Green Tea Catechins and Vitamin E Inhibit Angiogenesis of Human Microvascular Endothelial Cells Through Suppression of IL-8 Production

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Abstract: Epidemiological and animal studies have indicated that consumption of green tea and high vitamin E intake are associated with a reduced risk of developing certain forms of cancer. However, the inhibitory mechanism of green tea catechins and vitamin E in angiogenesis, an important process in tumor growth, has not been well established. In the present study, α-tocopherol and several major catechins of green tea (catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate) were tested for their ability to inhibit tube formation in vitro using a model in which human microvascular endothelial cells were exposed to a constant rate of a physiologically low level of H₂O₂. In this model, the production of interleukin (IL)-8 by human microvascular endothelial cells at a low level of H₂O₂ was required for angiogenesis, as assessed by tube formation in three-dimensional gel in culture. Vitamin E (α-tocopherol, 40 μM) in the culture media significantly reduced IL-8 production and angiogenesis. Among the green tea catechins, epigallocatechin (0.5–1 μM) was the most effective in reducing IL-8 production and inhibiting angiogenesis. These results suggest that consumption of green tea catechins or supplemental intake of vitamin E may have preventive effects on tumor development, mediated, at least in part, through inhibition of angiogenesis via suppression of IL-8 production.

Introduction

Angiogenesis, the formation of new blood vessels from preexisting blood vessels, is involved in the pathogenesis of many chronic diseases, such as cancer, rheumatoid arthritis, and diabetic retinopathy (1–4). The process of angiogenesis includes basement membrane degradation, endothelial proliferation and migration, extracellular matrix remodeling, and vascular tube formation (1). The development of new blood vessels is well controlled by angiogenic and antiangiogenic factors that are produced by normal and tumor cells (5). Several angiogenic factors have been characterized, including vascular endothelial growth factor (VEGF), fibroblast growth factor, epidermal growth factors, transforming growth factors, and platelet-derived endothelial cell growth factor, as well as angiogenin, pleiotrophin, angiotrophin, and interleukin (IL)-1, IL-6, and IL-8 (5–7). On stimulation by angiogenic factors, endothelial cells in tumor blood vessels divide rapidly, forming new blood vessels within tumor parenchyma and providing nutrition and oxygen for malignant cells (1).

Several antiangiogenic factors have been identified, including interferon-α (8,9), inhibitors of matrix metalloproteases, inhibitors of plasminogen activators (10), endostatin (11), and angiostatin (12,13). In physiological conditions, the proliferation of endothelial cells is tightly regulated and self-limited (5). In certain pathological conditions, however, angiogenesis significantly increases and loses its capacity to regulate itself (14). From a clinical perspective, the most important manifestation of pathological angiogenesis may be observed during the development of solid tumors (15).

Epidemiological studies and clinical trials have indicated that dietary antioxidants may decrease cancer risk. Green tea catechins and vitamin E, which have antioxidant properties, appear to be effective in reducing cancer risk (16–19). A prospective cohort study in Japan indicated that regular green tea consumption reduces the incidence of cancer (16). Epidemiological studies have indicated that regular green tea consumption and high vitamin E intake are associated with a lower risk of certain forms of cancer, including sarcomas, head and neck tumors, and intestinal cancers (16–20). Similar results have been found in vitro and in vivo animal models. Vitamin E has been shown to inhibit experimental carcinogenesis and tumor development (21). Consumption of green tea and vitamin E has been shown to inhibit tumor growth in animals (19,21,22). Catechins of green tea inhibited the growth of a human lung cancer cell line (23). Recently, Cao and Cao (24) reported that the green tea catechin epigallocatechin gallate (EGCG) suppressed bovine endothelial cell proliferation in culture and that...
drinking green tea suppressed VEGF-induced angiogenesis in a mouse corneal model (24).

Shono et al. (25) showed that treatment of human microvascular endothelial cells (HMVEC) with a single bolus dose of H$_2$O$_2$ (100–500 μM) resulted in microvascular tube formation in culture. H$_2$O$_2$ is a source of reactive oxygen species (ROS), which are believed to be important in the development of cancer, diabetes, and rheumatoid arthritis (26, 27). ROS are generated under various physiological and pathological conditions, including inflammation, ischemia and reperfusion, sepsis, and ultraviolet irradiation (28). Although high levels of ROS are cytotoxic, low levels are necessary, inasmuch as they mediate some biological processes (29). For example, several transcription factors use H$_2$O$_2$ as a second messenger for their activation (30). Studies have shown that antioxidants including α-tocopherol and polyphenols can influence cellular redox status, which in turn modulates signaling pathways involved in the regulation of gene expression, including that of IL-8 (31–35).

