the apoptosis ligand TRAIL, which is reported to have neurotoxic properties (25) and involve the activation of NF-κB (26). In both constellations, preincubation with EGCG 3 h before damage significantly protected against neuronal cell death (Fig. 5C). Next, to unravel the underlying mechanisms, we investigated the impact of EGCG on HT22 hippocampal neurons. As in slices, TRAIL induces cell death in HT22 neurons which can be inhibited by EGCG (Fig. 5D).

However, in contrast to our findings in T cells (Fig. 4B), no accumulation of IκB-α was observed (Fig. 5E). Therefore, we hypothesized that due to its structure containing two trihydroxybenzoyl groups, EGCG may exert its neuroprotective activity directly as a free radical scavenger (27). In fact, we could show in a model of glutathione depletion-induced neurotoxicity (20) that EGCG is able to inhibit the formation of ROS in neurons. HT22 neurons were incubated with BSO, an inhibitor of glutathione synthetase, thereby inducing glutathione depletion (20), which subsequently results in neuronal cell death. Treating HT22 neurons with BSO induced a 2-fold increase in ROS, which could be prevented by EGCG application (Fig. 5F). These results demonstrate that EGCG acts downstream of glutathione synthetase and, therefore, directly targets ROS formation.

**Discussion**

We have shown that orally applied EGCG, the major polyphenolic compound of green tea, suppressed inflammation in vivo, inhibiting proliferation and TNF-α synthesis of T cells, and effectively...
FIGURE 6. Proposed model for signal transduction pathways modified by EGCG. EGCG is capable of inhibiting both catalytic activities of the proteasome, including NF-κB activation, and the amount of ROS. In lymphocytes, this leads to decreased proliferation and production of the proinflammatory cytokine TNF-α, while in neurons, it results in less damage. Additionally, the antioxidative effects of EGCG on neurons might involve the NF-κB pathway as well, since ROS can induce NF-κB, which regulates the expression of a variety of molecules contributing to cell proliferation, inflammation, and neuronal damage.

Since the available treatment only consists of immunomodulatory agents, green tea compounds such as epigallocatechin-3-gallate are candidates for the treatment of MS patients, as they are administered orally, are well tolerated (34), and exhibit synergistic beneficial effects, unraveled here, in disorders with both pathological elements, inflammation and neuronal injury (Fig. 6). One may speculate that the biological properties described here contribute to the lower prevalence rates of MS in Asian populations with traditional consumption of green tea (35).

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References


