Dietary Prevention of Cancer: Anticancer and Antiangiogenic Properties of Green Tea Polyphenols

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Abstract: Both epidemiological and laboratory studies have suggested an inverse association between consumption of green tea and the prevalence of some cancers. The anti-tumorigenicity of green tea has been related to its content of specific polyphenols. The molecular mechanisms underlying the anticancer and antiangiogenic effects of green tea polyphenols (GTPs) are currently under intensive investigation. The purpose of this article is to update a previous review on the effects and biological activities of GTPs in relation to their therapeutic usefulness in preventing cancer in humans [1]. GTPs mainly consist of catechins (3-flavanols), of which epigallocatechin gallate (EGCG) is the most abundant in green tea and the most extensively studied. Moreover, the biological effects reported for GTPs have been mainly associated to EGCG. New perspectives on the applications of dietary GTPs as potential therapeutic and preventive agents against cancer are presented.

GREEN TEA CATECHINS AS ANTICANCER AND ANTIANGIOGENIC AGENTS

Tea (Camellia sinensis) originated in southern China and is the second most commonly consumed beverage in the world. Monks and physicians have noted the ability of green tea to offer refreshment, increase alertness, and stave off disease for thousands of years. For example, an old Chinese proverb proclaims "One cup does all disorders cure; with two your troubles will be fewer; three to the bones more vigor give; with four forever you will live as young as on your day of birth, a true immemorial on the earth" [2]. Over the past century, pharmacologists, chemists, physicians, nutritionists and others in health care sciences have recognized the benefits associated with green tea consumption. Accumulating evidence shows that daily intake of green tea is protective against several lethal diseases including cancer. The chemical composition of green tea is complex but of its components polyphenols (catechins) have been found to possess the most of the biological effects. Recently, a growing number of studies have been published about the molecular events regulating the bioavailability and the biological activities of green tea polyphenols (GTPs). In fact, more than 175 articles have been published in the 18 months since a previous review on GTPs as novel antitumor and antiangiogenic compounds [1]. The present update gives an overview of new data obtained regarding various aspects of GTPs including their chemical content in green teas, metabolism and distribution as well as their anticancer and antiangiogenic properties.

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CATECHIN CONTENTS, METABOLISM AND TISSUE DISTRIBUTION

1. Determination of Polyphenol Contents in Green Teas

Traditionally, high performance liquid chromatography (HPLC) has been the most useful approach for determination of the catechins and caffeine in aqueous and biological samples [3, 4]. Tea polyphenols have been measured by HPLC, using a photodiode array detector, in 45 different teas commercially available in Spain [5], while the factors affecting levels of tea polyphenols and caffeine in tea leaves have also been determined by HPLC in 31 commercial teas [6]. Results from both studies confirm that the contents of the four major catechins (EGCG; epigallocatechin gallate, EGC; epigallocatechin, ECG; epicatechin gallate, and EC; epicatechin) vary greatly between individual teas. The content of GTPs varies strongly depending on tea vintage, according to: variety of tea plant, the soil for tea cultivation, the method of processing tea leaves for manufacturing tea material and the season for harvesting [5]. As mentioned previously, green tea has a higher content of EGCG and total catechins than either oolong or fermented teas. For example, the EGCG content is around 86 mg/g in green tea, samples compared to 31 mg/g for black teas, 12 mg/g for oolong teas and less than 5 mg/g for red teas [5]. Red teas, which may taste like some black teas, are however obtained from the leaves of a different plant, African rooibos. Moreover, the level of EGCG and total catechins in teas is higher in old leaves compared to young leaves from green teas, while an inverse correlation is observed for caffeine [6]. It also appears that the caffeine content varies in the order black tea > oolong tea > green tea [6]. In addition, tea samples extracted with 75% ethanol, compared to boiling water extraction, could yield higher levels of EGCG and total catechins [6]. The use of lignocellulose, prepared from sawdust, for the purification of a raw decaffeinated tea

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polyphenol fraction from tea extracts was also investigated [7]. Following lignocellulose treatment, the caffeine/EGCG ratio was reduced from 0.696 to 0.004. This result showed that lignocellulose chromatography provides a useful and convenient process for purification of a tea polyphenol fraction (mainly polyphenols having gallate residues; EGCG and ECG) with a low caffeine content.

Microemulsion electrokinetic chromatography (MEKC), based on 2-hexanol and cyclohexanol as cosurfactants, has been developed and validated for the separation and quantification of bioactive catechins and caffeine present in commercial green tea products [8]. The results obtained were in good agreement with the published literature [3, 4]. More specifically, a generally higher content of GTPs and caffeine was found in green organic Indian tea, with EGC, EGCG and caffeine as the main components. Recently, it has been suggested that Matcha tea, a special powdered green tea used in the Japanese tea ceremony, has greater potential health benefits than do other green teas [9]. Using MEKC analysis, one study demonstrated that the concentration of EGCG obtained by drinking Matcha is 137 times greater than that the amount of EGCG available from China Green Tips green tea, and at least three times higher than the highest published EGCG content for other green teas [9]. However, the production of Matcha, a specially ground Hikicha, represents less than 1% of total tea consumption.

EGCG is susceptible to oxidation and, during this process, EGCG alkalinizes and changes from non-colored to yellow in aqueous solution [10]. The pH-dependent oxidation of EGCG, analyzed by liquid chromatography/ electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS), allowed the identification of an oxidation species called M+14 (where M corresponds to the molecular weight of EGCG). This oxidized analog has two hydrogen atoms removed and one oxygen atom added to the galloyl moiety on the B-ring of EGCG. Using Sephadex LH-20 column chromatography fractionation prior to HPLC-MS and proton nuclear magnetic resonance (^{1}H-NMR), two analogs of methylated EGCG have also been identified in green tea [11]. These two forms, methylated at the 3-*O*-position of the gallic moiety, have been identified as (-)-epigallocatechin-3-(3-*O*-methylgallate) and epigallocatechin-3-(4-*O*-methylgallate).

