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Neutrophil Restraint by Green Tea: Inhibition of Inflammation, Associated Angiogenesis, and Pulmonary Fibrosis[1]

Massimo Donà,* Isabella Dell'Aica,* Fiorella Calabrese,[†] Roberto Benelli,[‡] Monica Morini,[‡] Adriana Albini,[‡] and Spiridione Garbisa[2]*

Neutrophils play an essential role in host defense and inflammation, but the latter may trigger and sustain the pathogenesis of a range of acute and chronic diseases. Green tea has been claimed to exert anti-inflammatory properties through unknown molecular mechanisms. We have previously shown that the most abundant catechin of green tea, (−)epigallocatechin-3-gallate (EGCG), strongly inhibits neutrophil elastase. Here we show that 1) micromolar EGCG represses reactive oxygen species activity and inhibits apoptosis of activated neutrophils, and 2) dramatically inhibits chemokine-induced neutrophil chemotaxis in vitro; 3) both oral EGCG and green tea extract block neutrophil-mediated angiogenesis in vivo in an inflammatory angiogenesis model, and 4) oral administration of green tea extract enhances resolution in a pulmonary inflammation model, significantly reducing consequent fibrosis. These results provide molecular and cellular insights into the claimed beneficial properties of green tea and indicate that EGCG is a potent anti-inflammatory compound with therapeutic potential.   *The Journal of Immunology,* 2003, 170: 4335–4341.

Neutrophils are our most abundant bodyguard cells; they spend only a few hours in the bloodstream before being recruited toward inflammatory districts, where they survive for 1–2 days before undergoing spontaneous apoptosis; exposure of surface factors (e.g., phosphatidylserine) eventually leads to their engulfment by macrophages (1). In the tissue, they act as phagocytic cells and also release a variety of reactive oxygen species (ROS)[3] and proteases. These mechanisms confer to neutrophils a key role in host defense against bacteria and parasites, but paradoxically neutrophil-led inflammation may also be central to the pathogenesis of a range of chronic syndromes (2), ranging from bronchitis and asthma through emphysema, fibrosis, and tumor progression (3).

One of the proteolytic activities used by neutrophils at the site of inflammation is neutrophil elastase (NE), which has the potential to preferentially disrupt the elastic network, although natural substrates also include other extracellular matrix and circulating molecules (4). Neutrophils also require NE to kill some phagocytosed Gram-negative bacilli (5), and this activity is mediated through activation of proteases by K^+ flux into the phagocytic vacuoles caused by the $O_2^{\cdot-}$-generating system (6). However, ROS and myeloperoxidase activity are also independently important in the killing process (7) as well as in neutrophil apoptosis (2, 8), where they play an important role in the resolution of inflammation (9).

Evidence for a role for oxidative stress in the induction of apoptosis comes mainly from the numerous observations that antioxidants inhibit/delay the onset of apoptotic cell death in different systems (10, 11).

A number of cytokines (IL-1β (12), IL-2 (13), TNF-α (14), and GM-CSF (15)) have been shown to modulate neutrophil functions and death, although some reports are contradictory (12, 16). Flavonoids have been shown to scavenge NO radicals (17) and to protect U937 cells against NO-induced cell cycle arrest and apoptosis (18).

Green tea is rich in flavonoids and indeed epidemiological in vitro, and animal-model studies have associated green tea consumption with health benefits, including decreased risk of inflammation (19, 20). These effects have largely been attributed to the most prevalent polyphenol contained in green tea, the catechin or flavanol (−)epigallocatechin-3-gallate (EGCG). This phytofactor has been shown to influence numerous cellular mechanisms, including a number of proteolytic activities instrumental in alteration of extracellular matrix; it represses the activity of some gelatinases (matrix metalloproteinases MMP-2 and MMP-9) frequently overexpressed in cancer (21–24), angiogenesis (25), and inflammation (26). In addition, it inhibits metallo-elastase and serine-elastase, which are secreted by macrophages (MMP-12) (24) and neutrophils (NE) (27), respectively. EGCG also reduces neutrophil transmigration through monolayers of endothelial cells (28) and lowers the level of several markers of oxidative stress (29, 30).

Considering both the essential role of neutrophils in host defense and the role of inflammation in the pathogenesis of a range of acute and chronic diseases, we investigated whether and to what extent EGCG could modulate neutrophil 1) ROS activity, apoptosis, and 2) migration in vitro. Further, 3) we investigated in vivo whether oral green tea extract or EGCG solution could favorably affect the resolution of inflammation induced in s.c. and lung models, which by default resolves in angiogenesis (31) and fibrosis (32) respectively.

Materials and Methods

Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), except rabbit polyclonal anti-myeloperoxidase Ab (code A0398; DAKO, Glostrup, Denmark), and EGCG was code E 4143. Green tea decaffeinated

extract (GTE) was supplied lyophilized by SOFAR (Trezzano Rosa, Milan, Italy), and contained 59% EGCG, 86% total catechins, and 0.5% caffeine (HPLC titration by SOFAR).

