

Mechanisms of Inhibition of Tumor Angiogenesis and Vascular Tumor Growth by Epigallocatechin-3-Gallate

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ABSTRACT

Purpose: Green tea consumption has been linked to a reduced occurrence of some tumor types. Current data indicate that the principal mediator of this chemopreventive effect is epigallocatechin-3-gallate (EGCG), the most abundant polyphenol found in dried tea leaves. Here, we examined the effects of this compound on the two key cell populations typically involved in tumor growth: tumor cells and endothelial cells.

Experimental Design: The effects of green tea and EGCG were tested in a highly vascular Kaposi's sarcoma (KS) tumor model and on endothelial cells in a panel of *in vivo* and *in vitro* assays.

Results: EGCG inhibited KS-IMM cell growth and endothelial cell growth, chemotaxis, and invasion over a range of doses; high concentrations also induced tumor cell apoptosis. EGCG inhibited the metalloprotease-mediated gelatinolytic activity produced by endothelial cell supernatants and the formation of new capillary-like structures *in vitro*. Green tea or purified EGCG when administered to mice in the drinking water inhibited angiogenesis *in vivo* in the Matrigel sponge model and restrained KS tumor growth. Histological analysis of the tumors were consistent with an anti-angiogenic activity of EGCG and green tea.

Conclusions: These data suggest that the green tea gallate or its derivatives may find use in the prevention and treatment of vascular tumors in a chemoprevention or adjuvant setting.

INTRODUCTION

Tea is the most popular beverage in the world, next to water; inhabitants of oriental countries regularly consume green tea, whereas those in Europe and the United States preferentially use the fermented form of tea leaves (black tea). Epidemiological studies have suggested that the low occurrence of some tumors in Asian countries could be linked to regular drinking of green tea infusions, which conserve most of the crude content of the tea plant. In particular, in areas of green tea production, a significantly lower death rate for all types of cancers, especially for gastrointestinal tumors (stomach, esophagus, and liver cancer), has been reported (1).

Green tea extract derives from dried leaves of the tea plant (*Camelia sinensis*). The chemical composition of green tea extract is similar to that extracted from fresh leaves; in contrast, in manufacturing black tea, the flavonols undergo the fermentation process, resulting in oxidation of simple polyphenols to a more complex, condensed polyphenol (2). The polyphenols present in green tea are flavonols, commonly known as catechins; desiccated tea leaves contain high levels of these polyphenols (30% of the total weight of tea infusion; Ref. 1), which show diverse chemical and biological activities. These include an antioxidant function (2), carcinogen metabolism modulation (3, 4), inhibition of tumor growth (5), cell proliferation and cell cycle arrest (6, 7), induction of apoptosis (8, 9), inhibition of invasion and metastasis (10–12), and inhibition of angiogenesis (13). Most of the properties of green tea have been ascribed to the most abundant polyphenol, epigallocatechin-3-gallate (EGCG), which represents the 67% of the total polyphenols in tea extract.

The antioxidant activity of EGCG may represent only a marginal role in the control of tumor progression by this molecule. Plasma levels of EGCG reach 0.1–5 μM after moderate green tea consumption (14), and recent studies suggest that consumption of higher levels of EGCG does not give substantially higher plasma concentrations, reaching a C_{max} in the plasma of 1 μM (15). It is likely that the antioxidant activity would be insufficient to result in tumor suppression. Several studies indicate that *in vitro* growth factor receptor signaling (1, 16) and cell cycling (6, 17) can be inhibited by EGCG, blocking tumor cell proliferation. EGCG has shown anti-invasive properties as a potent inhibitor of the gelatinases [matrix metalloprotease (MMP)-2 and MMP-9], enzymes associated with invasive potential that are frequently overexpressed in cancer (18–21) and inflammation (22). EGCG inhibited these type IV collagenases at low concentrations (MMP-2 and to a slightly lesser extent MMP-9) with IC_{50} values that approximate the

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levels detected in the serum of moderate green tea drinkers, suggesting another mechanism by which EGCG could prevent or treat some tumors. Neutrophil elastase is even more sensitive to inhibition by EGCG (23), and recently, EGCG has been found to inhibit replication of two different HIV strains in peripheral blood lymphocytes, probably by interfering with reverse transcriptase activity (24).

Invasion of surrounding tissues and colonization of different sites are markers of tumor malignancy, whereas neovascularization is necessary for tumor growth and its metastatic dissemination. Kaposi's sarcoma (KS) is a highly angiogenic lesion, frequently associated with AIDS, which appears to consist largely of vascular cells recruited from the host. The products of KS cells are highly angiogenic *in vivo* and induce both inflammatory and endothelial cell migration and invasion *in vitro* (25). In the present study, we used this model to investigate the effects of EGCG on KS and endothelial cell invasion *in vitro* and angiogenesis *in vivo*.

MATERIALS AND METHODS

Cell Cultures. KS-IMM cells, a spontaneously immortalized line obtained from a KS biopsy as previously described (26), were cultured in RPMI 1640 (BioWhittaker, Caravaggio, Bergamo, Italy) supplemented with 10% FCS (Seromed, Milan, Italy) and 1% glutamine. Human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HM-VECs) were cultured in gelatin-coated flasks in medium 199 (Seromed) containing 10% FCS, 1% glutamine, 10 ng/ml acidic fibroblast growth factor, 10 ng/ml basic fibroblast growth factor (PeproTech, Inc., Rocky Hill, NJ), 10 ng/ml epidermal growth factor, 100 μ g/ml heparin sodium salt (ICN Pharmaceuticals, Inc., Costa Mesa, CA), and 1 μ g/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO). For treatment with EGCG (purchased from Sigma Chemical Co.), a 1 mM stock solution was freshly prepared in culture medium and added to cell culture at the indicated concentrations.

