Differential Effects of Green Tea–Derived Catechin on Developing Versus Established Atherosclerosis in Apolipoprotein E–Null Mice

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Background—Oxidative stress has been implicated in vascular injury and atherogenesis, and antioxidant treatment has shown favorable results in preclinical studies. Despite this, antioxidant therapy has failed to show benefit in clinical trials. Failure of antioxidants in clinical trials may be partly because such therapy is started after atherosclerosis is already well established, whereas the benefits in animal models may be results from early initiation of antioxidants while atherosclerosis is still evolving.

Methods and Results—To test this hypothesis, we evaluated the effect of epigallocatechin gallate (EGCG), the main antioxidant derived from green tea, on evolving and established atherosclerotic lesions in hypercholesterolemic apolipoprotein E–null mice. Established native aortic atherosclerotic lesions and evolving atherosclerotic lesions produced by periadventitial cuff injury to carotid arteries were assessed in mice after 21 and 42 days of treatment with daily intraperitoneal injections of EGCG (10 mg/kg) or PBS. EGCG treatment resulted in an increase in the antioxidant capacity in local vascular tissue and systemic circulation and reduced vascular smooth muscle cell proliferation and redox-sensitive gene activation in vitro. EGCG reduced cuff-induced evolving atherosclerotic plaque size at 21 and 42 days by 55% and 73%, respectively, compared with PBS treatment ($P<0.05$). Conversely, EGCG had no effect on established lesions in the aortic sinuses or the rest of the aorta.

Conclusions—Our data suggest that antioxidant EGCG differentially reduces evolving atherosclerotic lesions without influencing established atherosclerosis in the apolipoprotein E–null mice. (Circulation. 2004;109:2448-2453.)

Key Words: atherosclerosis ■ catechin ■ antioxidant

Tea contains many biologically active polyphenolic flavonoid, commonly known as catechins, which make up 30% of the dry weight of its leaves.1 These catechins include epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG), with EGCG being the principal constituent. EGCG is a well-characterized antioxidant2–4 with antitumorigenic, anti-inflammatory, and antiangiogenic properties.5–7 Because oxidative stress has been implicated in vascular injury and atherogenesis and because of these beneficial effects, tea has recently received attention as a prospect for dietary intervention in cardiovascular diseases.8–11

Although antioxidant therapies have been shown to modulate atherosclerosis in experimental animals, similar therapies have failed to show consistent clinical benefit.12 Failure of antioxidants in clinical settings may be because of the following: (1) short duration of treatment, (2) failure to use the correct antioxidant, or (3) initiation of antioxidant therapy too late in the evolution of atherosclerosis to have an impact.12 Despite negative results in clinical trials, it has been argued that antioxidant therapy still might be effective in reducing the initial stage of human atherosclerosis if therapy is started before atherosclerosis is established.12

Animal experiments evaluating the effect of antioxidants usually started treatment when animals were young,13–15 supporting the speculation that antioxidant treatment affects the initial stage of atherogenesis. This is quite different from the clinical trials in which adult patients with more advanced plaques were enrolled in randomized trials. It is not clear whether antioxidants would be effective if the treatment were started in adult animals with established plaques. In this study, we used a pericarotid cuff injury model in hypercholesterolemic apolipoprotein E–null (apoE$^{-/-}$) mice to answer the question whether EGCG treatment modulates early-stage atherosclerosis.

Received December 5, 2003; accepted February 6, 2004.

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Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000128034.70732.C2

2448
atherosclerosis and established plaques differently. Although this model is an accelerated atherogenesis model,\textsuperscript{16} it allows for evaluation of the effects of pharmacological agents on plaques of different stages (early cuff-induced carotid plaques versus established aortic sinus plaques) in the same mouse. We also examined the effect of EGCG on proliferation and redox-sensitive gene activation in vitro.

