Green tea compounds inhibit tyrosine phosphorylation of PDGF β-receptor and transformation of A172 human glioblastoma

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Abstract The effect of the green tea compounds 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3,5,7-triol (catechin), epicathechin (EC), epigallocathechin-3 gallate (EGCG), epicathechin-3 gallate (ECG) and catechin-3 gallate (CG) on the tyrosine phosphorylation of PDGF β-receptor (PDGF-Rβ) and on the anchorage-independent growth of A172 glioblastoma cells in semisolid agar has been investigated. Treatment of A172 glioblastoma with 50 μM CG, ECG, EGCG and 25 μM Tyrphostin 1296 resulted in an 82 ± 17%, 77 ± 21%, 75 ± 8% and 55 ± 11%, respectively (mean ± S.D., $n = 3$) inhibition of the PDGF-BB-induced tyrosine phosphorylation of PDGF-Rβ. The PDGF-Rβ downstream intracellular transduction pathway including tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1) and phosphatidylinositol 3′-kinase (PI 3′-K) was also inhibited. Spheroid formation was completely inhibited by 50 μM ECG, CG, EGCG and by 25 μM Tyrphostin 1296. We conclude that catechins of the green tea possessing the gallate group in their chemical structure act as anticancer agents probably partly via their ability to suppress the tyrosine kinase activity of the PDGF-Rβ.

Key words: Catechin; A172 glioblastoma; PDGF-Rβ; PLC-γ1; PI 3′-K; Tumorigenicity

1. Introduction

Several epidemiological studies suggest that black and especially green tea consumption is associated with a reduced risk of several forms of cancer in human populations [1,2]. Furthermore, there is evidence from experimental animal studies that consumption of green tea inhibits several tumor types [3–5]. Green tea consists mainly of catechins such as 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3,5,7-triol (catechin), epicathechin (EC), epigallocathechin-3 gallate (EGCG), epicathechin-3 gallate (ECG) and catechin-3 gallate (CG). In the last years, major attention has been focused on the anticancer activity of the green tea compound EGCG. In order to explain the anticancer activity of EGCG, different mechanisms have been postulated, e.g. EGCG might inhibit enzyme urokinase (uPA) activity, one of the most frequently overexpressed enzymes in human cancers [6]. However, the used effective concentration of EGCG seems to be physiologically too high (1 to 10 mM) for anticancer prevention observed by green tea [6]. More recently, it has been reported that EGCG may prevent cancer by inhibition of angiogenesis [7]. In this context, a remarkable suppression of the vascular endothelial growth factor (VEGF)-induced vascularization by oral green tea consumption in the mouse cornea has been demonstrated. Also, EGCG effectively inhibited the angiogenesis in the chick chorioallantoic membrane assay [7]. In an attempt to offer a possible explanation for the anticancer activity of EGCG, we recently demonstrated that colony formation of the *sis*-transfected NIH 3T3 cells and spheroid's formation of human glioblastoma cells (A172) in semisolid agar were effectively inhibited by 50 μM EGCG and Tyrphostin 1296 [8]. Tyrphostin 1296 is a selective inhibitor of the tyrosine phosphorylation of the PDGF-Rβ [9,10]. PDGF-BB propagates mitogenic signals through autophosphorylation of its PDGF-Rβ on tyrosine residues. Autophosphorylation of PDGF-Rβ results in tyrosine phosphorylation of different substrate proteins such as the phospholipase C-γ1 (PLC-γ1), $p21^{ras}$ GTPase activating protein (GAP) and phosphatidylinositol 3′-kinase (PI 3′-K) carrying Src homology region 2 (SH_2) domains that are capable of binding to specific regions of the phosphorylated PDGF-Rβ [11,12]. In addition, activation of Ras occurs through binding of the adapter protein complex Shc/Grb2/Sos to the tyrosine phosphorylated PDGF-Rβ resulting in an activation of the Raf/MAPKK and MAP kinase pathway [13]. In the last years major attention has been focused on EGCG and little is known about the anticancer activity of other green tea compounds. Furthermore, little is known about the intracellular mechanisms explaining the anticancer activity of the green tea compounds. The ability of the cells to grow in an anchorage-independent fashion is considered to be the classic predictor of tumorigenicity [14]. Therefore, we examined the effect of EGCG, ECG, CG, catechin and EC on the anchorage-independent growth of A172 cells in semisolid agar [15]. Since autocrine activation of the PDGF-Rβ seems to be the initial cause of the development of the human A172 glioblastoma [16] we examined the effect of the different catechins on the PDGF-Rβ mediated intracellular transduction pathway.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL, Eggenstein, Germany. (−)-ECG, (−)-CG, (−)-EGCG, (−)-EC catechin and Sepharose-coupled anti-phosphotyrosine antibody were obtained from Sigma Chemical, Deisenhofen, Germany. Tyrphostin AG1296 was obtained from Calbiochem, Bad Soden, Germany. PDGF-BB was prepared as described previously [17]. Monoclonal mouse anti-PI 3′-K, mouse anti-PLC-γ1, mouse antibodies were obtained from Transduction Laboratories, Lexington, KY, USA. The anti-PDGF-Rβ and anti-EGF rabbit polyclonal antibodies were obtained from Santa Cruz (Heidelberg, Germany). The polyclonal anti-CL100 antibody was a gift from Dr. Dirk Bokemeyer, Medizinische Universitäts-Poliklinik, Bonn, Germany. The horseradish peroxidase-