2. Bioavailability and Metabolism of Green Tea Polyphenols

The absorption, tissue distribution and elimination of EGCG have been monitored in beagle dogs following administration by intravenous (i.v.) and oral routes [12]. After i.v. administration of 25 mg/kg 4-[^{3}H]-EGCG, the radioactivity within the blood remained predominantly in the plasma fraction. Distribution of EGCG occurred during the first hour, and the radioactivity in plasma declined with a mean half-life of approximately 7 hours. The apparent volume of distribution (0.65 l/kg) and the total body clearance (1.01 ml/min-kg) indicate a wide tissue distribution for EGCG. A subsequent single oral dose (250 mg/kg) was rapidly absorbed, with peak plasma levels detected at about 1 hour after administration, followed by elimination with a mean half-life of around 8.6 hours. The mean area under the curve for total radioactivity was approximately 20% of the value following i.v. administration. Tissue distribution determined after chronic consumption of tea showed that radioactivity is distributed to a variety of epithelial tissues; the highest concentrations are observed in the liver and gastrointestinal tract. Repeated oral administration of EGCG resulted in significantly lower blood radioactivity compared to the concentration following a single dose. These results indicate that EGCG is widely distributed within several tissues where it could exert chemopreventive effects.

Better knowledge regarding the bioavailability and biotransformation of EGCG in humans would greatly increase the understanding of its purportedly beneficial health effects. A randomized, double-blind, placebo-controlled study assessed the safety, tolerability and pharmacokinetics of a single oral dose of 94% pure crystalline bulk EGCG under fasting conditions in 60 healthy male volunteers [13]. In each group of 10 subjects, eight received oral EGCG in single doses of 50-1600 mg, and two received placebo. Kinetic analysis revealed rapid absorption displaying a single peak in plasma concentration over time, followed by a multiphasic decrease consisting of a distribution phase and an elimination phase. The mean area under the concentration-time curve from 0 to infinity of total EGCG varied between 442 and 10,368 ng/h/mL. The mean maximum plasma concentration values observed after 1.3-2.2 hours ranged from 130 to 3,392 ng/mL, whereas the mean terminal elimination half-life values ranged between 1.9 and 4.6 hours. Overall, a single oral dose of EGCG up to 1,600 mg is safe and well tolerated.

Since oral bioavailability of green tea catechins has been shown to be low in animals, and possibly in humans, the contribution of first-pass hepatic elimination to the low oral bioavailability of green tea was investigated [14]. Following intraportal infusion, a high proportion of green tea catechins escaped first-pass hepatic elimination, with 87.0, 108.3 and 94.9 % of EGCG, EGC and EC, respectively, available in the systemic blood. These results suggest that factors within the gastrointestinal tract, such as limited membrane permeability, transporter mediated intestinal secretion or gut wall metabolism, may contribute significantly to the low oral bioavailability of GTPs.

The bioavailability and excretion of both EGCG and the methylated catechin, 4',4''-di-*O*-methyl-EGCG (4',4''-DiMeEGCG), were measured by using LC/MS/MS in plasma and urine following green tea intake [15]. Both 4', 4''-DiMeEGCG and EGCG reached peak plasma values at 2 hours after dosage. The half-lives of 4', 4''-DiMeEGCG and EGCG were 4.1 ± 0.8 and 2.7 ± 0.9 hours, respectively. The cumulative urinary excretion of 4', 4''-DiMeEGCG during a 24 hour period was 140.3 ± 48.6 μg, about 5-fold higher than that of EGCG, but the excreted 4', 4''-DiMeEGCG and EGCG in urine only account for about 0.1 % of ingested EGCG. Also, the metabolites (-)-5-(3', 4', 5'-trihydroxyphenol)-γ-valerolactone (M4), (-)-5-(3', 4'-dihydroxyphenyl)-γ-valerolactone (M6) and an another possible ring-fission metabolite, (-)-5-(3',5'-dihydroxyphenyl)-γ-valerolactone (M6'), are detected in human urine after green tea intake [15]. The combined excretion of the three ring-fission

metabolites accounted for 1.5-1.6% of ingested catechins. M4, M6 and M6' are all observed after the intake of pure EGCG or EGC by human subjects, whereas only M6 is produced after EC intake. Known methylated EGCG metabolites and ring-fission products exist in substantial quantities and may contribute to the biological activities of tea.

The metabolism of EGCG is an important issue that remains to be clearly established in humans. An initial study revealed the biosynthesis and structures of six EGCG and EGC glucuronides [16]. Among them, (-)-EGCG-4''-*O*-glucuronide is the major EGCG glucuronide formed. In addition, the catalyzed glucuronidation of EGCG is much faster than that of EGC. However, the metabolism seems to vary between species since the glucuronidation of EGCG and EGC in mice appeared more similar to that in humans than to rats. Some of these catechin glucuronides retained the activities of their parent compounds in radical scavenging and in inhibiting the release of arachidonic acid from HT-29 human colon cancer cells.

The methylation of EGCG and EGC by cytosolic catechol-*O*-methyltransferase (COMT), a central enzyme in the metabolic inactivation of neurotransmitters and neuroactive xenobiotics possessing a catechol motif, was investigated in humans, mice and rats [17]. EGCG is readily methylated by liver cytosolic COMT to 4''-*O*-methyl-EGCG and then to 4', 4''-di-*O*-methyl-EGCG whereas EGC is methylated to 4'-*O*-methyl-EGC. The small intestine has a lower specific activity than does the liver in the methylation of EGCG and EGC. Glucuronidation of the B-ring or the D-ring of EGCG greatly inhibits methylation of the same ring, but glucuronidation on the A-ring of EGCG or EGC did not affect their methylation. Since EGCG is methylated by COMT during this metabolic process, these results suggest that EGCG, by interacting with COMT, may inhibit methylation of other endogenous and exogenous compounds by competing for COMT.