**Methods**

**Animal Protocol**

Apoe\textsuperscript{−/−} male mice (C57BL/6J, Jackson Laboratories, Bar Harbor, Me) were housed in an animal facility accredited by the American Association of Accreditation of Laboratory Animal Care and kept on a light/dark (12/12-hour) cycle. The mice were fed a high-cholesterol diet (0.15% cholesterol, 21% fat, TD 88137 from Harlan-Teklad) at 7 weeks of age until they were euthanized and received food and water ad libitum. At the age of 28 weeks, a nonocclusive periadventitial collar was placed on the right common carotid artery after mice were anesthetized with Avertin (0.016 mL/g of 2.5% solution IP) as described previously.\textsuperscript{17} EGCG (10 mg/kg) was injected intraperitoneally starting the day of cuffing and every day thereafter (5 d/wk) until death. Mice receiving PBS injection served as controls. Mice were euthanized by inhalation of Enflurane at 3 days (day 3 mice), or 21 days (day 21 mice), or 42 days (day 42 mice) after cuffing. Blood samples were collected in EDTA tubes before injury or immediately before death. Mice receiving PBS injection served as controls. Mice were euthanized by inhalation of Enflurane at 3 days (day 3 mice), or 21 days (day 21 mice), or 42 days (day 42 mice) after cuffing. Blood samples were collected in EDTA tubes before injury or immediately before death by retro-orbital bleeding. This experimental protocol was approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center.

**Tissue Harvesting and Sectioning**

After the heart and aortic tree had been perfused with normal saline at physiological pressure, the right common carotid was excised, embedded in OCT compound (Tissue-Tek), frozen, and sectioned. Serial sections 6 μm thick were collected from the injured area of the carotid. Typically, 3 consecutive sections were on 1 slide, and every fifth slide was grouped for staining. The base of the heart containing the aortic root was excised, embedded in OCT, and frozen-sectioned. Serial sections 10 μm thick were collected from the appearance of at least 2 aortic valves to the disappearance of the aortic valve leaflets. Three consecutive sections were on 1 slide, with a total of 25 to 30 slides collected from 1 animal. Every fifth slide was grouped for staining.

**Immunohistochemistry and Histomorphometry**

Sections of the cuffed carotid artery were stained with hematoxylin and eosin for morphometric analysis. Sections of aortic sinus were stained with MOMA-2 antibody (Serotec) for macrophage immunoreactivity or stained with trichrome to identify collagen content by standard protocols. Computer-assisted morphometry was performed with Image Pro-Plus (Media Cybernetics) as described previously.\textsuperscript{18} MOMA immunoreactivity and collagen content are presented as percentage of positive-stained area of the total aortic sinus plaque area.

**En Face Preparation of Descending Thoracic and Abdominal Aorta**

After the descending thoracic and abdominal aorta was cleaned of surrounding tissue and fat, the aorta was carefully removed and opened longitudinally, followed by overnight fixation in Histochrome (Amresco). The aorta was stained with oil red O and then placed luminal up on a slide freshly coated with egg albumin solution. The extent of aortic atherosclerosis was measured by computer-assisted morphometry.

**Measurement of Superoxide Generation by DHE Staining**

The oxidative fluorescent dye dihydroethidium (DHE, Molecular Probes) was used to measure in situ production of superoxide. Fresh unfixed frozen sections from the cuffed carotid artery of mice treated with EGCG (n = 6) or PBS (n = 3) for 3 days were placed on a glass slide. DHE (0.25 μmol/L) was applied topically to each tissue section, and the sections were then incubated at 37°C for 30 minutes. Slides were briefly washed in PBS, and coverslips were placed over the sections. Images were obtained with fluorescence microscopy. Data are presented as percent positive stained area of total medial area.

**Plasma Cholesterol Level and Antioxidant Potential Measurement**

Plasma total cholesterol levels were measured with a commercially available kit (Sigma), and the manufacturer’s instructions were followed. Plasma samples were diluted 1:2 with PBS before the assay. Plasma antioxidant potential was measured with a commercially available kit (Bioxxytech Aop-490, OxisResearch).

**Plasma EGCG Concentration Measurement**

Plasma catechin was extracted by mixing 500 μL plasma with 200 μL Vc-EDTA (20% ascorbic acid, 0.1% Na₂-EDTA, 0.4 mol/L NaH₂PO₄, H₂O and 10 mmol/L propylgallate, adjusted to pH 3.6, all final concentration) and 50 μL β-glucuronidase/sulfatase (5000/250 U/mL). The sample was flushed with argon and kept at 37±1°C in a water bath for 40 minutes, mixed every 10 minutes. The reaction was ended with the addition of 1000 μL of 1:1 methanol:sodium chloride (5% wt/vol) solution. The samples were vortexed for 30 seconds and centrifuged for 5 minutes at 3300g, after which the water layer was removed. The pellet was reextracted with 1 mL sodium chloride (5% wt/vol), vortexed for 30 seconds, and centrifuged for 5 minutes at 3300g. The water phases were combined. To the water phase, 4 mL ethylacetate was added, which was vortexed for 30 seconds and centrifuged at 3300g. The ethylacetate (upper) phase was removed and the water phase reextracted with 4 mL ethylacetate according to the same procedure. The ethylacetate phases were combined and stored at −20°C for 24 hours at most before further analysis.