Evidence indicates that H$_2$O$_2$ induction of HMVEC tube formation is mediated by the increased IL-8 production (25). Earlier, we showed that IL-1-stimulated IL-8 production by human aortic endothelial cells is suppressed by supplementing cells with α-tocopherol (36). Therefore, we hypothesized that the suppression of angiogenesis by antioxidants such as vitamin E or green tea catechins is mediated in part by the inhibition of IL-8 production under oxidative stress conditions. Therefore, in the present study, we sought to investigate the effect of five major green tea catechins with varying degrees of antioxidant activity in comparison to vitamin E on IL-8 production and HMVEC tube formation in culture. Our results demonstrate for the first time that green tea catechins or vitamin E dose dependently inhibits oxidative stress-induced tube formation, in part through inhibition of IL-8 production.

**Materials and Methods**

**Reagents and Antibodies**

MCDB-131 medium and amphotericin B were obtained from Sigma Chemical (St. Louis, MO); phenol red-free medium 199 (M199), fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin from Life Technologies (Gaithersburg, MD); HMVEC, recombinant human epidermal growth factor, bovine brain extract, and hydrocortisone from Clonetics (San Diego, CA); and IL-8, monoclonal anti-IL-8 antibody, and VEGF from R & D Systems (Minneapolis, MN). Type I collagen was prepared from rat tail tendons. Type I collagen was diluted to mainly type I collagen, the major constituent of the pericapillary connective tissue. The type I collagen was seeded on the collagen-coated wells. After stimulation with H$_2$O$_2$ or VEGF for 72 h (see below), HMVEC were fixed with 0.5 ml of glutaraldehyde-paraformaldehyde mixture (2.5%) and stained with modified May-Gruenwald’s solution (0.25%) (25). Tube formations on 3-D gel were visualized under a phase-contrast microscope (×200), and photomicrographs were documented by a digital camera (Nikon, Tokyo, Japan). Recorded images were analyzed by the NIH Image analyzer program (Scion, Frederick, MD) using a personal computer for the numbers and the total length of tube formation. Tube formation was defined as straight cellular extensions joining two cells’ masses at branch points. Eight random fields per well were used for angiogenesis assessment.

**Green Tea Catechins and d-α-Tocopherol Supplementation**

Green tea is the product of fresh tea leaves steamed or dried at elevated temperatures. The high temperatures result in the tea’s polyphenolic compounds, including flavonols, remaining unoxidized. Catechins and flavonols constitute 35–52% of green tea solid extract. We tested the five major green tea catechins, including catechin, epicatechin, epicatechin gallate, epigallocatechin (EGC), and EGCG. We supplemented cell culture medium with each green tea catechin up to 2 μM, the highest concentration that can be achieved in the plasma by drinking four cups of green tea (38,39). We also supplemented cells with 20–60 μM vitamin E. The normal range of vitamin E in human plasma is 20–30 μM (40). The high concentration of 60 μM in plasma can be achieved by oral supplementation of 200–800 IU of vitamin E per day (41).

Before the induction of angiogenesis by the oxidative stress of H$_2$O$_2$, HMVEC monolayers were incubated with different doses (0, 0.5, 1, and 2 μM) of green tea catechins for 24 h. Green tea catechins were incorporated into FBS (the final concentration in the medium was 2%) for 30 min and mixed with phenol-red free M199. After incubation, cells were washed with phosphate-buffered saline before the induction of angiogenesis with H$_2$O$_2$. d-α-Tocopherol at 20, 40, and 60 μM was used to compare green tea catechins’ effect on a known antioxidant. d-α-Tocopherol was incorporated into FBS for 30 min and mixed with phenol-red free M199. HMVEC monolayers were incubated with media containing different doses of d-α-tocopherol (0, 20, 40, and 60 μM) for 24 h. Before stimulation for angiogenesis, cells were washed with phosphate-buffered saline.
Experimental Design

Control HMVEC monolayers and cells supplemented with \(\alpha\)-tocopherol or green tea catechins were stimulated for angiogenesis with \(\text{H}_2\text{O}_2\) for 72 h. Earlier studies used very high, bolus, and unphysiological levels of \(\text{H}_2\text{O}_2\) (100–500 \(\mu\text{M}\)) that cannot be achieved in vivo (25). Therefore, to better simulate the in vivo condition in our in vitro model, we used a glucose-glucose oxidase (G/GO) system (G: 5 mM; GO: 0.25 mU/ml) to produce a constant pathophysiological level (\(\approx 30\) \(\mu\text{M}\)) of \(\text{H}_2\text{O}_2\) during the stimulation period. This level simulates the in vivo level of \(\text{H}_2\text{O}_2\) produced by inflammatory cells at the site of inflammation. The production of \(\text{H}_2\text{O}_2\) in culture media was measured by the fluorescence method of Ruch et al. (42). VEGF (50 ng/ml) was used as a positive control to induce angiogenesis.