Neutrophil isolation and treatment

Neutrophils were isolated from buffy coats of healthy donors following a single-step separation procedure, as previously described (33). The cells, in DMEM without phenol red, were seeded and incubated at 37°C in 5% CO_2 in air. After 15 min, 5 μg/ml cytochalasin B was added, followed after 5 min by 100 nM fMLP (for ROS analysis, this treatment was performed before cell seeding, as described below). EGCG from a freshly prepared 10× solution in deionized water was added to the appropriate final concentration, and the incubation of neutrophils was continued for the periods stated below.

ROS determination

ROS activity was detected using carboxy-H_2-dichlorodihydro fluorescein diacetate (Molecular Probes, Eugene OR). The neutrophils were preincubated 45 min at 37°C with carboxy-H_2-dichlorodihydro fluorescein diacetate (50 μM); following rinsing and activation, 10^5 cells were seeded on 96-well plates and treated with EGCG (0.4–300 μM). Cell accumulation of 2′,7′-dichlorofluorescein was measured (34) at time intervals up to 24 h by the increase in fluorescence at 530 nm when the sample was excited at 485 nm.

Caspase-3 assay

Caspase activity in neutrophils was detected using the colorimetric CaspACE assay system (Promega, Madison WI), following the manufacturer's instructions. The cell lysate was centrifuged for 20 min at 12,000 × g and 4°C, the supernatant fraction was collected, mixed with caspase assay buffer and DEVD-p-nitroaniline substrate, and incubated for 6 h at 37°C; the absorbance was measured at 405 nm.

Neutrophil death detection

Neutrophil death was quantitatively determined using a Cell Death Detection ELISA Plus kit (Roche, Mannheim, Germany) following the manufacturer's protocol. The procedure, using a photometric enzyme immunoassay, determines cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after cell death. After 4 h at 0.4–100 μM EGCG, a loss of membrane integrity was revealed by propidium iodide permeability, while for detection of apoptosis, the In Situ Cell Death Detection Kit, Fluorescein (TUNEL; Roche) was used. Fluorescein labels were detected by fluorescence and confocal microscopy.

Neutrophil migration assay

The effect of EGCG on neutrophil chemotaxis was tested as previously described (35) in 48-microwell chemotaxis chambers (Costar-Nucleopore, Milan, Italy), using 5-μm pore size polyvinylpirrolidone-free polycarbonate filters. The lower compartment of each chamber was filled with 28 μl of IL-8; serum-free RPMI medium with 0.1% BSA was used as a control for random unstimulated migration. Neutrophils (5 × 10^6 cell/ml in 50 μl of serum-free RPMI medium), not treated with fMLP and cytocalasin B, were preincubated with EGCG for 30 min at the indicated concentrations and then added to the wells in the upper chamber; in additional control incubations, EGCG was present in the lower chamber only. Chambers were incubated for 45 min at 37°C in a 5% CO_2 humidified atmosphere. The filters were then removed and treated with 100% ethanol and toluidine blue. Nonmigrated cells were removed from the upper surface of the filter using filter paper, and migration was measured by densitometric analysis.

Macrophage inflammatory protein-2 (MIP-2) and LPS-induced s.c. angiogenesis

The in vivo Matrigel model of inflammatory angiogenesis described by Benelli et al. (31) was used. MIP-2 (50 ng/ml) or LPS (100 ng/ml) was added to liquid Matrigel containing heparin at 4°C to a final volume of 0.6 ml (heparin is always added to Matrigel samples to avoid cytokine/growth factor trapping by proteoglycans). The Matrigel suspension was slowly injected s.c. into the flanks of C57 Black mice using a cold syringe, and in vivo the gel was rapidly polymerized to form solid implants. GTE (6 mg/ml, equivalent to 3.5 mg/ml EGCG, 4 times the average reported in common green tea beverage (36)) or EGCG (0.5 mg/ml) solutions were given to mice instead of drinking water. Groups of six mice were given one of the beverages starting 2 days before Matrigel injection, while one group drank only plain water. Control animals received implants of Matrigel containing heparin only. Four days after injection, the gels were collected and

weighed. Some samples were subjected to analysis of hemoglobin content as previously described (37). Other samples were paraffin-embedded and stained with H&E for histological analysis or with a rabbit polyclonal anti-myeloperoxidase Ab for histochemical identification of neutrophils.