High-performance liquid chromatography/mass spectrometry analysis was performed by use of an HP1100 LC/diode array detector/mass specific detection with an orthogonal electrospray source (Hewlett-Packard) on a Symmetry C₁₈ column (150×2.1 mm, 3.5 μmol/L) (Waters Chromatography BV). The flow was 0.3 mL/min. The samples were eluted with eluent A (water+0.5% [vol/vol] acetonitrile+0.1% [vol/vol] formic acid) and eluent B (water/acetonitrile [50:50, vol/vol]+0.1% [vol/vol] formic acid) with the following elution gradient: 0 to 3 minutes, 5% eluent B; 3 to 12 minutes, 20% eluent B; 12 to 18 minutes, 5% eluent B. The mass spectrometer (MSD) was operated in the negative mode. EGCG was targeted at m/z 457.4. The following parameters were set: fragmentor voltage 100 V, capillary voltage-4000 V, drying gas flow 8 L/min (250°C), scan rate 2 scans/s, nebulizer pressure 60 psi. The DAD was set at 2 wavelengths, 210 and 280 nm.

**Isolation of Vascular Smooth Muscle Cells**

Aortic vascular smooth muscle cells (VSMCs) were cultured from explanted aortic segments from apoE\textsuperscript{−/−} mice and were positive for α-actin (Sigma). VSMCs between passage 6 and 12 were used and maintained in DMEM/F-12 with 20% FBS, 2 mmol/L L-glutamine, and antibiotics.

**Cell Growth Study**

VSMCs (10\textsuperscript{4} cells/well) were cultured with and without EGCG (10 or 30 μmol/L) in culture medium, and cell number was then determined 72 hours later. In a separate experiment, confluent and growth-arrested VSMCs were stimulated with platelet-derived growth factor (PDGF)-BB (10 ng/mL) for 24 hours with or without 24-hour EGCG (30 μmol/L) pretreatment for cell cycle analysis by flow cytometry (performed twice) or nuclear protein extraction for proliferating cell nuclear antigen (PCNA) determination by Western blot analysis. In another experiment, growth-arrested VSMCs treated with tumor necrosis factor (TNF)-α (10 ng/mL) for 30 minutes after
EGCG pretreatment (30 μmol/L) for 24 hours were analyzed for activating protein (AP)-1 binding activity by electromobility shift assay and for c-Jun expression by Western blot analysis. Growth-arrested VSMCs treated with TNF-α for 24 hours with or without EGCG (10 μmol/L) were analyzed for cytosolic inducible NO synthase (iNOS) expression by Western blot analysis.

Nuclear Protein Extraction Protocol
VSMCs were suspended in cytosolic lysis buffer (0.5 mmol/L DTT, protease inhibitor, NP40), incubated on ice (15 minutes), and centrifuged (13 000 rpm, 10 minutes, 4°C). The supernatant was saved as cytosolic protein extract; the nuclear pellet was washed with cytosolic lysis buffer and recentrifuged, and the supernatant was removed. The pellet was resuspended in nuclear lysis buffer, put through a freeze/thaw cycle (~80°C to room temperature 3 times), and spun (13 000 rpm, 5 minutes, 4°C). The supernatant was saved as the nuclear extract in ~80°C.

Western Blot Analysis
Equal amounts of cytosolic or nuclear protein preparations were run on an SDS-polyacrylamide gel (6% for cytosolic iNOS and 12% for nuclear PCNA and c-Jun). Protein samples were then electrophoresed to Hybond enhanced chemiluminescence nitrocellulose membrane and blotted with primary antibody against iNOS, PCNA, or c-Jun (Santa Cruz Biotechnology) followed by horseradish peroxidase–conjugated secondary antibody. Blots were developed using enhanced chemiluminescence plus reagent (Amersham Pharmacia Biotech).