3. Biological Interactions of Green Tea Polyphenols

GTPs have been reported to interact with lipid bilayers, and these interactions have been further investigated by using liposomes as model membranes [18]. In this study, the number of hydroxyl groups on the B-ring, the presence of the galloyl moiety, and the stereochemical structure of each catechin governs their affinity for lipid bilayers. Overall, this study revealed that the salt concentration, the electric charge of the membrane and the presence of other catechins critically affect tea catechin affinity for lipid bilayers.

Catechins have also been shown to reduce plasma cholesterol levels and the rate of cholesterol absorption. To investigate the biological interaction of EGCG with cholesterol metabolism, Wistar rats were fed with a diet high in cholesterol and fat, containing between 0-0.1% EGCG (0-0.7 g/day/kg) [19]. After 4 weeks of treatment, plasma levels of both total cholesterol and low-density lipoprotein were reduced in the group fed with 1.0% EGCG when compared to the control group. Plasma triglycerides and high-density lipoprotein levels are not significantly changed by this treatment. These results suggest that the cholesterol-lowering effect of green tea is mainly elicited by ECGC. It appears that, by interfering with the solubilization of cholesterol in the digestive tract, EGCG decreases cholesterol absorption.

Catechins are considered to be natural flavonoid inhibitors of lipoxygenases, a ubiquitous non-heme iron-containing enzymes that are involved in the metabolism of polyunsaturated fatty acids. Recently, X-ray analysis of the 3D structure of a complex between EGCG and soybean lipoxygenase-3 revealed the inhibitor depicting EGC that lacks the galloyl moiety suggesting a degradation of EGCG [20]. The A-ring is near the iron co-factor, attached to the C-terminus by hydrogen bonds and by van der Waals interactions formed with the surrounding amino acids and water molecules.

Basic, salivary proline-rich proteins are known to defend organisms against dietary polyphenols by precipitating them, leading to the oral phenomenon of astringency. The structure of the complex between a typical basic, proline-rich protein repeat (Gln-Gly-Arg-Pro-Pro-Gln-Gly) and the polyphenol EGCG has been determined by NMR analysis [21]. This study showed that the complex exists in one major conformation, in which the A ring of EGCG is positioned over the proline at position 5 (Pro5) and the D-ring (gallate moiety) is over the proline at position 4 (Pro4), with the B-ring frequently close to the arginine side chain. This structure is consistent with previous interaction models and suggests how polyphenol precipitation could occur.

Since recent study has revealed that polyphenol derivatives (glucuronides or methylated GTPs) and metabolites possess biological activity, knowledge about the quantities of these derivatives and metabolites in green tea and about the digestion, absorption and metabolism of tea polyphenols should be investigated in order to accurately assess the use of GTPs as cancer preventing agents.

BIOLOGICAL ACTIVITIES OF GREEN TEA POLYPHENOLS

1. Anticancer Properties

A large body of evidence has suggested GTPs as potential preventing agents. Recent *in vitro* and *in vivo* findings in animal and human studies have identified multiple molecular mechanisms affected by green tea catechins (Fig. **1**). Most of these studies highlight that, among green tea catechins, EGCG has the strongest effects. EGCG, which acts as a powerful antioxidant, can inhibit a number of tumor cell proliferation- and survival-related proteins including the proteasome, metalloproteinases, tumor-associated protein kinases such as the epidermal growth factor receptor, the platelet-derived growth factor receptor and mitogen-activated protein kinase. Tea polyphenols have also been found to inhibit some cancer-related proteins involved in DNA replication and transformation. Most of these findings show the cancer preventive effects of GTPs in which EGCG showed the most potent effects among these polyphenols .

i. Reversal of Multidrug Resistance

Since our first report on interactions between green tea catechins and the drug efflux pump, P-glycoprotein (P-gp) [22], this effect of EGCG has been further investigated. In