Crystal Violet (CV) Cell Growth Assay. Cytostatic and toxic effects of EGCG were tested using the colorimetric CV method. Cells were seeded at a concentration of 1500 cells/well in 96-microwell plates in 100 μ l of complete medium and treated with EGCG at the indicated concentrations. Complete RPMI was used as a control. Plates were incubated for 4 days, and the number of cells was assessed every 24 h by washing with PBS and fixing and staining with 50 μ l of CV solution for 30 min. Plates were then extensively washed in tap water and air-dried. The CV stain was eluted with a 50% ethanol and 0.1% methanol solution, and the absorbance at 595 nm was read with an automatic microtiter plate reader.

ELISA Assessment of Apoptosis. The Cell Death Detection ELISA assay (Roche, Mannheim, Germany) used is based on a quantitative sandwich enzyme immunoassay principle that uses mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Briefly, samples were placed into a streptavidin-coated microtiter plate, added with a mixture of anti-histone-biotin and anti-DNA-peroxidase and incubated. After 2 h, unbound components were removed by washing, and the amount

of peroxidase retained in the immunocomplex was determined with 2,2'-azino-di(3-ethylbenzothiazolinesulfonate) diammonium salt as a substrate in an ELISA reader at 405 nm. The assay was performed 24 h after EGCG treatment. Ten μ M vincristine apoptotic agent were used as a positive control.

Preparation of Conditioned Medium. NIH/3T3 cells, approximately at 80% confluence, were rinsed twice with serum-free medium (SFM) and incubated for 24 h with 8 ml of SFM in a T75 flask. The conditioned medium (NIH/3T3-CM) was successively collected, centrifuged at $200 \times g$ for 10 min to eliminate cell debris, and stored in aliquots at -20°C until used as a chemoattractant in migration and invasion assays.

Chemotaxis and Chemoinvasion Assays. The Boyden chamber chemoinvasion assay was performed as described previously (27). Cells were pretreated overnight in complete medium with EGCG at the concentrations indicated. Cells were harvested with trypsin, resuspended in SFM with 0.1% BSA, and placed in the upper compartment of a Boyden chamber (1.5×10^5 cells/800 μ l/chamber) with EGCG at the indicated concentrations. The compartments were separated by polycarbonate filters (Millipore; Vimodrone, Milan, Italy) with 8- μ m pores for KS-IMM cells or 12- μ m pores for HUVECs coated with Matrigel (12–20 μ g/filter). NIH/3T3-CM was used as a chemoattractant and SFM as a negative control. The chemotaxis assay was performed as for chemoinvasion assay, except for the filters that were coated with a solution of 5 μ g/ml gelatin for KS-IMM and 5 μ g/filter of collagen IV for HUVECs. Chambers were incubated for 6 h at 37°C , 5% CO_2 . Cells remaining on the upper side of the filter were removed, whereas those migrated to the lower side were fixed in ethanol and stained with Toluidine blue (Sigma Chemical Co.). Five to eight fields/filter were counted. Assays were performed in triplicate and repeated several times.

Gelatin Zymography. KS-IMM and HUVECs were incubated in SFM for 6 h without or with EGCG at the indicated concentrations. Supernatants were collected and concentrated, and gelatin zymographs were then performed as described previously (28). Briefly, enzyme-containing samples were electrophoresed in 7.5% SDS-PAGE gels containing copolymerized gelatin at a final concentration of 0.6 mg/ml. Gels were washed in 2.5% Triton X-100 for 30 min to remove SDS and incubated for 18 h at 37°C in collagenase buffer [40 mM Tris, 200 mM NaCl, 10 mM CaCl_2 (pH 7.5)]. In other experiments, HUVECs were incubated in SFM for 6 h, and the supernatants were collected, concentrated, and loaded onto a single wide lane. After electrophoresis, the single lane was divided into equal strips and incubated either in collagenase buffer alone or buffer containing EGCG. Gels were then stained in 0.1% Coomassie brilliant blue and destained. Enzyme-digested regions were observed as white bands against a blue background. Digestion bands were quantified by densitometric analysis.

Matrigel Morphogenesis Assay. Matrigel was thawed at 4°C in an ice-water bath, and 300 μ l/well were carefully added to a 24-microwell plate, prechilled at -20°C using a cold pipette. Matrigel was allowed to polymerize for 30' at 37°C . After polymerization, 7×10^4 cells/well in endothelial cell growth medium without serum were layered on top of polymerized gel in the presence or not of EGCG at the indicated concentrations. Plates were then incubated at 37°C in a 5%

CO₂-humidified atmosphere. The effects on morphogenesis of endothelial cells were evident after few hours. Wells at 7 and 24 h were photographed in a Leitz DR-IMB microscope with charge-coupled device optics and a digital analysis system (Image Pro Plus 3.0; Media Cybernetics, Silver Spring, MD).

Angiogenesis *in vivo*. The Matrigel sponge model of angiogenesis introduced by Passaniti and modified by Albini (29) was used to test the ability of EGCG to inhibit the formation of new blood vessels. KS-CM and heparin were added to unpolymerized liquid Matrigel at 4°C to a 600- μ l final volume. The resulting suspension was slowly injected s.c. into the flanks of C57/bl6N male mice (Charles River; Calco, Lecco, Italy) with a cold syringe. The Matrigel quickly polymerizes *in vivo* to form a solid gel. Mice were randomized into three groups, and controls were provided water *ad libitum*. Another group of animals was treated with EGCG in the drinking water at a concentration of 0.05%. Finally, a third group was provided