FITC-induced pulmonary fibrosis

Pulmonary fibrosis in mice was induced by intratracheal instillation of FITC as previously described (32). Here, 21 mg of FITC was dissolved in
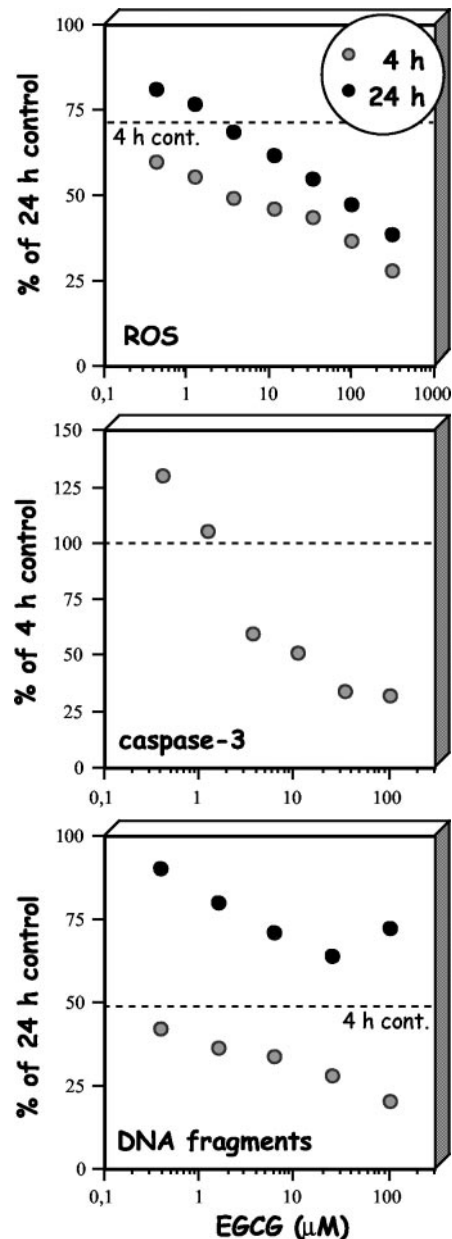
FIGURE 1. Incubation of neutrophils with micromolar concentrations of EGCG down-modulates ROS activity, caspase-3 expression, and DNA fragmentation. Each point in the ROS graph is the mean of eight determinations, with SD <5% (representative result of duplicate experiments; two donors). All EGCG-treated samples showed significantly less ROS activity ($p < 0.001$, by t test). The other two graphs show representative results of three experiments (donors), and each point is the mean of three determinations, with SD <10%. Samples treated with 3.7 μM EGCG or higher concentrations all showed significantly less caspase activity ($p < 0.005$, by t test), those treated with 1.56 μM EGCG or higher concentrations at 4 h or 6.25 μM EGCG or higher concentrations at 24 h showed significantly less DNA fragmentation ($p < 0.05$, by t test). The dashed line indicates the control level at 4 h.

FIGURE 2. DNA fragmentation is reduced in neutrophils incubated with EGCG. Hoechst staining reveals neutrophil nuclei (*A1* and *B1*), and TUNEL stains nuclei containing fragmented DNA (*A2* and *B2*). Magnification, ×100. Higher magnification (*C1* and *C2*) shows an example of an apoptotic neutrophil (TUNEL). An example of triplicate experiments (three donors) is shown.
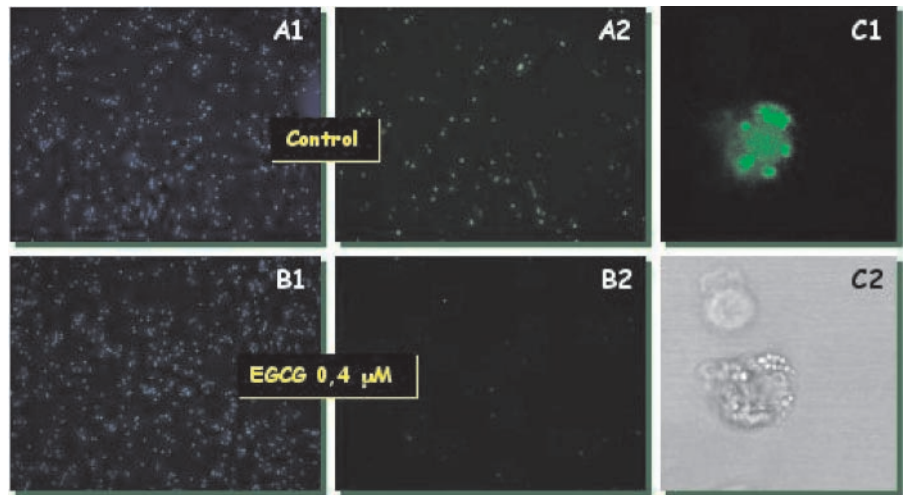
10 ml of sterile PBS, vortexed extensively, sonicated for 30 s, and vortexed again before each 50-μl aliquot was used. CD1 mice were anesthetized, the trachea was exposed and entered with a 29-gauge needle, intratracheal instillation was performed slowly, and the skin wound was sealed and treated with betadine solution. One group of mice was given GTE (as described above) starting 7 days before instillation; one group drank only plain water.

Preparation of the pulmonary tissues

The animals were anesthetized, heparinized, and exsanguinated via the femoral artery. The heart and lungs were removed en bloc, and the lungs were dissected away from the external vasculature and bronchi. The lungs were sectioned parasaggitally, superior to inferior, and specimens were either frozen at −80°C for biochemical analysis or fixed in buffered 4% paraformaldehyde for morphological studies.