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labeled anti-rabbit and anti-mouse antibodies were obtained from Amersham Life Sciences, Little Chalfont, UK.

2.2. Cell culture

A172 from human (male, 53 years old) were obtained from Interlab Cell Line Collection, Genoa, Italy. Cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), non-essential amino acids, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in the Steri-cult incubator from Forma Scientific in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Soft agar assay

The soft agar assay was performed as described previously [14]. Briefly, 35 mm petri dishes were underlain with 1 ml MEM supplemented with 0.7% agar, 10% FCS and EGCG. After trypsinization, 5×10^4 A172 cells were suspended in 1.5 ml MEM supplemented with 0.35% agar, 10% FCS and 10 or 50 μM of catechins and plated on the 0.7% agar underlay. Cells were fed once per week with 2 ml MEM supplemented with 10% FCS and 10 or 50 μM of catechins. Cells were photographed by phase-contrast light microscope after 1 h and 3 weeks.

2.4. Gel electrophoresis and immunostaining

Confluent cells in 75 cm^2 were preincubated in serum-free medium consisting of DMEM and Ham's F-10 (1:1, v/v), in the presence and absence of 50 μM of each catechin compound for 24 h. Then the medium was replaced with serum-free medium without catechins and A172 glioblastoma were stimulated with 50 ng/ml PDGF-BB. After removing the medium cells were lysed with a 1 ml RIPA buffer (50 mM NaCl, 20 mM Tris–HCl, 50 mM NaF, 10 mM EDTA, 20 mM $Na_4P_2O_7\cdot10H_2O$, 1% Triton X-100, pH 7.4) containing 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml antipain, and 0.023 TIU/ml aprotinin. After 10 min at 0°C, cell lists were centrifuged at $14\,000\times g$ for 2 min. Then supernatant was mixed with 80 μl Sepharose-coupled anti-phosphotyrosine antibody and shaken for minimum 2 h at 4°C. After elution of tyrosine phosphorylated proteins with 100 μl of lysis buffer containing 5 mM phenylphosphate protein determination was performed using the Bio-Rad DC Protein Assay. Proteins were separated in a 7.5% SDS–polyacrylamide gel (SDS–PAGE). Proteins were transferred to a PVDF membrane overnight at 100 mA with a buffer containing 25 mM Tris–HCl, 192 mM glycine and 20% methanol, pH 8.3. Enhanced chemiluminescence Western blotting analysis was performed with the chemiluminescence Western blotting system from NEN Life Science Products, Inc. (Boston, MA, USA), using primary monoclonal anti-PI 3′-K (1:5000), anti-PLC-γ1 (1:1000), or polyclonal anti-PDGF-Rβ (1:500) IgGs. Chemiluminescence detection of PDGF-Rβ was performed using secondary horseradish peroxidase-labeled anti-rabbit IgG (1:5000). Detection of PI 3′-K, PLC-γ1 was performed using secondary horseradish peroxidase-labeled anti-mouse IgG (1:5000).