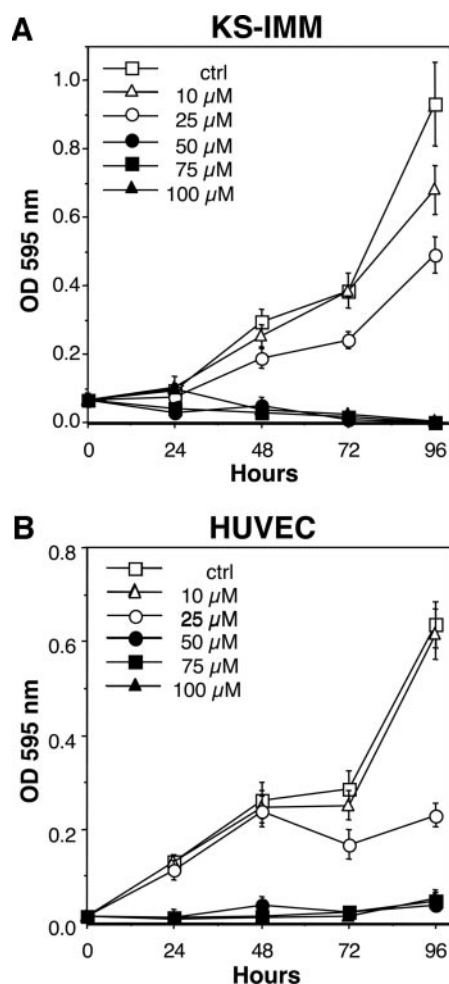


Fig. 1 Effects of epigallocatechin-3-gallate (EGCG) on endothelial and Kaposi's sarcoma (KS-IMM) cell growth measured by the crystal violet assay. Concentrations of EGCG of $\geq 25 \mu\text{M}$ significantly inhibited KS-IMM cell growth (A); $25 \mu\text{M}$ EGCG also showed a strong cytostatic effect after 48 h of treatment in human umbilical vein endothelial cells (HUVECs; B). Doses $> 25 \mu\text{M}$ EGCG completely blocked cell growth in both cell lines. Means \pm SD are shown.

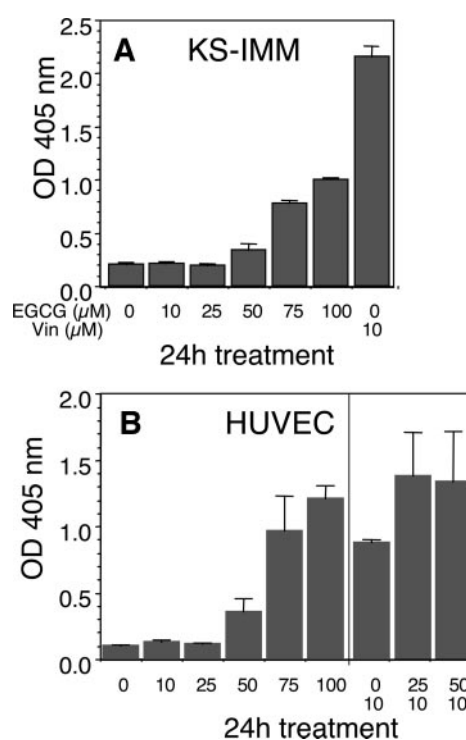


Fig. 2 Epigallocatechin-3-gallate (EGCG)-induced apoptosis as assessed by the cell death ELISA kit. EGCG dose dependently induced apoptosis in both Kaposi's sarcoma (KS-IMM) cells (A) and human umbilical vein endothelial cells (HUVECs; B) at a doses $> 25 \mu\text{M}$. EGCG did not modify the basal level of apoptosis and necrosis at low doses (1– $25 \mu\text{M}$) and was not able to prevent apoptosis induced by vincristine ($10 \mu\text{M}$; B). Data shown are representative of multiple experimental repetitions. On the x axis, upper level numbers are EGCG concentrations (μM), lower numbers vincristine (when present).

commercially available (China or Sri Lanka) green tea leaves that were steeped in 1 liter (12.4 g/liter) of boiled distilled water for 1–2 min. Animals received treatment p.o. starting 3 days before the Matrigel injection. Four days after injection, the gels were recovered, weighed, and either fixed in formalin and embedded in paraffin for histological examination or minced and diluted in water for hemoglobin content measurement with a Drabkin kit (Sigma Chemical Co.). Final hemoglobin concentration was calculated from a standard calibration curve.

***In vivo* Tumor Growth Assays.** Seven-week-old (CD-1)BR nude mice were purchased from Charles River and housed in pathogen-free conditions. KS tumors were obtained by s.c. injection of 5×10^6 KS-IMM cells mixed with liquid Matrigel (final volume, $250 \mu\text{l}$) in the flanks of nude mice as described previously (28). Animals were randomized into three groups. Controls were provided water *ad libitum*; one group was given EGCG (0.05%) and another group green tea extract (China or Sri Lanka, 12.4 g/liter, prepared as above) starting 3 days before cell injection). The infusion was cooled to room temperature, filtered, and stored at -20°C until use. Green tea was provided every other day to prevent dehydration due to its diuretic effects, as was EGCG for comparative purposes. Animals were weighed, and tumor growth was monitored for 30 days by measuring two tumor diameters every 2 days with calipers and

calculating the tumor volumes with the formula length \times width²/2.

Histochemistry. On day 30, animals were sacrificed and the tumors removed. Each sample was fixed in formalin and paraffin embedded. Four- μ m sections were rehydrated and stained with H&E for histological examination.

RESULTS

EGCG Effects on Endothelial and KS-IMM Cell Growth. The effects of EGCG were first tested on cell growth *in vitro*. Concentrations of EGCG of 25 μ M and over significantly ($P < 0.001$ at and beyond 48 h, two-way ANOVA) inhibited KS-IMM cell growth as measured by the CV assay (Fig. 1A), with an apparent reduction in total cell number at concentrations $> 50 \mu$ M. The EC₅₀ of KS-IMM growth inhibition by EGCG was $\sim 26 \mu$ M at all time points. Similar growth inhibitory effects of EGCG were observed for HUVECs; 25 μ M EGCG showed a strong cytostatic effect from 72 h of treatment (Fig. 1B; $P < 0.001$, two-way ANOVA). Higher doses of EGCG again completely blocked cell growth, with significant ($P < 0.001$, two-way ANOVA) inhibition of cell growth from 24 h. The EC₅₀ for EGCG growth inhibition of HUVECs decreased linearly with increasing time in culture from 40 μ M at 24 h to 22 μ M at 96 h of exposure.