Determination of hydroxyproline

The hydroxyproline content of mouse lung was determined by standard methods (32) with slight modifications. After rinsing with PBS, the upper left lung lobe was defatted, dried, weighed, and hydrolyzed 22 h at 110°C in 6 N HCl. Aliquots were then assayed by adding chloramine T solution for 20 min, 3.15 M perchloric acid for 5 min, and Erlich's reagent at 60°C for 20 min. Absorbance was measured at 561 nm, and the amount of hydroxyproline was determined against a standard curve.

Lung histology

Fixed tissue were embedded in paraffin using standard histological techniques, sectioned at 5 μm, and stained with H&E and Heidenhain Tri-Chrome. Fibrosis was measured in perivascular, peribronchial, and interstitial areas by computerized morphometry using an image analyzer system (TV camera 3CCD JVC; Olympus microscope; personal computer with Image-Pro Plus software version 4.1, Media Cybernetics, Silver Spring MD). The analysis was conducted on 30- to 50-μm^2 areas of lung parenchyma and 200- to 300-μm^2 areas of perivascular and conducting airway structures in fibrotic areas and in anatomically matched controls. The values were expressed as a percentage of the whole analyzed area. Slides from the two groups of mice were randomized, coded, and examined without knowledge of the treatment.

Statistics

Data are expressed as the means of multiple determinations (eight for ROS assay (two donors separately); six for migration (three donors separately) and angiogenesis (six animals per group) assays; three for hydroxyproline (data averaged from four lung lobes per group of animals), caspase-3, and DNA fragmentation assays (three donors separately); eight fields per six sections (data averaged from four animals per group) for morphometric analysis of lung tissue). Comparisons were conducted by one-way ANOVA, with significance set at $p < 0.05$.

Results

Biochemical assays

Following isolation and activation, neutrophils were analyzed for three parameters describing oxidant activity and apoptosis: ROS,

caspase-3, and DNA fragmentation. In the presence of increasing concentrations of EGCG, the level of ROS measurable in neutrophils declined in a dose-dependent manner; this effect was already evident at 0.4 μM after 1 h (not shown), but progressively increased from 4 or 24 h and reached a reduction of ~70% with respect to controls at 300 μM (Fig. 1).

Of the two measured apoptosis parameters, caspase-3 levels, measured after 4 h of EGCG, fell progressively by 40% at 3 μM and 70% at 30 μM EGCG (Fig. 1). This effect was paralleled by a similar dose-response reduction of the DNA fragmentation after 4 and 24 h, up to 20 μM EGCG. After 24 h, the fragmentation was always (in triplicate experiments) higher at 100 μM than at 20 μM EGCG (Fig. 1).

Fluorescence microscopy

Inhibition of DNA fragmentation was detected using a TUNEL assay, which showed a marked reduction in the number of fluorescence-positive cell nuclei (DNA fragments) even after 4 h at 0.4 μM EGCG (Fig. 2). Cell death followed both necrosis and the apoptosis pathway (the former <1/10th in different experiments); different phases of apoptosis were morphologically revealed by either chromatin condensation (not shown) or chromatin partition into multiple bodies (Fig. 2), and loss of membrane integrity was revealed by propidium iodide permeability (not shown). In both cases, EGCG showed a dose-response protective effect that paralleled the results obtained with the DNA fragmentation assays.

In vitro migration assay

The effect of EGCG on human neutrophil chemotaxis in response to IL-8 was tested in Boyden chambers, an in vitro assay that mimics their recruitment. Neutrophils showed a 2.3-fold increase in migration to IL-8 over unstimulated controls. When the cells were briefly pretreated with EGCG, and EGCG was included in the migration chamber, neutrophil migration was inhibited in a dose-dependent manner, with an IC$_{50}$ <1 μM (Fig. 3). At 3 μM, EGCG reduced PMN migration almost to background levels. In this test no matrix barrier was used, so EGCG appears to directly influence neutrophil migration. Controls with EGCG in the lower chamber only excluded that the EGCG preparation itself has chemotactic activity that might be complicating the results (not shown).

Induced s.c. angiogenesis

Neutrophil activation in response to chemokines or inflammatory stimuli can culminate in neutrophil-dependent angiogenesis (31).
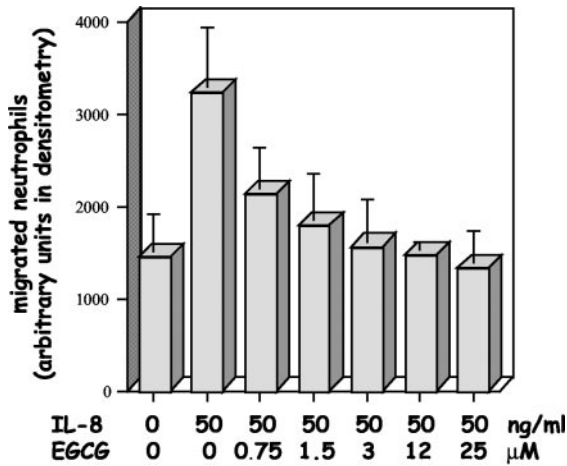
FIGURE 3. Migration of neutrophils in vitro (Boyden chamber) is inhibited by EGCG. Neutrophil chemotaxis toward IL-8 (in the lower chamber) is restrained in a dose-response manner by the catechin present in the upper chamber, with an $IC_{50} < 1\ \mu M$. An example of triplicate experiments (three different donors) is shown; each column represents the mean of six determinations.