Electromobility Shift Assay for AP-1 Binding Activity
Redox-sensitive AP-1 binding activity was determined by an electromobility shift assay using standard protocol with nuclear protein extract from apoE−/− VSMCs incubated with an oligonucleotide containing the AP-1 binding sequence.

Preparation of EGCG
(−)-Epigallocatechin-3-gallate (>95% purity), a generous gift from Lipton, Inc, was dissolved in PBS and filtered with a 0.2-μm filter before in vivo or in vitro use.

Statistics
All values are expressed as mean±SD. Number of animals and statistics used in each experiment are listed in texts, tables, or figure legends. Unless otherwise stated, all statistical analysis was done by t test for comparison between 2 groups or by ANOVA followed by paired group comparison for comparison among multiple groups. A value of P<0.05 was considered statistically significant.

Results
Plasma Cholesterol, EGCG Levels, and Antioxidant Potential
Mice euthanized at 42 days after carotid injury showed no difference in body weight or plasma cholesterol level between the EGCG group and control groups (Table). There was no difference in body weight or plasma cholesterol level among mice euthanized at day 21 (data not shown). Plasma EGCG concentration measured ~1 hour after intraperitoneal injection was 138±44 ng/mL (n=5).

Plaque size, % total area 21 0.02 NS 19 5 NS
Aortic lesions, flat prep
Plaque size, % total area 21 0.23 0.056 NS 19 0.19 NS

Effect of EGCG on Mature Atherosclerotic Plaque
EGCG treatment did not affect aortic sinus plaque size or the aortic plaque size from en face measurement after 42 days of

By Mann-Whitney test.

To demonstrate the antioxidant property of EGCG, plasma antioxidant potential was measured, and it was higher in the EGCG-treated group (1.15±0.58 versus 0.26±0.29 mmol/L uric acid equivalents, P<0.05, n=7 in each group by t test). EGCG treatment also resulted in a significant reduction of superoxide generation (EGCG group, 2.6±2.5%, n=5, versus PBS group, 10.1±6.4%, n=3; P=0.05) as assessed by DHE staining in the media of the cuffed carotid of day 3 mice (Figure 1).

Effect of EGCG on Evolving Carotid Atherosclerotic Plaques
At 21 days after pericarotid cuffing, there was a clear neointimal formation in the cuffed carotid segment with positive oil red O staining, characteristic of early fatty streak formation (Figure 2, A and C). At 42 days after injury, such intima became more abundant and larger (Figure 2, D and F), indicating progression of atherosclerotic plaque formation in the control group. Conversely, daily treatment of EGCG significantly reduced such plaque formation 21 and 42 days after injury (Figure 2, B and E). The size of atherosclerotic plaques in the day 42 mice is similar to the plaque size from day 21 mice in the groups treated with EGCG (Figure 2G), suggesting that EGCG treatment significantly reduced the progression of atherosclerotic plaque formation. The intima/media ratio was significantly smaller in the EGCG-treated mice than the PBS-treated mice (Table).

Figure 1. Superoxide staining by DHE in sections from right common carotid of apoE−/− mice euthanized 3 days after injury. Mice treated with EGCG had significantly less superoxide staining than mice treated with PBS (see text for statistical analysis). Representative sections of DHE staining from PBS-treated (A) or EGCG-treated (B) mice were shown. L indicates lumen; A, adventitia; arrows, internal elastic lamina.
Collagen content in the aortic sinus plaque as assessed by trichrome staining was comparable. Similarly, there was no statistically significant difference in macrophage content, as shown by MOMA-2 staining (Table).

**Cell Growth Study**

EGCG treatment resulted in a significant reduction of cell growth at 30 μmol/L (Figure 3A) and PCNA expression induced by PDGF-BB (Figure 3B). Cell cycle analysis...
revealed that PDGF-BB treatment mobilized a significantly higher percentage of growth-arrested VSMCs into S phase compared with control (18.6±4.9% versus 10.6±4.0%), whereas concomitant EGCG treatment arrested VSMCs in the G₁ phase, and the percentage of cells in the S phase was similar to that of control (11.3±0.6%).

c-Jun Expression, AP-1 Activation, and iNOS Expression
Redox-sensitive gene activation and reactive oxygen species have been implicated in cellular proliferation and activation of atherogenic proinflammatory molecules. iNOS is a potential source of reactive oxygen species and is involved in the tissue proliferative repair process. Hence, in our in vitro experiments, we tested whether EGCG reduces the activation of redox-sensitive transcription factor AP-1 and iNOS expression. Pretreatment with EGCG 30 μmol/L significantly reduced nuclear translocation of c-Jun and subsequent AP-1 activation in VSMCs treated with TNF-α (Figure 3C). TNF-α-induced iNOS expression was also significantly reduced by EGCG treatment (Figure 3D).