Apoptosis. Because the effects of EGCG on cell growth suggested a potential apoptotic or cytotoxic action, EGCG ef-

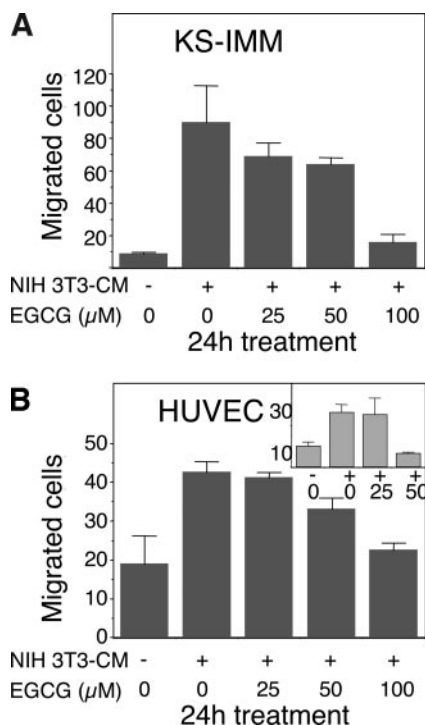


Fig. 3 Effects of epigallocatechin-3-gallate (EGCG) on cell responses to angiogenic factors measured by chemotaxis and chemoinvasion assays. EGCG potently inhibited migration of KS-IMM cells at high concentrations (100 μ M; A), whereas both human umbilical vein endothelial cell (HUVEC) migration and invasion were inhibited by 50 μ M EGCG (B and *insert*). Means \pm SD are shown.

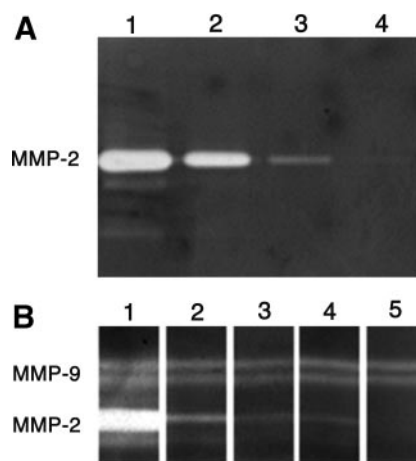


Fig. 4 Gel zymography of the activity of the collagenase IV metalloprotease matrix metalloprotease (MMP)-2. Human umbilical vein endothelial cells were treated with epigallocatechin-3-gallate (EGCG) in serum-free medium for 6 h. A, Lane 1, control; Lane 2, 25 μ M EGCG; Lane 3, 50 μ M EGCG; Lane 4, 100 μ M EGCG. Treatment with EGCG inhibited dose dependently the release of MMP-2 by the cells. Complete inhibition was reached at 100 μ M. B, Lane 1, control; Lane 2, 12.5 μ M EGCG; Lane 3, 25 μ M EGCG; Lane 4, 50 μ M EGCG; Lane 5, 100 μ M EGCG. Treatment with EGCG resulted in a strong inhibition of MMP-2 (and partially of MMP-9) also at low doses (12.5 μ M) when EGCG was added directly to the collagenase buffer, showing the ability of gallate to interfere with MMP enzymatic activity.

fects on apoptosis were examined. EGCG after 24 h of treatment did not modify the basal level of apoptosis and necrosis at low doses (10–25 μ M) in either Kaposi's or endothelial cells, as assessed by the Cell Death Elisa kit. At doses $> 25 \mu$ M, EGCG dose dependently induced apoptosis in both KS-IMM and HUVECs (Fig. 2, A and B); a 50 μ M concentration appeared to be borderline in both cell lines. In HUVECs, the level of apoptosis attained was similar to that induced by the typical apoptotic agent vincristine (10 μ M; Fig. 2B). However, EGCG was not additive with nor was it able to prevent the apoptosis induced by vincristine (Fig. 2b).

EGCG Effects on HUVEC and KS-IMM Cell Chemotaxis and Invasion. Key steps in the process of angiogenesis and tumor metastasis are migration and invasion through extracellular matrix barriers. Chemotaxis and chemoinvasion assays were used to measure the effects of EGCG on cell responses to the strong angiogenic factors contained in conditioned medium from NIH/3T3 cells used as a chemoattractant. Treatment with EGCG dose dependently and significantly ($P = 0.0128$ for 25 μ M, $P = 0.0015$ for 50 μ M, and $P < 0.0001$ for 100 μ M; Mann-Whitney U test) inhibited migration of KS-IMM cells. Although high concentrations (100 μ M; Fig. 3A) of EGCG essentially blocked migration, no overt effects on invasion in the chemoinvasion assay were observed (data not shown). EGCG also significantly inhibited endothelial cell migration over the same dose range ($P = 0.001$ for 50 μ M and $P < 0.0001$ for 100 μ M, Mann-Whitney U test; Fig. 3B). Furthermore, HUVEC invasion was significantly ($P < 0.0001$, Mann-Whitney U test) inhibited by 50 μ M EGCG (Fig. 3B, *insert*).

EGCG Inhibits Gelatinolytic Activity in Endothelial Cells. The gelatinases/collagenase IV metalloproteases MMP-2 and MMP-9 are involved in the invasion processes of both cancer

and angiogenesis. Because EGCG inhibited endothelial cell invasion and has been previously reported to directly inhibit MMP-2 (18, 19), we tested its effects on the MMP activity produced by endothelial cells. Gelatin zymography revealed the activity of the metalloprotease MMP-2 produced by HUVECs, and treatment of the cells with EGCG resulted in a dose-dependent decrease in the release of MMP-2 that reached complete inhibition at 100 μM EGCG (Fig. 4A). When EGCG was added directly to the collagenase buffer to examine the ability of this molecule to interfere with MMP enzymatic activity, a strong inhibition of MMP-2 (and partially of MMP-9, which was poorly expressed in HUVECs) even at low doses (12.5 μM) was observed (Fig. 4B).

Effects of EGCG on Morphogenesis. Migration, invasion, and matrix remodeling are all required for the morphogenesis of endothelial cells into capillaries. The morphogenesis on Matrigel assay provides an indication of the ability of endothelial cells to reorganize and differentiate into capillary-like structures: here, we tested both HUVECs and HMVECs. Untreated HUVECs and HMVECs showed formation of the typical cellular network 7 h after plating (Fig. 5, A and C); 50 μM EGCG had little effect on the morphogenesis of HUVECs (Fig. 5B). In contrast, treatment of the HMVECs with 50 μM EGCG inhibited the formation of the capillary-like structures (Fig. 5D).