We examined the ability of GTE and EGCG to inhibit neutrophil-dependent angiogenesis in vivo. Oral administration of GTE (containing 3.5 mg/ml EGCG) or EGCG (pure, at 0.5 mg/ml) produced a strong inhibition of the angiogenic reaction and inflammatory infiltration caused by the injection with Matrigel sponges containing either the angiogenic chemokine MIP-2 or the inflammatory stimulus LPS. The mean hemoglobin level was significantly higher in gels containing MIP-2 or LPS alone vs pellets in animals treated with GTE or EGCG (Fig. 4, *A* and *B*). Control implants showed no cell infiltration and displayed no angiogenic response (not shown).

Immunohistochemical examination of the implants showed, by myeloperoxidase staining, massive infiltration of neutrophils in response to these stimuli (Fig. 4). The presence of these leukocytes was accompanied by the appearance of primitive vessels lined by endothelial cells. The invasion of degranulating neutrophils often appeared to precede and lead monocyte and endothelial cell migration in response to the angiogenic stimuli (31). Treatment with GTE or EGCG resulted in an almost complete block of cell recruitment and vessel formation (Fig. 4).

Induced pulmonary fibrosis

Intratracheal instillation of FITC solution in mice produced, within 4 wk, mixtures of both acute and chronic inflammatory cells in fibrotic areas, as previously reported (32). We therefore examined the potential protective effect of a GTE beverage on FITC-induced pathology. Mouse weight was checked twice per week, and no significant differences were registered compared with controls; no other side effects were noted throughout the experiment.
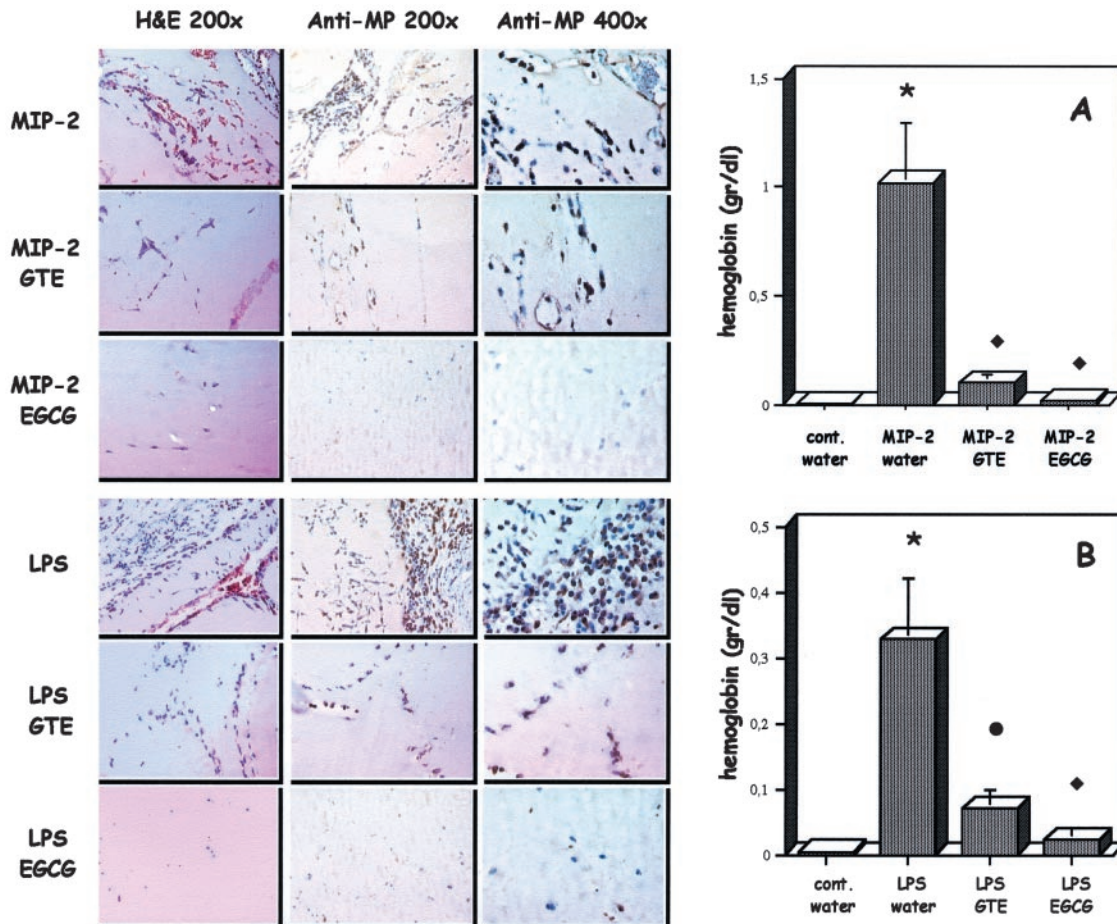