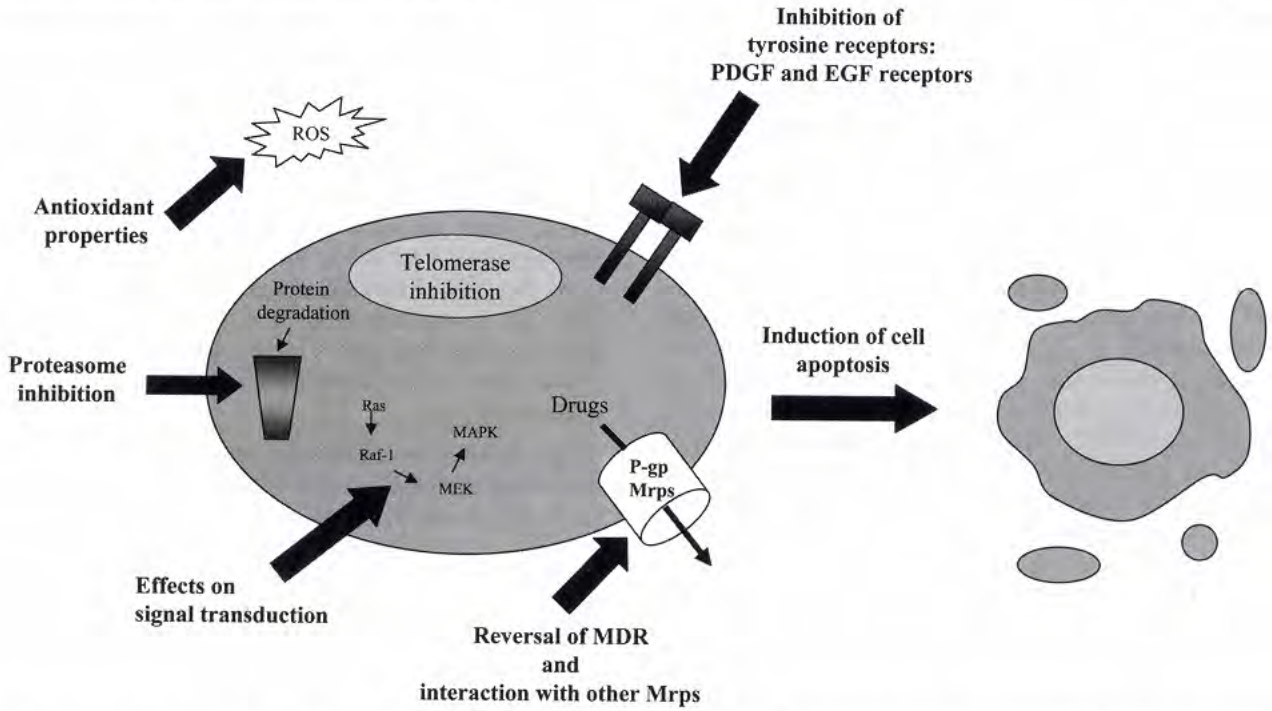

Fig. (1). Anticancer activities of green tea polyphenols. Antioxidant properties of GTPs protect the cell against DNA damage by scavenging reactive oxygen species (ROS). GTPs, mainly EGCG, modulate key players in various signal transduction pathways such as AP-1, MAPK, PI 3'-K, p70S6-K and Akt. GTPs also reduce the activity of tyrosine kinase receptors (PDGF-Rβ, EGF-R) that contribute to the malignant proliferation of tumor cells and induce cell apoptosis in tumor cells. In addition, GTPs reverse the MDR phenotype by blocking P-gp efflux of anticancer drugs and interact with MRPs. New GTP activities include inhibition of both telomerase and the chymotrypsin-like activity of the proteasome.

human multidrug resistant (MDR) carcinoma cells, EGCG reversed the MDR phenotype involving doxorubicin [23]. Moreover, both tea polyphenols and EGCG showed reversal of the MDR phenotype in KB-A-1 cells, leading to an increase in the cytoxocity of doxorubicin by 2.5 fold at 10 μg/mL [24]. A study on the stability, cellular uptake, biotransformation and efflux of EGCG in HT-29 human adenocarcinoma cells suggested that this catechin is metabolized by the cell and that its metabolites are pumped out by multidrug-resistant protein (MRP) transporters [25]. Overall, these studies reported that EGCG might act as a reversal agent and that P-gp and other efflux pumps, such as MRPs, might be involved in the bioavailability and biodistribution of catechins including EGCG.

ii. Apoptosis and Cell Cycle Arrest

Apoptosis is a continuous physiologic process for programmed cell death and is one of the most active fields of biomedical research [26]. This process can occur through the activation of two major pathways: 1) the extrinsic pathway, which involves the binding of specific ligands to a cell surface receptor and 2) the mitochondria-associated (intrinsic) pathway, which leads to the release of cytochrome c. Multiple mechanisms and proteins involved in apoptosis have been identified as potential molecular targets for GTPs in various cancer cells.

Since the previous review on green tea catechin as novel antitumor and antiangiogenic compounds [27], many studies have showed that GTPs could protect against apoptosis in normal cells keratinocytes and induce apoptosis of cancer cells. In normal keratinocyte cells, for example, it was reported that EGCG promotes survival and inhibits UV-induced apoptosis via phosphorylation of Ser112 and Ser136 of Bad protein through Erk and Akt pathways, and by increasing the Bcl-2 to Bax ratio [27]. Aged keratinocytes with low basal cellular activities renewed DNA synthesis and activated succinate dehydrogenase after exposure to EGCG or GTPs, suggesting that they may be used for the treatment of wounds or certain skin conditions involving altered cellular activities or metabolism [28]. GTPs have been shown to induce apoptosis of prostate [29, 30], colon [31], cervical [32], brain [33], liver [34] and blood cancer cells [35, 36]. This induction was provoked by affecting various molecular mechanisms such as shifting the balance between pro- and antiapoptotic proteins [29, 33], cell-cycle arrest [30, 32], increasing JNK-mediated apoptosis [31, 35]. The concentrations of GTPs usually used (>25 μM) for these *in vitro* studies are not generally relevant to *in vivo* situations or epidemiological observations. However, despite the high concentrations of ECGC required to induce apoptosis, these studies indicate that this GTP has anticancer properties against various types of cancer cells *in vitro* including skin, leukemia, brain, liver and prostate cancers.

iii. Telomerase Inhibition by EGCG Derivatives

The proliferative capacity of human cells is regulated by telomerase and a critical role for telomerase activation in tumor progression and tumor maintenance has been well established by numerous studies [37, 38]. In fact, most (80-90%) cancer cells possess telomerase activity. EGCG, at low concentrations corresponding to plasma levels obtained after

drinking a few cups of green tea, has been shown to inhibit telomerase [39, 40]. More recently, newly synthesized compounds of EGCG-related moieties (MST-312, MST-295 and MST-199) have been identified as more effective inhibitors than EGCG. Continuous treatment of human monoblastoid leukemia cells (U937) with a nontoxic dose of each of these EGCG-related moieties lead to a progressive telomere shortening and eventually to a reduction of growth rate. In the case of MST-312, the required effective dose of stable was 15- to 20-fold lower than that of EGCG (1 μM), suggesting that these compounds may play an important part in chemotherapeutic strategies [41].

iv. Inhibition of Tumor Growth by Green Tea Polyphenols in Animal Models

In UVB-induced skin tumor mice, topical applications of EGCG reduced the numbers of nonmalignant and malignant tumors per mouse by 55% and 66%, respectively, by increasing apoptosis as measured by the number of caspase 3-positive cells [42]. Furthermore, by inducing apoptosis, EGCG inhibited carcinogenesis in the mouse viral mammary epithelial model RIII/MG [43]. From these results, it has been suggested that EGCG could be used as a chemopreventive agent by affecting the growth of pre-cancerous mammalian cells [43]. Using the transgenic adenocarcinoma of the mouse prostate model which closely emulates human disease, it has been shown that oral infusion of GTPs at a human achievable dose inhibits prostate cancer development and increases survival of these mice [44]. Thus, these *in vivo* studies support the claim arising from *in vitro* studies that EGCG has anticancer properties against both skin and breast cancers.