EGCG Inhibits Angiogenesis *in vivo*. The *in vitro* data all suggested that EGCG could inhibit KS cell-induced angiogenesis *in vivo*. Subcutaneous injection of Matrigel rapidly produces a three-dimensional sponge *in vivo*; if angiogenic factors such as KS-CM are included in the Matrigel, the sponges become rapidly vascularized *in vivo* (Fig. 6A). The extent of angiogenesis can be estimated by measurement of the hemoglobin content in the Matrigel sponges recovered after 4 days (Fig. 6B). Treatment of the animals with EGCG or green tea extract, beginning 3 days before Matrigel injection, drastically and sig-

nificantly ($P < 0.003$ and 0.005, *t* test) reduced the hemoglobin content of the recovered gels. The reduction was calculated as 42- and 9-fold for EGCG and tea extract treatment, respectively (Fig. 6b). Histological analysis of the recovered gels indicated rare infiltrating stellar cells in the green tea- or EGCG-treated samples, whereas control samples showed formation of extensive vascular lacunae (data not shown).

EGCG Reduces KS Tumor Growth. Finally, given the ability of EGCG to suppress KS cell growth and angiogenesis, we examined whether EGCG or green tea was able to inhibit vascular tumor growth *in vivo*. The immortalized KS cell line KS-IMM forms highly angiogenic tumors when injected s.c. in male nude mice. Treatment in drinking water with EGCG or green tea extract every other day starting 3 days before KS-IMM cell injection significantly ($P < 0.05$ and 0.001, two-way ANOVA, respectively) reduced the tumor growth as compared with controls receiving water alone (Fig. 7). All treated mice developed slow growing tumors limited in size, whereas the controls showed large tumors in 90% of the animals. No differences were noted in animal body weights, indicating limited or no toxicity of the EGCG or green tea treatments, and no differences were observed for the two sources of green tea used. Mice treated with EGCG showed a 50% reduction in tumor size; interestingly, the reduction was even more evident in animals treated with green tea extract.

Histochemical analysis (Fig. 8A–C) of the recovered tumors indicated a significant ($P < 0.001$, one-way ANOVA) reduction in the number of vessels in tumors from mice after treatment with EGCG and green tea extracts (Fig. 8D).

DISCUSSION

A wide variety of different mechanisms of action of green tea or green tea flavanols on diverse physiological or patholog-

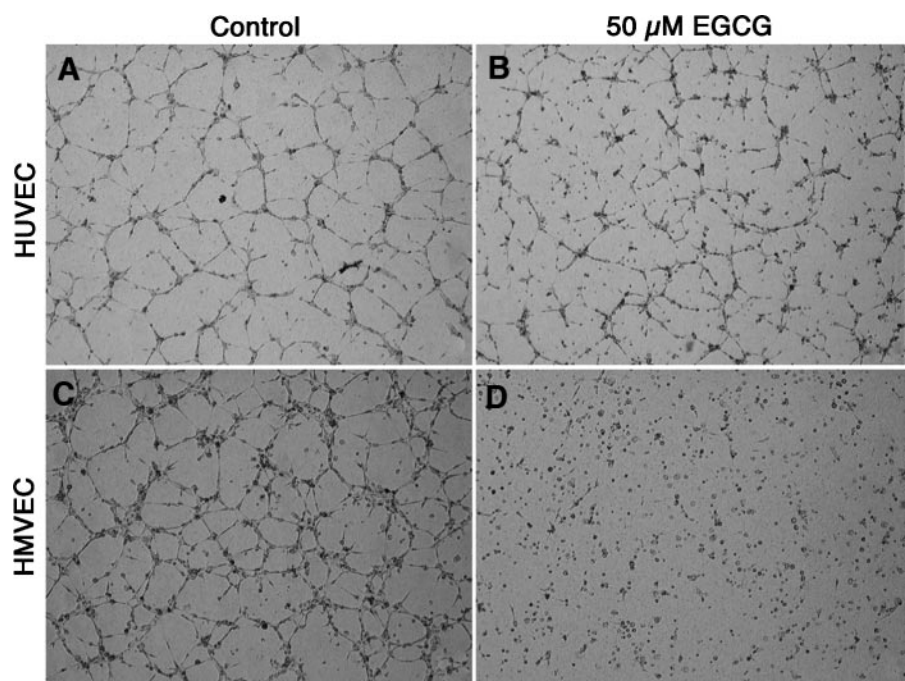


Fig. 5 Effects of epigallocatechin-3-gallate (EGCG) on morphogenesis. A and C, untreated human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMVECs), respectively; B and D, 50 μM EGCG treated HUVECs and HMVECs, respectively. All pictures represent the morphology of cells 7 h after plating and treatment, cultured in the absence of serum. Untreated HUVECs and HMVECs showed formation of the typical cellular network 7 h after plating (A and C); 50 μM EGCG had little effect on the morphogenesis of HUVECs (B) but inhibited the formation of the capillary-like structures in microvascular endothelial cells (D). Nominal magnification, $\times 25$.

ical situations have been proposed. Makimura *et al.* (30) showed that the steric structure of 3-O-gallate group is important for the inhibition of collagenase activity. It has been suggested that catechins can block free radicals through their polyphenol group (2). Moreover, a pyrogallol-type B-ring structure is the minimal requirement for induction of apoptosis by catechins, and a 3-O-gallate group in *cis*-configuration to the B ring could enhance this activity. Catechins without a pyrogallol-type structure showed no activity (31). Protection against chemical carcinogenesis and UV irradiation appears to be exerted both by green and black tea and mediated by direct sequestration of free radicals and activation of antioxidative responses, according to Xu and Dashwood (32). The great majority of *in vitro* studies on EGCG have shown activities at doses from 10 to 100 μM , substantially higher than those obtained *in vivo*. Inhibition of tumor cell proliferation appears to be mediated either through modulation of the activities of several key G1 regulatory pro-