FIGURE 4. MIP-2- and LPS-induced neutrophil recruitment and eventual angiogenesis are reduced in mice drinking GTE (6 mg/ml) or EGCG (0.5 mg/ml) compared with water-drinking mice. The animals received the treatments 2 days before s.c. injection of a Matrigel sponge containing either MIP-2 or LPS. Four days after injection, the sponges were removed, and neutrophil infiltration (micrographs) and angiogenesis were analyzed; the latter was quantitated by measuring the hemoglobin content (*A* and *B*); $p < 0.01$ for ♦ vs *, $p < 0.05$ for ● vs *. Staining was with H&E and anti-myeloperoxidase (MP) Ab.
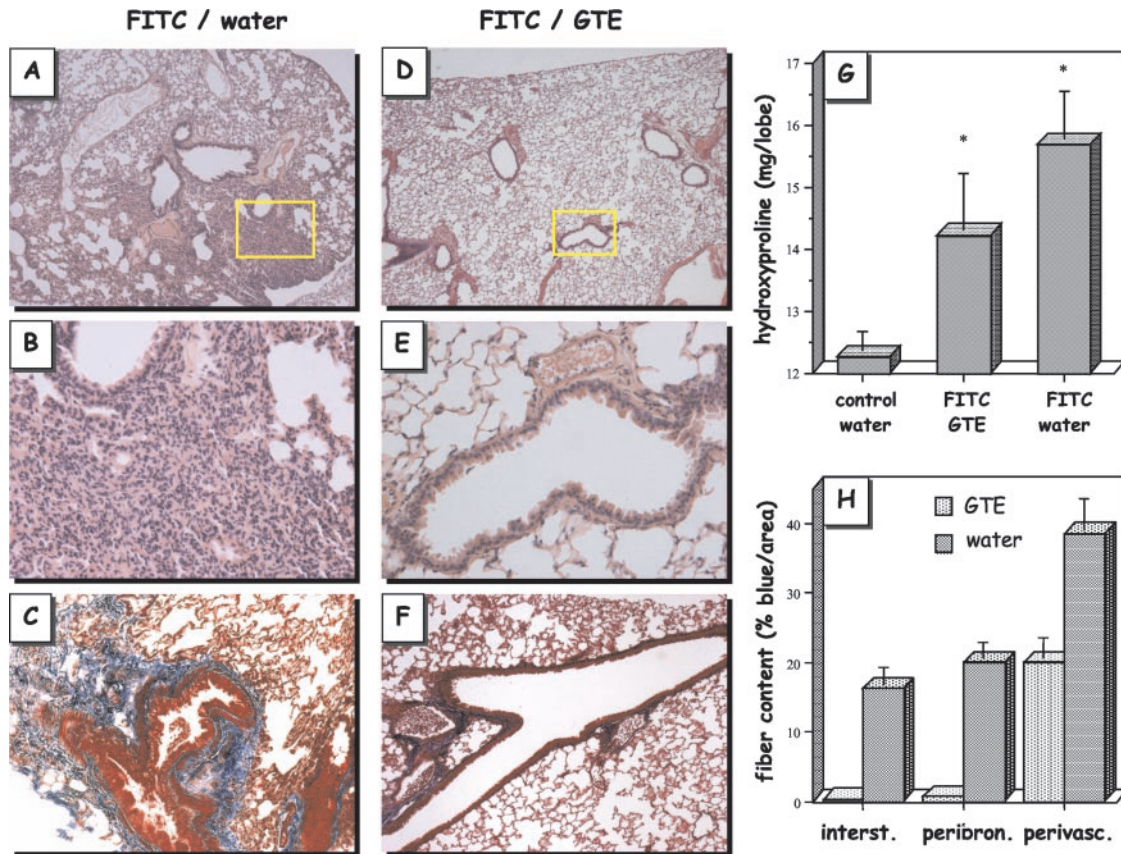
FIGURE 5. Histology of lungs from mice given intratracheal instillation of FITC and drinking GTE (6 mg/ml) or plain water. Foci of inflammatory infiltration (*A* and *B*; H&E; magnification, ×30 and ×165) and peribronchial and interstitial fibrosis (*C*; Heidenhain Tri-Chrome; magnification, ×203) are clearly visible in lungs of FITC-treated mice, while reduced or normal parenchyma is seen in FITC/GTE-treated animals after 4 wk (*D* and *E*: H&E; magnification, ×22 and 330; *G*: Heidenhain Tri-Chrome; magnification, ×101). *G*, Compared with control animals, in water-drinking mice the hydroxyproline content in the upper left lung lobe was increased 28% following FITC treatment, but only 16% in those drinking GTE (∗, $p = 0.010$). Each column represents three determinations per sample averaged for four animals. *H*, Image analysis quantitation of the blue-stained fibers confirms the reduction of FITC-induced fibrosis (interstitial, $p = 0.012$; peribronchial, $p = 0.0078$; perivascular, $p = 0.0067$) in GTE-drinking mice. Each column represents the mean of 192 fields (eight fields per six sections per four animals).