To investigate the role of IL-8 on oxidative stress-induced angiogenesis, HMVEC monolayers and cells supplemented with \(\alpha\)-tocopherol or green tea catechins were stimulated with \(\text{H}_2\text{O}_2\) (\(\approx 30\) \(\mu\text{M}\)) for 20 h, and IL-8 released into the supernates was measured by enzyme-linked immunosorbent assay. Cellular protein was measured by the bicinchoninic acid Lowry protein assay (Pierce, Rockford, IL). Angiogenesis was also investigated when exogenous IL-8 (20 ng/ml) was added to the culture media for 72 h or when anti-IL-8 monoclonal antibody (1 \(\mu\text{g/ml}\)) was co-administered with an \(\text{H}_2\text{O}_2\)-generating system for 72 h.

Statistics

Each experiment was conducted in triplicate and repeated twice. Data were analyzed by unpaired Student’s \(t\)-test and expressed as means \(\pm\) SEM. Significant differences were determined at \(P < 0.05\).

Results

Effects of \(\text{H}_2\text{O}_2\) and IL-8 on Tube Formation

Exposure of HMVEC to constant low levels of \(\text{H}_2\text{O}_2\) (\(\approx 30\) \(\mu\text{M}\)) produced by the G/GO system for 72 h induced tube formation (Fig. 1A). The addition of IL-8 (20 ng/ml) to the culture medium also induced tube formation (Fig. 1A). The effect of \(\text{H}_2\text{O}_2\) or IL-8 on tube formation was comparable to that of VEGF (50 ng/ml), which was used as a positive control in this study (Fig. 1A). The addition of anti-IL-8 monoclonal antibody (1 \(\mu\text{g/ml}\)) to the medium inhibited \(\text{H}_2\text{O}_2\)-induced tube formation (Fig. 1B). These data indicate that IL-8 is an important mediator of low-level \(\text{H}_2\text{O}_2\)-induced tube formation in HMVEC.

Effect of Vitamin E on Tube Formation and IL-8 Production

After supplementation of HMVEC with increasing doses of \(\alpha\)-tocopherol (20, 40, and 60 \(\mu\text{M}\)) for 24 h, \(\text{H}_2\text{O}_2\)-induced tube formation was reduced and reached a statistically sig-

Figure 1. Tube formation induced by pathophysiological levels of \(\text{H}_2\text{O}_2\), interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF). A: confluent human microvascular endothelial cells (HMVEC) were stimulated by \(\text{H}_2\text{O}_2\) (~30 \(\mu\text{M}\)), IL-8 (20 ng/ml), or VEGF (50 ng/ml) for 72 h, and total length of tube formation was determined by image analyzer program. B: to study role of IL-8, anti-IL-8 antibody (anti-IL-8 Ab, 1 \(\mu\text{g/ml}\)) was coadministered with \(\text{H}_2\text{O}_2\) to confluent HMVEC for 72 h, and total length of tube formation was determined. Values are means \(\pm\) SEM of 8 randomly selected fields in each culture dish, each performed in triplicate and repeated twice. *, \(P < 0.05\) compared with control.
pressed tube formation, but the results were not significantly different from those observed with 0.5 μM EGC. Production of IL-8 by HMVEC was significantly reduced with EGC supplementation in a dose-dependent manner (Fig. 3B). Representative photomicrographs of tube formation in control and H2O2-treated HMVEC are shown in Fig. 4, A and B, respectively. Figure 4C represents H2O2-stimulated HMVEC supplemented with 2 μM EGC. Because the cell culture medium contained low levels of FBS, which contains residual amounts of growth factors, low levels of tube formation were also observed in control and EGC-supplemented cells (Fig. 4, A and C, respectively).

**Role of IL-8 in Oxidative Stress-Induced Tube Formation**

To demonstrate that EGC inhibits tube formation by reducing IL-8 production, IL-8 was administered with the H2O2-generating system in EGC-supplemented HMVEC. As shown in Fig. 5, addition of exogenous IL-8 reversed the inhibitory effect of EGC on oxidative stress-induced tube formation. This finding suggests that the inhibitory effect of EGC on oxidative stress-induced tube formation occurs via suppression of endogenous IL-8 production.