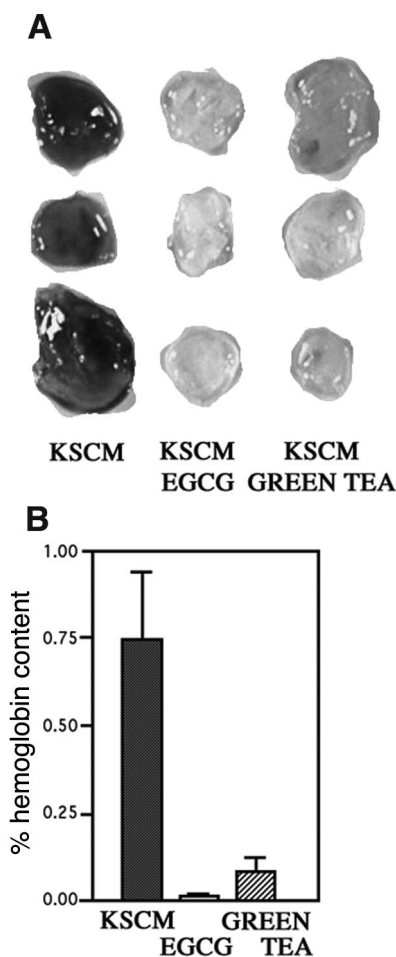


Fig. 6 Epigallocatechin-3-gallate (EGCG) inhibited angiogenesis *in vivo*. Matrigel injected s.c. with Kaposi's sarcoma conditioned medium (KS-CM) and heparin angiogenic factors, produced rapidly vascularized sponges *in vivo* (A). Treatment with EGCG and green tea extract caused a strong and significant ($P < 0.003$ and $P < 0.005$, *t* test) reduction ($\times 42$ and $\times 9$, respectively) in angiogenesis as measured by hemoglobin content in tumors recovered after 4 days (B). Means \pm SEM are shown.

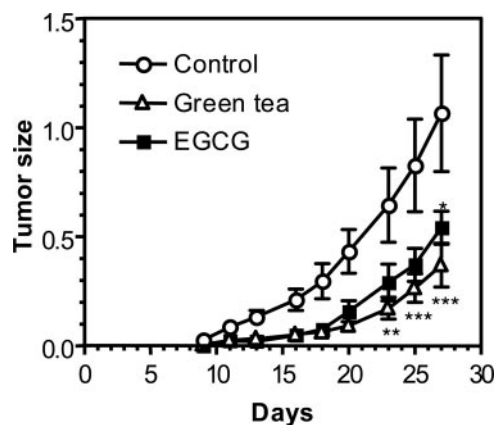


Fig. 7 Epigallocatechin-3-gallate (EGCG) repressed Kaposi's sarcoma tumor growth. Mice receiving, after s.c. injection of highly angiogenic tumor-forming KS-IMM cells, EGCG or green tea extract p.o. showed a $\geq 50\%$ reduction in tumor size, as compared with controls receiving water alone. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, two-way ANOVA. Means \pm SEM are shown.

teins such as cyclin-dependant kinase Cdk2 and Cdk4 or through induction of cyclin-dependant kinase inhibitors p21 and p27 (33). EGCG has been reported to induce apoptosis and growth inhibition in a variety of tumor cell lines at doses from 20 to 100 μM (34–39). Inhibition of the tyrosine kinase activities of growth factor receptors such as epidermal growth factor receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor has been observed for EGCG with an IC_{50} of 1–2 μM in A431 human epidermoid carcinoma cells (40). Several studies show that EGCG at higher concentrations (5–100 μM) also perturbs downstream signaling, repressing the mitogen-activated protein kinase pathway, activator protein-1, and nuclear factor- κB (41–48), although other studies have indicated EGCG increased mitogen-activated protein kinase and activator protein-1 activity (37, 49). Decreases in nitric oxide production by EGCG was observed, apparently caused by reduction of inducible nitric oxide synthase gene expression at concentrations of 1–10 μM (50).

EGCG has been reported to inhibit matrix metalloproteases, in particular MMP-2 (18, 51) at relatively low doses (EC_{50} values on the order of 10–20 μM). Our data demonstrate that endothelial cell-derived MMP-2 and MMP-9 show a similar range for inhibition by EGCG. Both the flavanol skeleton and the galloyl moiety are necessary for the inhibitory activity of these gelatinases, and the block of tumor cell invasion did not appear to be due to cytoskeleton or cell motility impairment but to MMP inhibition (19).

Green tea polyphenols are becoming increasingly recognized for their antiangiogenic properties. Relatively high levels of EGCG (80–90 μM) have been found to reduce vascular endothelial growth factor production in breast carcinoma cell lines (52), and EGCG has been reported to interfere with vascular endothelial growth factor receptor-2 activity (5–10 μM ; Ref. 53) and with vascular endothelial growth factor receptors expression (10–50 μM ; Ref. 54) *in vitro*. Higher concentrations of EGCG (5–50 μM) were found to interfere with HMVEC

the millennia that green tea has been consumed, adverse effects have been reported for specific tumor types such as bladder cancer (63). These data suggest that we should use caution when proposing high doses of tea polyphenols for therapy of established cancer (51, 64). Although over the last few years several clinical trials have been carried out, including Phase II trials, the results in the inhibition of established tumor growth have not been completely satisfactory (65). We suggest that greater attention should be given to the chemopreventive aspect of green tea derivatives (64, 66, 67). Consequently, additional effort is need to fully understand the mechanisms of action of EGCG and green tea extracts, which could be efficiently used as low cost, low side effect chemicals to flank standard clinical therapies.