The lungs from the animals treated with FITC/GTE showed normal architecture with intact parenchyma, vessels, and airways (Fig. 5, *D–F*). No inflammation or fibrosis was observed in this group. The lungs of all the animals treated with FITC/water showed multiple scattered foci of fibrosis and inflammation (Fig. 5, *A–C*): in these areas, the architecture of the lung was distorted, and the air spaces were separated or compressed by bands of connective tissue or inflammatory cell infiltration.

Morphometry results revealed diffuse areas of fibrosis in the lungs of FITC-treated mice, whose extension was severalfold that measured in the interstitial and peribronchial zones and doubled in the perivascular districts of the FITC/GTE-treated animals (Fig. 5*H*). To quantitatively determine the extent of fibrosis, hydroxyproline content was measured as a surrogate for lung collagen deposition. Four weeks after FITC instillation, the increase in lung hydroxyproline in GTE-drinking mice was 33% lower ($p = 0.01$) than that in animals drinking plain water (Fig. 5*G*).

Discussion

This study demonstrates that neutrophils in vitro readily respond to micromolar concentrations of EGCG, the major flavanol in green tea, by down-modulating ROS activity, caspase-3 activity, DNA fragmentation, as well as migration toward a chemotactic stimulus. Furthermore, we showed that consumption of GTE with 4 times the amount of EGCG as that in the regular beverage (36) inhibits in vivo neutrophil recruitment and inflammation-driven angiogenesis as well as pulmonary fibrosis. Similar results were also obtained with animals given water containing EGCG at a concentration two-thirds that in normal green tea infusion.

In vitro, the effect of the catechin was already measurable after 4 h of incubation at concentrations close to those reported in the blood of moderate green tea drinkers (1–10 μM) (36); it persisted and increased after 24 h and was dose dependent. Only at concentrations ≥100 μM, which are essentially not obtainable in humans, does EGCG become cytotoxic. At this concentration, caspase-3 activity and DNA fragmentation rise, and the neutrophils die as previously described for other cell types (22).

Numerous observations report that antioxidants inhibit or delay the onset of apoptotic cell death (10, 11), but other studies show that oxidants are also able to inhibit apoptosis under certain conditions (38–40). Recently, two different modes of cell death have been specifically proposed for neutrophils: 1) a constitutive pathway involved in the turnover of senescent neutrophils (the majority), which follows the characteristic caspase-dependent pathway; and 2) a pathway restricted to the small number of neutrophils involved in pathogen-induced responses, which requires an alternative oxidant-dependent pathway, since ROS production may prevent caspases from functioning (8). In our model system the antioxidant property of EGCG (17) and the parallel down-modulation of ROS and caspase-3 activity registered in cytochalasin

B/fMLP-activated neutrophils fit with the former reports (delay of apoptosis) and confirm that the first hypothesized mode of cell death was prevalent.

Persistent neutrophil accumulation in any given tissue could nevertheless be the cause of persistent injury of normal architecture and functions, mediated by the local release of proteases overwhelming local antiprotease defenses, and could eventually lead to abnormal repair. However, previous studies have shown that EGCG reduces neutrophil transmigration through monolayers of endothelial cells (28), and that neutrophil elastase, a potent proteolytic molecule, is inhibited by EGCG at concentrations easily obtainable in the blood of moderate green tea drinkers (27). Furthermore, it has recently been reported that UVB-induced inflammatory responses in human or mouse skin are reduced by topical application of EGCG (20, 29, 30).

In these studies EGCG reduced the levels of several markers of oxidative stress, and UV-induced infiltration of inflammatory leukocytes, particularly monocytes/macrophages and neutrophils, was inhibited by EGCG pretreatment. However, whether the reduced infiltration of leukocytes was the cause or the effect of the ROS decrease observed with EGCG treatment was not determined. We have now addressed this question.

Since neutrophil recruitment can be largely mimicked in vitro by testing the ability of these cells to migrate, the effect of EGCG on the chemotaxis of purified neutrophils in response to IL-8 was tested in Boyden chambers. The chemokine IL-8 is one of the most powerful chemoattractants for neutrophils, able to induce massive neutrophil recruitment without causing complete activation of these phagocytes leading to ROS production (41). In the presence of the chemokine, neutrophils showed a 2.3-fold increase in migration compared with unstimulated controls. When the cells were briefly pretreated with EGCG, and EGCG was maintained in the migration chamber, the migration of phagocytes was inhibited in a dose-dependent manner. At 3 μM, EGCG was able to reduce neutrophil migration almost to background levels. In this test no extracellular matrix barrier was used, so EGCG appears to directly influence neutrophil migration, also in the absence of complete activation and ROS production.