Discussion
Our results demonstrate the following: (1) intraperitoneal injection of EGCG enhanced systemic and local antioxidative capacities; (2) EGCG effectively reduced the progression of accelerated atherosclerotic carotid plaque formation induced by cuff injury, whereas it had no effect on advanced aortic plaques from the same animals; and (3) EGCG inhibited VSMC proliferation in vitro, possibly via antioxidative modulation of redox-sensitive gene expression.

Previous studies have suggested that green tea and its polyphenolic extracts are antiatherogenic; however, this effect was modest. For instance, supplementation of green tea through drinking water achieved a non–statistically significant reduction of aortic plaque content in hypercholesterolemic rabbits. Similarly, apoE/−/− mice given drinking water supplemented with green tea extract had reduced aortic atheromatous area and tissue cholesterol content, but no difference was observed in the aortic sinus plaque. Pure catechin was not used in these studies, which may explain the modest antiatherogenic effect observed. We chose to use EGCG with >95% purity instead of tea extract, which has a lower content of catechin. We also measured plasma EGCG concentration to ensure that the plasma level was adequate. With the amount of EGCG administered, there were significantly higher antioxidant capacities both in the systemic circulation and in local vascular tissue.

Extravascular arterial manipulation has been used to induce local plaque formation in both rabbits and mice. The placement of a perivascular collar in apoE/−/− mice causes rapid atherosclerotic lesion development in the carotid artery. After 6 weeks of cuff placement, a fully developed, advanced

Figure 3. A, Cell number after serum stimulation of aortic VSMCs from apoE/−/− mice cultured with (10 or 30 μmol/L) or without EGCG (n=4; *P<0.05 by ANOVA). B, Western blot analysis of PCNA of aortic VSMCs from apoE/−/− mice stimulated with PDGF-BB with or without concomitant treatment of EGCG. C, Western blot analysis of nuclear c-Jun expression (top) and electromobility shift assay of AP-1 (bottom) in aortic VSMCs from apoE/−/− mice pretreated with EGCG (24 hours) and stimulated with TNF-α (2 hours). TNF-α treatment induced nuclear translocation of c-Jun and subsequent nuclear AP-1 binding, whereas EGCG treatment prevented such an event. D, Western blot analysis of cytosolic protein from VSMCs from apoE/−/− mice treated with TNF-α with or without EGCG. TNF-α treatment for 24 hours induced abundant expression of iNOS, whereas EGCG treatment reduced such events.
atherosclerotic plaque was observed. Our findings from 21- and 42-day mice are consistent with this reported observation.

In the present study, EGCG treatment was started late when atherosclerotic lesions in aortic sinus were already at an advanced stage. Our observations that EGCG reduced the progression of evolving carotid atherosclerotic plaques but had no effect on the mature plaques in aortic sinus and aorta suggest a differential effect of this pharmacological intervention on early-stage versus late-stage events in atherogenesis. This observation is further supported by several experimental studies in which antioxidant therapy was beneficial when such therapy was initiated before mice were 10 weeks old. Taken together, these observations suggest that antioxidant therapy would be effective only when initiated during a critical window in the temporal evolution of atherosclerotic plaque.

Potential mechanisms for this inhibitory effect exhibited by EGCG in our study and others include its antiproliferative properties and its ability to inhibit redox-sensitive signal transduction pathways related to cell growth on VSMCs. We demonstrated that EGCG treatment resulted in reduction of VSMC growth and arrested VSMCs in the G1 phase of the cell cycle. PCNA, a marker of cell growth, was also to the outcome. The observed benefits of pretreatment with antioxidant therapy for atherosclerosis suppression.

In summary, our study suggests that EGCG treatment significantly reduces progression of evolving atherosclerotic lesions without affecting advanced lesions. This differential effect highlights the importance of time of initiation of antioxidant therapy for atherosclerosis suppression.

References
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Circulation. 2004;109:2448-2453; originally published online May 10, 2004;
doi: 10.1161/01.CIR.0000128034.70732.C2

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/20/2448

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