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REFERENCES

- Lin JK, Liang YC, Lin-Shiau SY. Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. *Biochem Pharmacol* 1999;58:911–5.
- Mukhtar H, Ahmad N. Green tea in chemoprevention of cancer. *Toxicol Sci* 1999;52:111–7.
- Yang CS, Wang ZY. Tea and cancer. *J Natl Cancer Inst* (Bethesda) 1993;85:1038–49.
- Yang CS, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Ann Rev Nutr* 2001;21:381–406.
- Wang ZY, Huang MT, Ho CT, et al. Inhibitory effect of green tea on the growth of established skin papillomas in mice. *Cancer Res* 1992;52:6657–65.
- Ahmad N, Cheng P, Mukhtar H. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem Biophys Res Commun* 2000;275:328–34.
- Chen C, Yu R, Owuor ED, Kong AN. Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death. *Arch Pharm Res* 2000;23:605–12.
- Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst* (Bethesda) 1997;89:1881–6.
- Gupta S, Ahmad N, Nieminen AL, Mukhtar H. Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. *Toxicol Appl Pharmacol* 2000;164:82–90.
- Bracke M, Vyncke B, Opendakker G, Foidart JM, De Pestel G, Mareel M. Effect of catechins and citrus flavonoids on invasion *in vitro*. *Clin Exp Metastasis* 1991;9:13–25.
- Sazuka M, Murakami S, Isemura M, Satoh K, Nukiwa T. Inhibitory effects of green tea infusion on *in vitro* invasion and *in vivo* metastasis of mouse lung carcinoma cells. *Cancer Lett* 1995;98:27–31.
- Taniguchi S, Fujiki H, Kobayashi H, Go H, Miyado K, Sadano H, Shimokawa R. Effect of (-)-epigallocatechin gallate, the main constituent of green tea, on lung metastasis with mouse B16 melanoma cell lines. *Cancer Lett* 1992;65:51–4.
- Cao Y, Cao R. Angiogenesis inhibited by drinking tea. *Nature (Lond.)* 1999;398:381.
- Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutr* 2000;130:2073S–85S.
- Chow HHS, Cai Y, Hakim IA, et al. Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenone in healthy individuals. *Clin Cancer Res* 2003;9:3312–9.
- Sachinidis A, Seul C, Seewald S, Ahn H, Ko Y, Vetter H. Green tea compounds inhibit tyrosine phosphorylation of PDGF beta-receptor and transformation of A172 human glioblastoma. *FEBS Lett* 2000;471:51–5.
- Liberto M, Cobrinik D. Growth factor-dependent induction of p21(cip1) by the green tea polyphenol, epigallocatechin gallate. *Cancer Lett* 2000;154:151–61.
- Garbisa S, Biggin S, Cavallarin N, Sartor L, Benelli R, Albini A. Tumor invasion: molecular shears blunted by green tea [letter]. *Nat Med* 1999;5:1216.
- Garbisa S, Sartor L, Biggin S, Salvato B, Benelli R, Albini A. Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate. *Cancer (Phila.)* 2001;91:822–32.
- Maeda-Yamamoto M, Kawahara H, Tahara N, Tsuji K, Hara Y, Isemura M. Effects of tea polyphenols on the invasion and matrix metalloproteinases activities of human fibrosarcoma HT1080 cells. *J Agric Food Chem* 1999;47:2350–4.
- Demeule M, Brossard M, Page M, Gingras D, Beliveau R. Matrix metalloproteinase inhibition by green tea catechins. *Biochim Biophys Acta* 2000;1478:51–60.
- Corbel M, Belleguic E, Boichot E, Lagente V. Involvement of gelatinases (MMP-2 and MMP-9) in the development of airway inflammation and pulmonary fibrosis. *Cell Biol Toxicol* 2002;18:51–61.
- Sartor L, Pezzato E, Garbisa S. (-)Epigallocatechin-3-gallate inhibits leukocyte elastase. *J Leukoc Biol* 2002;71:73–9.
- Fassina G, Buffa A, Benelli R, Varnier OE, Noonan DM, Albini A. Polyphenolic antioxidant (-)-epigallocatechin-3-gallate from green tea as a candidate anti-HIV agent. *AIDS* 2002;16:939–41.
- Albini A, Fontanini G, Masiello L, et al. Angiogenic potential *in vivo* by Kaposi's sarcoma cell-free supernatants and HIV-1 tat product: inhibition of KS-like lesions by tissue inhibitor of metalloproteinase-2. *AIDS* 1994;8:1237–44.
- Albini A, Paglieri I, Orengo G, et al. The beta-core fragment of human chorionic gonadotrophin (hCG) inhibits growth of Kaposi's sarcoma-derived cells and a new immortalized Kaposi's sarcoma cell line. *AIDS* 1997;11:713–21.
- Albini A, Iwamoto Y, Kleinman HK, et al. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987;47:3239–45.
- Albini A, Morini M, D'Agostini F, et al. Inhibition of angiogenesis-driven Kaposi's sarcoma tumor growth in nude mice by oral *N*-acetylcysteine. *Cancer Res* 2001;61:8171–8.
- Albini A. Tumor and endothelial cell invasion of basement membranes. The Matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. *Pathol Oncol Res* 1998;4:230–41.
- Makimura M, Hirasawa M, Kobayashi K, et al. Inhibitory effect of tea catechins on collagenase activity. *J Periodontol* 1993;64:630–6.
- Isemura M, Saeki K, Kimura T, Hayakawa S, Minami T, Sazuka M. Tea catechins and related polyphenols as anti-cancer agents. *Biofactors* 2000;13:81–5.
- Xu M, Dashwood RH. Chemoprevention studies of heterocyclic amine-induced colon carcinogenesis. *Cancer Lett* 1999;143:179–83.
- Liang YC, Lin-Shiau SY, Chen CF, Lin JK. Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (-)-epigallocatechin-3-gallate. *J Cell Biochem* 1999;75:1–12.
- Masuda M, Suzui M, Weinstein IB. Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression and chemosensitivity in human head and neck squamous cell carcinoma cell lines. *Clin Cancer Res* 2001;7:4220–9.
- Masuda M, Suzui M, Lim JTE, Weinstein IB. Epigallocatechin-3-gallate inhibits activation of Her-2/neu and downstream signaling pathways in human head and neck and breast carcinoma cells. *Clin Cancer Res* 2003;9:3486–91.