v. Clinical Trials Using Green Tea

Recently, a clinical study was conducted to determine the safety and pharmacokinetics of green tea polyphenols after 4 weeks of daily p.o. administration of EGCG or Polyphenon E (a defined, decaffeinated green tea polyphenol mixture) [45]. Adverse effects reported during the 4-week treatment period included excess gas, upset stomach, nausea, heartburn, stomach ache, abdominal pain, dizziness, headache and muscle pain. However, the incidence of these effects in the polyphenol-treated groups was not more than that reported for the placebo group. No significant change was observed in blood counts or blood chemistry profile after repeated administration of green tea polyphenol products. Thus, it may be concluded that it is safe for healthy individuals to take green tea polyphenol products in amounts equivalent to the EGCG content found in 8-16 cups of green tea once a day or in divided doses twice a day for 4 weeks. There was a >60% increase in the systemic availability of free EGCG after chronic green tea polyphenol administration at a high daily bolus dose (800 mg EGCG or Polyphenon E once daily).

A phase II clinical trial showed that green tea carries limited antineoplastic activity in the treatment of patients with androgen-independent metastatic prostate carcinoma, as defined by a decline in prostate-specific antigen (PSA) levels [46]. In this later study, the median time of green tea intake was only one month since it had little effect on the early rise of PSA levels. Because the antineoplastic or antiangiogenic mechanisms of activity for GTPs in laboratory studies may require prolonged exposure, a longer treatment period may be needed to ultimately produce a tumor response. Thus, further studies are still required to establish whether green tea might exert anticancer effects in patients with hormone–sensitive prostate carcinoma, reduce the risk of disease recurrence or decrease the risk of developing prostate carcinoma.

2. Antiangiogenic Properties

It is now recognized that the formation of a new blood vessel network within a tumor represents an absolute requirement for the maintenance and progression of most solid tumors [47-49]. Tumor angiogenesis has thus become one of the most promising therapeutic targets in cancer medicine. Accordingly, tremendous efforts have been made to identify antiangiogenic molecules with antitumor properties. This has led to the development of a variety of molecules that are directed against critical cellular aspects of angiogenesis such as cell adhesion, extracellular matrix degradation and the stimulation of endothelial cell by angiogenic cytokines or growth factors. More than 49 antiangiogenic molecules are currently undergoing clinical trials (www.angio.org). There is also evidence in the literature indicating that GTPs can contribute to cancer prevention not only by the reduction of tumor cell growth, migration and invasion, but also by the inhibition of angiogenesis.

i. Angiogenic Targets for Green Tea Polyphenols

As previously mentioned, the antiangiogenic effect of green tea was first suggested by the observation that green tea extracts, and more particularly EGCG, inhibit angiogenesis in the chick embryo neovascularization assay [50]. However, the molecular mechanisms involved in this inhibition remain, for the most part, unknown. Key players in angiogenesis have been identified as potential targets for EGCG (Fig. **2**). These targets include the hydrolytic activities of matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9) [51], the mechanisms regulating their secretion and activation are through a membrane type-1 (MT1-) MMP-dependent process [52], as well as the phosphorylation of the vascular endothelial growth factor receptor-2 and the capacity of endothelial cell to form capillary-like structures [53]. The effects of catechins were tested using *in vitro* models of angiogenesis, such as growth, migration and tube formation of human umbilical vein endothelial cells (HUVECs) induced by the vascular endothelial growth factor (VEGF) [54]. Four tea catechins (EC, ECG, EGC, EGCG) inhibited these processes *in vitro* at concentrations ranging from around 1.6 to 100 μM. EGCG was the most potent inhibitor among these four catechins. Binding assays were further performed with various concentrations of EGCG or Polyphenon E, a mixture of four tea catechins. Both EGCG and Polyphenon E inhibited the binding of VEGF to its cell surface receptor in a concentration-dependent manner. Moreover, when the four catechins were individually compared, only EGCG effectively inhibited the binding while the other three catechins had almost no effect. Thus, EGCG alone can reduce the binding of VEGF to its receptors.