**Table 1. Inhibitory Effect of Green Tea Catechins on Oxidative Stress-Induced IL-8 Production**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>EC</th>
<th>ECG</th>
<th>EGC</th>
<th>EGCG</th>
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<tbody>
<tr>
<td>IL-8, pg/μg protein</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>2.10 ± 0.42</td>
<td>2.35 ± 0.36</td>
<td>2.11 ± 0.42</td>
<td>2.26 ± 0.31</td>
<td>2.35 ± 0.36</td>
</tr>
<tr>
<td>H2O2</td>
<td>3.71 ± 0.5*</td>
<td>4.62 ± 1.61*</td>
<td>4.36 ± 0.34*</td>
<td>3.43 ± 0.12*</td>
<td>4.62 ± 1.61*</td>
</tr>
<tr>
<td>Catechin (0.5 μM) + H2O2</td>
<td>3.58 ± 0.20</td>
<td>4.31 ± 0.33</td>
<td>4.48 ± 0.21</td>
<td>2.88 ± 0.31†</td>
<td>3.8 ± 0.86</td>
</tr>
<tr>
<td>Catechin (1 μM) + H2O2</td>
<td>2.87 ± 0.29†</td>
<td>2.37 ± 0.73†</td>
<td>2.91 ± 0.45†</td>
<td>1.88 ± 0.45†</td>
<td>2.95 ± 1.23†</td>
</tr>
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*a:* Values are means ± SE. Experiments were performed in triplicate and repeated twice. Confluent human microvascular endothelial cells were incubated with 0, 0.5, or 1 μM catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), or epigallocatechin gallate (EGCG) at 37°C for 24 h. Cells were then stimulated with H2O2 (~30 μM) for 20 h, and interleukin-8 (IL-8) was measured by enzyme-linked immunosorbent assay.

*b:* Statistical significance is as follows: *, *P* < 0.05 compared with control; †, *P* < 0.05 compared with H2O2.
Discussion

In this study, we used an in vitro model of angiogenesis to demonstrate that α-tocopherol and green tea catechins, particularly EGC, inhibit H$_2$O$_2$-induced tube formation by HMVEC via suppression of IL-8 production. These findings provide mechanistic insights into the effects that supplementation with vitamin E and green tea might have on cancer prevention through suppression of angiogenesis. Our findings on green tea catechins’ inhibition of tube formation by HMVEC in culture are in line with a reduced risk of cancer with drinking green tea in human and experimental studies (16,43,44), in particular, the recent report demonstrating the inhibition of angiogenesis by drinking green tea extract in the mouse corneal model of angiogenesis (24). Our observation on α-tocopherol’s inhibition of H$_2$O$_2$-induced tube formation by HMVEC on a 3-D gel is novel and suggests an additional mechanism by which this vitamin may reduce the risk of certain forms of cancer (17–21).

Green tea catechins and vitamin E, in addition to being capable of scavenging oxygen free radicals (31,45), might influence tumor development by modulating the production of mediators such as IL-8, as indicated by our data. IL-8 plays a role in the formation of new capillaries during tumor growth. Studies have indicated that IL-8 may be an important modulator of angiogenesis-related diseases, including cancer and arthritis (46–48). A high bolus dose of H$_2$O$_2$ has been reported to be angiogenic by increasing IL-8 production (25). Endogenous IL-8 has an autocrine or a paracrine effect on the proliferation and migration of endothelial cells and the development of new capillaries. IL-8 is produced by other cells as well, including lymphocytes, monocytes, and cancer cells (49,50). Indeed, expression of IL-8 by human melanoma cells correlates with their metastatic potential in vivo (47). Moreover, human recombinant IL-8 was shown to be potent angiogenic when implanted in rat corneas (46). Our data clearly demonstrate that IL-8 production by HMVEC was increased when cells were exposed to a low but constant level of H$_2$O$_2$ in culture and support the notion that IL-8 is an important factor in angiogenesis. The increase in IL-8 production by H$_2$O$_2$ in HMVEC in the present study was associated with an increase in tube formation. This was further supported by an increase in tube formation when recombinant IL-8 was added into the HMVEC culture without H$_2$O$_2$ stimulation (Fig. 1A). Furthermore, the addition of anti-IL-8 antibody to HMVEC culture, which was stimulated by H$_2$O$_2$ (Fig. 1B), inhibited tube formation. Thus our data clearly demonstrate that production of IL-8 is required for tube formation in this in vitro model of angiogenesis.