36. Jung YD, Kim MS, Shin BA, et al. EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells. *Br J Cancer* 2001;84:844–50.
37. Chen C, Shen G, Hebbbar V, Hu R, Owuor ED, Kong AN. Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis (Lond.)* 2003;24:1369–78.
38. Gupta S, Hastak K, Afaq F, Ahmad N, Mukhtar H. Essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor kappaB and induction of apoptosis. *Oncogene* 2003;1–16.
39. Hastak K, Gupta S, Ahmad H, Agarwal MK, Agarwal ML, Mukhtar H. Role of p53 and NF-kappaB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cell. *Oncogene* 2003;22:4851–9.
40. Liang YC, Lin-shiau SY, Chen CF, Lin JK. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J Cell Biochem* 1997;67:55–65.
41. Sah JF, Balasubramanian S, Eckert RL, Rorke EA. Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway: evidence for direct inhibition of ERK 1/2 and AKT kinases. *J Biol Chem* 2003; 279:12755–62.
42. Chung JY, Huang C, Meng X, Dong Z, Yang CS. Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: structure-activity relationship and mechanisms involved. *Cancer Res* 1999;59:4610–7.
43. Dong Z, Ma W, Huang C, Yang CS. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (-)-epigallocatechin gallate, and theaflavins. *Cancer Res* 1997;57:4414–9.
44. Chen W, Dong Z, Valcic S, Timmermann BN, Bowden GT. Inhibition of ultraviolet B-induced c-fos gene expression and p38 mitogen-activated protein kinase activation by (-)-epigallocatechin gallate in a human keratinocyte cell line. *Mol Carcinog* 1999;24:79–84.
45. Barthelman M, Bair, WBR, Stickland KK, et al. (-)-Epigallocatechin-3-gallate inhibition of ultraviolet-B-induced AP-1 activity. *Carcinogenesis (Lond.)* 1998;19:2201–4.
46. Ahn SC, Kim GY, Kim JH, et al. Epigallocatechin-3-gallate, constituent of green tea, suppresses the LPS-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and NF-kappaB. *Biochem Biophys Res Commun* 2004;313:148–55.
47. Okabe S, Ochiai Y, Aida M, et al. Mechanistic aspects of green tea as a cancer preventive: effect of components on human stomach cancer cell lines. *Jpn J Cancer Res* 1999;90:733–9.
48. Ahmad N, Gupta S, Mukhtar H. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells. *Arch Biochem Biophys* 2000;376:338–46.
49. Balasubramanian S, Efimova T, Eckert RL. Green tea polyphenol stimulates a ras, MEKK1, MEK3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. *J Biol Chem* 2002;277:1828–36.
50. Chan MM, Fong D, Ho CT, Huang HI. Inhibition of inducible nitric oxide synthase gene expression and enzyme activity by epigallocatechin gallate, a natural product from green tea. *Biochem Pharmacol* 1997;54:1281–6.
51. Benelli R, Venè, R, Bisacchi D, Garbisa S, Albini A. Anti-invasive effects of green tea polyphenol epigallocatechin-3-gallate (EGCG), a natural inhibitor of metallo and serine proteases. *Biol Chem* 2002;383:101–5.
52. Sartippour MR, Shao ZM, Heber D, et al. Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *J Nutr* 2002;132:2307–11.
53. Lamy S, Gingras D, Bèliveau R. Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation. *Cancer Res* 2002;62:381–5.
54. Kojima-Yuasa A, Hua JJ, Kennedy DO, Matsui-Yuasa I. Green tea extracts inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors. *Life Sci* 2003;73:1299–313.
55. Tang FY, Nguyen N, Meydani M. Green tea catechins inhibit VEGF-induced angiogenesis *in vitro* through suppression of VE-cadherin phosphorylation and inactivation of AKT molecule. *Int J Cancer* 2003;106:871–8.
56. Leone M, Zhai D, Sareth S, Kitada S, Reed JC, Pellicchia M. Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. *Cancer Res* 2003;63:8118–21.
57. Biroccio A, Candiloro A, Mottolose M, et al. Bcl-2 overexpression and hypoxia synergistically act to modulate vascular endothelial growth factor expression and *in vivo* angiogenesis in a breast carcinoma line. *FASEB J* 2000;14:652–60.
58. Donà M, Dell'Aica I, Calabrese F, et al. Neutrophil restraint by green tea: inhibition of inflammation, associated angiogenesis, and pulmonary fibrosis. *J Immunol* 2003;15:4335–41.
59. Yang CS. Inhibition of carcinogenesis by tea. *Nature (Lond.)* 1997;389:134–5.
60. Chen L, Lee MJ, Li H, Yang CS. Absorption, distribution, and elimination of tea polyphenols in rats. *Drug Metab Dispos* 1997;22:1045–50.
61. Muto S, Yokoi T, Gondo Y, Katsuki M, Shioyama YKF, Kamataki T. Inhibition of benzo[*a*]pyrene-induced mutagenesis by (-)-epigallocatechin gallate in the lung of *rpsL* transgenic mice. *Carcinogenesis (Lond.)* 1999;20:421–4.
62. Lee MJ, Maliakal P, Chen L, et al. Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiol Biomark Prev* 2002;11:1025–32.
63. Lu CM, Lan SJ, Lee YH, Huang JK, Huang CH, Hsieh CC. Tea consumption: fluid intake and bladder cancer risk in southern Taiwan. *Urology* 1999;54:823–8.
64. Pisters KM, Newman RA, Coldman B, et al. Phase I trial of oral green tea extract in adult patients with solid tumors. *J Clin Oncol* 2001;19:1830–8.
65. Jatoi A, Ellison N, Burch PA, et al. A Phase II trial of green tea in the treatment of patients with androgen independent metastatic prostate carcinoma. *Cancer (Phila.)* 2003;97:1442–6.
66. Kamat AM, Lamm DL. Chemoprevention of bladder cancer. *Urol Clin N Am* 2002;29:157–68.
67. Fujimoto N, Sueoka N, Sueoka E, et al. Lung cancer prevention with (-)-epigallocatechin gallate using monitoring by heterogeneous nuclear ribonucleoprotein B1. *Int J Oncol* 2002;20:1233–9.