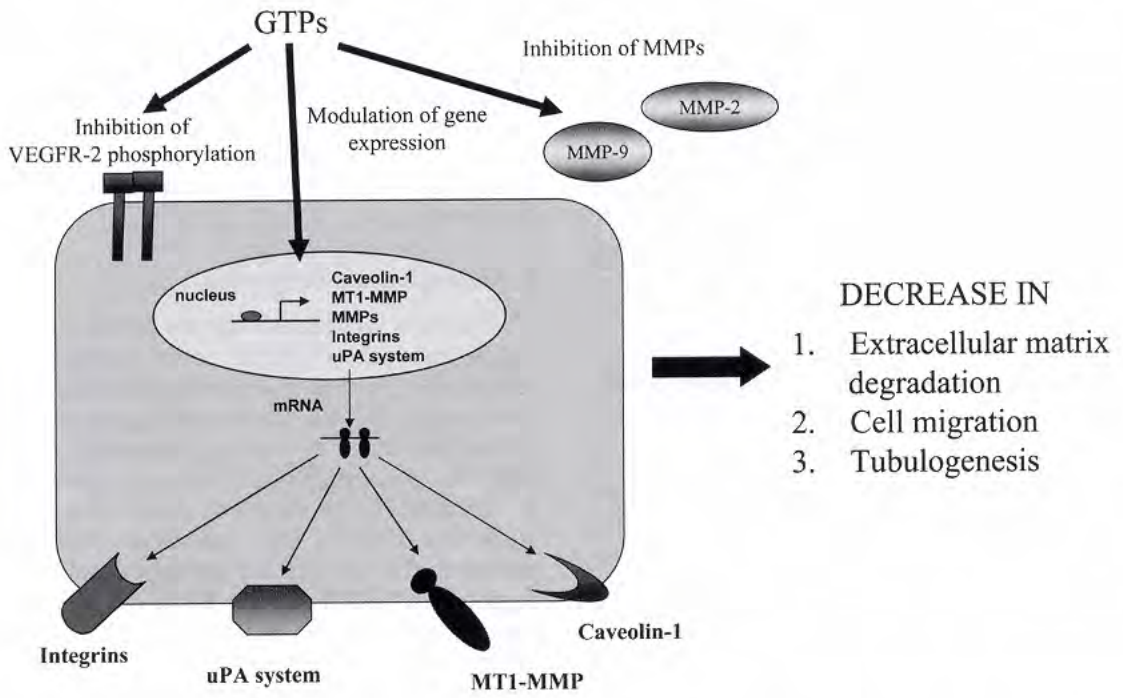

Fig. (2). Antiangiogenic activities of green tea polyphenols. Several molecular mechanisms involved in angiogenesis are targeted by GTPs, particularly by EGCG, even at very low concentrations (<1 μM). The activity of VEGFR-2 as well as the expression of MT1-MMP, integrins, the uPA system and the secretion of MMP-2 are inhibited by GTPs. Catechins also inhibit the activity of MMP-2, MMP-9 and MMP-12. These actions of GTPs lead to the inhibition of three phenomena associated with angiogenesis: 1) extracellular matrix degradation, 2) cell migration and 3) tubulogenesis.

Recent studies have provided evidence that green tea extract (GTE) and EGCG downregulate the enhanced secretion of VEGF from normal human keratinocytes (NHK) [55] and from human breast cancer cells (MDA-MB231) [56]. In the first study, low concentrations of GTE (0.001 to 0.05%) and EGCG (0.1 to 10 μM) inhibited VEGF release from NHK, which were stimulated by the proinflammatory cytokine TNFα, in a dose-dependent manner. In the other study, 40 mg/L GTE or EGCG greatly inhibits VEGF protein secretion in conditioned media. This inhibition occurred at the level of transcriptional regulation, which was manifested by a decrease in transcript levels and a decrease in VEGF promoter activity. They also showed that GTE decreased the levels of c-fos and c-jun RNA transcripts, suggesting that activator protein (AP)-1-responsive regions present in the human VEGF promoter [57] may be involved in the inhibitory effect of GTE. Furthermore, GTE suppressed the expression of protein kinase C, another modulator of VEGF transcription in breast cancer cells [58]. VEGF production was also inhibited by EGCG in head, neck and breast carcinoma cells [59].

Tang *et al.* [60] have evaluated the use of GTPs, specifically EGCG, as an inhibitory agent that can potentially suppress tyrosine phosphorylation at adherence junction sites, as well as inhibit the activation of the Akt survival pathway following stimulation by VEGF. They found that GTPs dose-dependently inhibited VEGF-induced angiogenesis in human microvascular endothelial cells (HMVECs). At a concentration of 2 μM, EGCG suppressed VEGF-induced angiogenesis by 39% whereas 20 μM completely abolished the effect of VEGF on angiogenesis. They also demonstrated that inhibition of tube formation by EGCG is, in part, mediated through suppression of VE-cadherin tyrosine phosphorylation and inhibition of Akt activation during VEGF-induced tube formation. Akt, which is a kinase that operates downstream of PI3-kinase, is involved in VEGF-induced growth and survival. It was demonstrated that stimulation of HMVECs with VEGF increased Akt phosphorylation, whereas EGCG supplementation downregulated VEGF-induced Akt activation. Because VE-cadherin is reported to be colocalized with VEGF receptors [61], however, it is possible that the observed inhibition of VE-cadherin phosphorylation directly resulted from EGCG inhibition of VEGFR-2 phosphorylation [53]. Another study demonstrated that GTE inhibits angiogenesis of HUVECs through reduction of expression of the VEGF receptors VEGFR-1 and VEGFR-2 [62]. This study demonstrated that the expression of VEGF receptors is reduced in a dose-dependent manner by GTE (5-25 μg/ml). Overall, these reports support the hypothesis that one of the mechanisms by which GTPs exert their antiangiogenic effect is through the inhibition of VEGF receptors.

The effect of GTPs on angiogenic processes induced by other angiogenic growth factors has also been investigated. Angiogenin, a 14 kDa protein homologous to pancreatic ribonuclease, is one of the most potent stimulators of blood vessel formation [63, 64]. GTPs reduce the neovascularization induced in the chicken chorioallantoic membrane (CAM) assay by an angiogenin-like protein [65].

Neutrophil activation, in response to angiogenic chemokines or to inflammatory stimuli, can also stimulate angiogenesis [66]. Oral EGCG (0.5 mg/ml) and GTE (containing 3.5 mg/ml EGCG) block neutrophil-mediated angiogenesis *in vivo* in an inflammatory angiogenesis model in which Matrigel plugs in mice are used [67]. Sphingosine-1-phosphate (S1P), a platelet-derived bioactive lysophospholipid [68], strongly stimulates both endothelial cell migration and cell wound healing [69]. EGCG antagonizes S1P-induced migration of bone marrow-derived stromal cells as well as migration of transfected COS-7 cells overexpressing the recombinant receptor for S1P, EDG-1 [70]. The angiogenic basic fibroblast growth factor (bFGF) also seems to be affected by GTPs. Effectively, 40 μg/ml GTE or EGCG decreased the levels of bFGF in both HUVECs and in MDA-MB231 cells [71]. Furthermore, GTE and EGCG decrease the transcript levels of acidic and basic fibroblast growth factors (aFGF and bFGF) in these cells. Taken together, these studies strongly suggest that GTPs may interact with several aspects of tumor angiogenesis.

cDNA array technology has been used to determine the effects of GTPs on genes associated with metastasis- and/or angiogenesis-related pathways in a transgenic mouse model of prostate adenocarcinoma [72]. This gene array analysis allowed the identification of seven genes, related to metastasis pathways, whose expression was increased by at least five-fold by GTPs. These included MMP-2, MMP-9, TIMP-1, TIMP-2, VEGF, uPA and uPA receptor and five other genes whose expression decreased by at least five-fold (E-cadherin, integrin-α3, integrin-α6, laminin-β2 and H-Ras). For angiogenesis-associated genes, a large increase is observed for ten genes (MMP-2, MMP-9, TIMP-1, VEGF, VEGF-receptor, uPA, uPA receptor, EGF, angiopoietin-1 and metalloproteinases with thrombospondin motifs-1 and -8) whereas four genes are found to be repressed (E-cadherin, tenascin C, integrin-αV, laminin-β3).