These in vitro results suggest that EGCG, by independently restraining neutrophil recruitment as well as ROS activity, apoptotic pathway activation, and eventual cell death, in addition to neutrophil elastase (27), has the effect of attenuating the molecular responses of these potentially deleterious cells. This down-modulation may in vivo facilitate the safe clearing of neutrophils from an inflammatory site by macrophages (1), whose complete differentiation following extravasation takes a few days, with minimal damage to surrounding tissues (20); this would be advantageous when neutrophil recruitment and activity could trigger and sustain chronic pathological responses.

To verify this possibility, we have chosen two in vivo experimental model systems in which neutrophil recruitment eventually resolves in measurable angiogenesis and pulmonary fibrosis, respectively.

Angiogenesis

Inhibition of vascular endothelial growth factor-induced angiogenesis has been reported (25) in green tea-drinking mice, a model system that is essentially inflammation independent. The present approach made use of a recently optimized model (31) in which the inflammatory infiltrate is responsible for induction of the angiogenic process, in that neutrophil depletion abrogated the angiogenic response induced by CXCR2 ligands. Using chemokines or bacterial inflammatory stimuli, we were able to induce a rapid and intense infiltration of neutrophils into Matrigel plugs in mice, ac-

companied by angiogenesis. When the animals were given GTE or EGCG beverage (with EGCG concentrations, respectively, equivalent to 4× and 0.66× that in normal green tea infusion), neutrophil recruitment was strongly inhibited. These data are consistent with the chemotaxis results. The inhibitory effect of GTE and EGCG on neutrophil migration, in turn, apparently blocks the recruitment of vascular cells in vivo, as angiogenesis was weak or absent in GTE- or EGCG-treated animals. Although a direct effect of EGCG on endothelial cells (42) cannot be excluded, the blocking of neutrophil recruitment directly correlates with the inhibition of the angiogenic response, as observed in our previous report (31). These data indicate that GTE and EGCG should be considered suitable drugs for inhibiting inflammatory angiogenesis.

Pulmonary fibrosis

Chronic inflammation of the alveolar space is thought to mediate the development of pulmonary fibrosis, a disorder with an aggressive course (5-year survival of <50%) that is characterized by fibroblast proliferation and extracellular matrix remodeling, resulting in irreversible distortion of the lung's architecture (43). Intratracheal FITC challenge is a model of acute lymphocyte-independent lung injury resulting in areas of patchy and chronic inflammation, with production of cytokines, chemokines, and growth factors that mediate the eventual subpleural, peribronchiolar, and perivascular deposition of extracellular matrix and scar tissue formation that are characteristic of pulmonary fibrosis (32). Protection from FITC-induced pulmonary fibrosis in chemoattractant chemokine receptor knockout (CCR2$^{-/-}$) mice, in comparison with wild type, was shown to be independent from differential recruitment of inflammatory cells, and no statistical differences were registered in the neutrophil populations (44). Nevertheless, the extravasated neutrophil total number increased shortly after FITC injury (24 h) and remained elevated up to conclusion of the experiment (4 wk).

Here, histological staining and automated image analysis clearly show that in animals given GTE in the drinking water starting 1 wk before FITC instillation, both the inflammatory cell infiltration and the patchy fibrosis were clearly reduced. Interstitial and peribronchial fibrosis was reduced by >99%, and perivascular fibrosis was reduced by ~50%. This corresponds to a greater reduction of fibrosis in those areas (peribronchial and interstitial) that usually represent the setting mainly involved in postinflammatory pulmonary fibrosis. Quantitative determination of hydroxyproline (a marker of collagenous proteins) confirmed the reduction of inflammation-triggered extracellular matrix collagen fiber deposition; the less pronounced result (−33%) may in part originate from the increased lung mass due to the inflammatory infiltrate, which lowers the estimated collagen relative content.

A contributory factor to the antifibrotic effect might well be the inhibition exerted by EGCG on gelatinases involved in the damage to lung extracellular matrix, MMP-2 and MMP-9 (21–24, 26, 45), whose level significantly increases in induced pulmonary fibrosis concomitantly with collagen deposition (43). The multiple inhibition exerted by EGCG (neutrophil migration, ROS, apoptosis, elastase, activation of gelatinases, gelatinase activity, and angiogenesis) may also eventually lower the high incidence of lung cancer in patients with pulmonary fibrosis (46, 47), but we are not aware of specific epidemiological studies about this topic.

Conclusions

Whether both the antiangiogenic and antifibrosis effects are mainly the result of the restraint in the neutrophil default activity and turnover (ROS activity, apoptosis), their recruitment, or both and to what extent modulation of other cells by EGCG (e.g. macrophages (17) and endothelial cells (25, 42)) may contribute to the in

vivo effects remain to be determined. Certainly, EGCG and green tea are potent inhibitors of neutrophil activities in vitro and in vivo, and are orally available pharmacological agents that may be effective in preventive treatment of individuals exposed to risk of inflammation in general and pulmonary fibrosis and tumor neoangiogenesis in particular.

Acknowledgments

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