To best simulate the in vivo condition of angiogenesis, we selected several criteria for our in vitro system, including 1) the cell culture matrix we used was a preparation of 3-D gels with type I collagen, the predominant collagen in the pericapillary connective tissue (37); 2) for the induction of oxidative stress, in contrast to an earlier study where the bolus dose of H$_2$O$_2$ was used (25), we used the G/GO system to generate a constant level of H$_2$O$_2$; 3) we also used a pathophysiological level (≥ 30 μM), the level that would be
found during chronic inflammation (51), whereas the earlier study used very high levels (100–500 μM) of H2O2, a condition not representative of an in vivo situation for angiogenesis and tumor development (25); 4) we supplemented cells with media containing levels of green tea catechins comparable to those found in plasma after the consumption of four cups of green tea per day (38,39); 5) the cell media were supplemented with the levels of α-tocopherol comparable to plasma levels that are achievable after supplementation with vitamin E at 200–800 IU on a regular basis (40,41). Inclusion of these criteria provided a more realistic in vitro model to investigate the effects of green tea catechins and vitamin E on angiogenesis.

Our data provide the first direct evidence of an anti-angiogenic effect of vitamin E and green tea catechins via suppression of IL-8. Vitamin E reduced production of IL-8 by H2O2-stimulated HMVEC in a dose-response manner. This effect was observed when HMVEC were supplemented with 20–60 μM α-tocopherol in their culture media. Vitamin E suppression of tube formation was significant when cells were supplemented with 40 or 60 μM, the levels comparable to those found in plasma with vitamin E supplementation of 200–800 IU/day.

The observed inhibition of H2O2-induced IL-8 production and angiogenesis with vitamin E or EGC supplementation was not due to their direct antioxidant effect on H2O2. In a preliminary experiment, we measured the level of H2O2 production by the G/GO system in the supernatant of cells supplemented with vitamin E or EGC. Even though the level of H2O2 at the end of the incubation period was low (≤15 μM), we did not find a dose-dependent reduction of H2O2 by increasing vitamin E (from 20 to 60 μM) or EGC (from 0.5 to 2 μM) levels, whereas we found that IL-8 production and tube formation were reduced dose dependently by an increase in the level of these compounds in HMVEC (Figs. 2 and 3). In addition, we have shown that the inhibition of IL-8 production can be observed when human aortic endothelial cells are supplemented with vitamin E and stimulated with a proinflammatory cytokine such as IL-1, rather than H2O2 (36). Therefore, vitamin E and EGC inhibition of oxidative stress-induced tube formation is, in part, mediated through the suppression of IL-8 production, rather than a direct interaction of these antioxidants with H2O2 or with the enzyme system.

Thus vitamin E may inhibit tumor development not only by its antioxidant activity (which could prevent DNA damage) but also through the inhibition of angiogenesis via IL-8 suppression. Therefore, the results of this study support the epidemiological data indicating an association of a low risk of certain forms of cancer with a high intake of vitamin E.

In the present study, all the green tea catechins reduced IL-8 production by H2O2-stimulated HMVEC. However, EGC was the most effective catechin tested. At 0.5 μM, EGC reduced H2O2-induced angiogenesis by 67%. This was comparable to the effect of vitamin E (74% reduction) on tube formation when cells were supplemented with 40 μM vitamin E. Thus, on a molar basis, EGC appears to be more effective than vitamin E. EGC at 2 μM totally abolished H2O2-induced tube formation (Fig. 3A). The addition of exogenous recombinant IL-8 to the stimulated cells fully abrogated the effect of EGC, further demonstrating that inhibition of IL-8 is one mechanism by which EGC inhibits H2O2-induced tube formation (Fig. 5). These data further support the notion that EGC’s inhibition of tube formation is not mediated solely through its antioxidant effect and elimination of H2O2, but also by suppression of IL-8 production.

Our data provide mechanistic insights for the effect of green tea and vitamin E on the reduction of certain forms of cancer. Although we have not yet investigated the effects of a mixture of green tea catechins or a mixture of green tea catechins with vitamin E, evidence from this study indicates that a dietary regimen containing green tea catechins and supplemented with vitamin E might be effective in suppressing IL-8 production and angiogenesis. Vitamin E and green tea are dietary components with numerous health benefits and can be easily incorporated into a daily regimen. Vitamin E at 40 μm in plasma is attainable with supplemental intake of vitamin E (41,52). Furthermore, 0.5–1 μm EGC in plasma can be achieved by drinking four cups of green tea per day (38,39).

Acknowledgments and Notes

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