Although inhibition of angiogenesis represents a new, promising therapeutic approach for a wide variety of cancers, including brain tumors, pre-clinical data indicate that antiangiogenic modalities, when used as a single therapy, only slow tumor growth. Appropriate patient selection and relevant biological end points are thus required, as well as careful design of therapeutic intervention [73]. Thus, the combination of antiangiogenic agents with cytotoxic chemotherapy or vascular targeting agents represent a promising option for increasing the efficacy of antitumoral therapies [73].

ii. Combined Ionizing Radiation/Antiangiogenesis Therapies

One innovative approach, which is now under intense investigation, is the combined utilization of antiangiogenic agents, aimed at blocking the formation of new blood vessel network within a tumor, with radiotherapy. According to the National Cancer Institute database there are currently more than 10 clinical trials that are using antiangiogenic molecules, often in combination with conventional approaches such as radiotherapy or chemotherapy. Single doses of ionizing radiation (IR) have recently been shown to preferentially damage the endothelium [74-77] and could have profound implications for cancer therapy. Recent evidence shows that combining IR with angiostatin, a proteolytic fragment of plasminogen, improved tumor eradication [78-80]. Most of the recent data, however, documented the use of synthetic agents in combination with radiotherapy. An alkylating agent such as temozolomide was shown to prevent irradiation-induced glioma cell invasion [81], while the orally available VEGF receptor inhibitor PTK787 [82], combined with IR, was shown to decrease both endothelial cell proliferation and the number of microvessels in tumor xenografts. Similarly, other antiangiogenic agents such as SU5416 (an inhibitor of VEGF receptor) and SU6668 (an inhibitor for VEGF, FGF and PDGF receptors) were also recently shown to increase the antitumor effects of fractionated IR [79]. Some other promising synthetic agents include thalidomide [83], gemcitabine, paclitaxel, docetaxel, irinotecan and vinorelbine [84], as well as rofecoxib (Vioxx), a specific COX-2 inhibitor that was found to inhibit endothelial cell function in combination with IR [85]. However, the radiosensitizing ability of these agents has so far shown limited efficacy in standard treatments for patients with a number of types of cancer.

We have recently reported that the naturally occurring green tea catechin EGCG effectively inhibits the VEGF receptor-2 tyrosine kinase activity in endothelial cells [53]. The clinical potential of green tea for decreasing the incidence of several cancers through its multiple anticancer and antiangiogenic properties was recently reviewed [1, 86]. Moreover, we have recently reported that the irradiation-induced tubulogenesis in endothelial cells was antagonized by EGCG [87]. It is thus tempting to hypothesize that such inhibitory mechanisms may be specifically responsible for the actions of EGCG and other VEGFR inhibitors in synergy with IR. The recent discovery that several naturally occurring polyphenols, including EGCG from green tea, inhibited tumor angiogenesis has shed light on the beneficial effects of these natural products. The fact that these natural polyphenols could be used in combination with radiotherapy is appealing as it was recently investigated [87]. The *in vitro* effects of IR on HUVEC migration and tubulogenesis were first assessed, the latter angiogenic function being a prerequisite for neovascularization to occur (Fig. **3**). It was then demonstrated that IR upregulates specialized angiogenic markers, namely MT1-MMP and caveolin-1, both known to regulate endothelial cell migration and tubulogenesis. The IR-induced increase in caveolin-1 directly correlates with the increase in capillary-like structure formation in HUVEC and is in agreement with caveolin-1 involvement in tubulogenesis [88]. Moreover, integrin β_3 transcript and protein expression were also upregulated by IR while that of cell surface tissue transglutaminase (tTG) was decreased. Pretreatment of endothelial cells with low concentrations of EGCG specifically antagonized the IR-induced effects. Collectively, these observations are suggestive of crucial protein interactions involving MT1-MMP/caveolin-1/integrins $\alpha v\beta_3$ within a limited caveolar microenvironment at the cell surface, by which angiogenic functions could be specifically downregulated by combined antiangiogenic/radiotherapeutic treatments. These observations should prompt the development of combined synergistic radiotherapy and antiangiogenic approaches to clinical

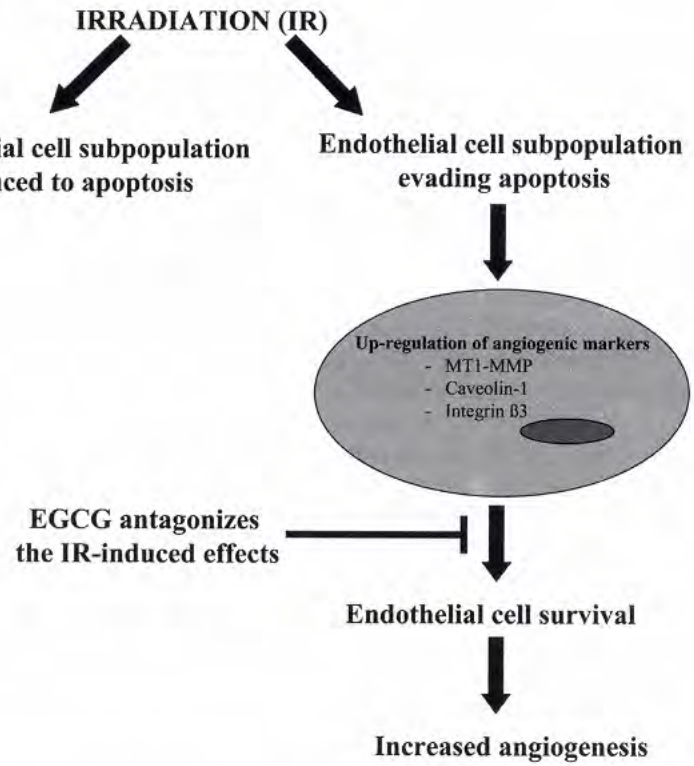

Fig. (3). Combined irradiation and green tea polyphenol treatments. Irradiation has been shown to preferentially damage the endothelium. In particular, low irradiation doses affect the expression of angiogenic markers in a subpopulation of endothelial cells that escape irradiation-induced apoptosis. This leads to an increased capacity of these cells to migrate and to form capillary-like structures (tubulogenesis), which could favor tumor neovascularization and hence tumor progression. Pre-treatment with EGCG antagonized all the IR-induced effects on cell migration and endothelial cell tubulogenesis, two phenomena associated to angiogenesis.

treatment in order to target those tumor-derived endothelial cells that escape IR-induced apoptosis.

In light of the observations that IR induces HUVEC adhesion to fibronectin, which is reversed by EGCG [87], one may assume that EGCG specifically modulates the cell's interaction with fibronectin through specific cell surface integrins. Moreover, modulation of signal transduction pathways by tea polyphenols has also helped elucidate many molecular mechanisms of cancer chemoprevention by green tea. Of particular interest is the suppression by tea polyphenols of various tumor biomarkers and related transducing proteins including growth factor receptor tyrosine kinases, cytokine receptor kinases, PI3K, and the serine/threonine Akt (protein kinase B) [59]. Since the PI3K/Akt signal transduction pathway was recently shown to be activated in HUVEC by IR [89], it is tempting to suggest that this same pathway may well be inhibited by the actions of low EGCG concentrations, and may profoundly modulate adhesion receptor signaling.

Since combined antiangiogenic/radiotherapy treatments are already underway as Clinical Phase I trials in adult patients, it was proposed that the cooperative antitumoral effects of naturally occurring GTP possessing antiangiogenic properties, combined with irradiation treatments, will help optimize the present clinical treatments. The synergistic effects obtained when radiotherapy is combined with low-dose metronomic antiangiogenic therapy should thus provide the underpinnings for the development of new therapeutic strategies using a nutraceutical approach that would include natural compounds such as GTP or EGCG in combination with radiotherapy.

iii. Bone Marrow-Derived Stromal Cells as Potential Targets for Green Tea Polyphenols During Angiogenesis

The multiple inhibitory activities of EGCG, that affect soluble MMP secretion [52], MT1-MMP-mediated cell migration [90] and tubulogenesis [53], were recently reported to also affect the angiogenic properties of bone marrow-derived stromal cells (BMSC) [70, 91]. The recently reported unorthodox plasticity and endothelial cell-like phenotype of BMSC, which represent a subpopulation of nonhematopoietic pluripotent cells within the bone marrow microenvironment [92-94], may provide new insights into their potential role in neo-vascularization as well as in microvascular network remodeling. Interestingly, recent studies suggest that BMSC participate in angiogenesis and arteriogenesis *de novo* [91], as well as in the vascularization of a subcutaneously implanted U-87 glioma-derived tumor [95]. As EGCG was shown to target MT1-MMP-dependent activation of proMMP-2 [52] as well as to antagonize three-dimensional capillary-like structure formation in BMSC [91], it is conceivable that green tea extracts may have the capacity to target crucial angiogenic properties of early progenitor cells that may exhibit an endothelial cell-like phenotype.

These EGCG inhibitory activities in bone marrow-derived cells may affect future clinical use because EGCG

specifically inhibited growth of leukemic cell lines while normal bone marrow-derived hematopoietic progenitors remained unaffected. Moreover, it was also observed that BMSC can be recruited to active sites of angiogenesis in response to tumor-derived growth factors [95] and to selectively reach tumor sites, proliferate there, and participate in the formation of tumor stroma [96].

Synergistic Action of EGCG with Other Natural Compounds

Synergistic actions between green tea and other agents have been documented. The antineoplastic drugs doxorubicin [97], tamoxifen [98], phytic acid [99] and sulindac [98, 100-102] have all been reported to exert synergistic activities with either green tea or EGCG. In light of these published studies, the synergistic effects of green tea with other natural compounds have been investigated for cancer-prevention activity. The synergy between a decaffeinated green tea concentrate and a vanilloid-containing Capsicum preparation have been reported [103]. This combination is 100-fold more potent than green tea alone for the inhibition of tNOX, a cancer-specific growth protein. Moreover, the synergistic effects between these two natural extracts also produced stronger growth inhibition of human cervical carcinoma and mouse mammary cancer cells in culture. Co-treatment with EGCG and Sulindac, examined using a human cancer cDNA expression array, induced modulation of gene expression in a human lung cancer cell line (PC-9) [104]. This co-treatment upregulated the expression of the GADD153 and WAF1 genes whereas the expression of tissue-type plasminogen activator, TIMP3, IL-1β and integrin-β4 genes was downregulated. The combination of soy phytochemical concentrate (SPC) and green tea synergistically prevented the progression and metastasis of androgen-sensitive prostate tumors *in vivo* [105]. This synergistic inhibition by the green tea and SPC combination is associated with effective reductions of serum levels of both testosterone and dihydrotestosterone, suggesting that modulation of androgen levels is an important mechanism for the synergistic prevention of prostate cancer progression by the soy/green tea combination. Overall, these results demonstrate that the combined use of green tea polyphenols and cancer preventive agents is a practical way to enhance the beneficial potential of cancer preventive agents. The molecular mechanisms involved in the synergistic effects remain to be investigated in order to understand the efficacy and safety of many chemopreventive compounds with future therapeutic potential.

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