Immunoprecipitation and detection of mitogen-activated protein kinase phosphatase-1 (MKP-1), the mouse homologue of CL100 (97% identity), were performed as we previously described [18]. Briefly, confluent cells in 10 cm (diameter) culture dishes were incubated in serum-free medium consisting of a mixture of DMEM and Ham's F-10 medium (1:1) in the presence and absence of 50 μM EGCG for 24 h. Cells were then stimulated with 50 ng/ml PDGF-BB for 60 min. After removal of the medium, cells were lysed in 400 μl Triton X-100 lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM

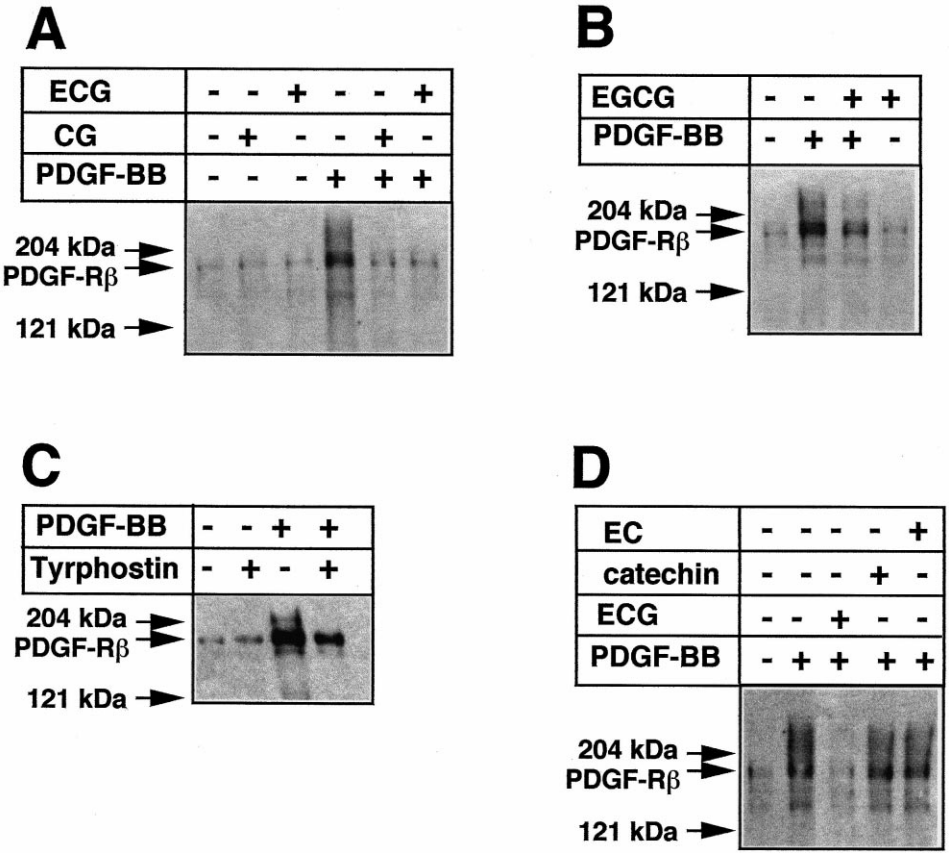

Fig. 1. Effect of PDGF-BB on tyrosine phosphorylation of PDGF-Rβ in A172 glioblastoma treated with 50 μM (−)-ECG, (−)-CG, (−)-EGCG, (−)-EC, catechin, and Tyrphostin 1296. Confluent cells in 75 cm^2 were preincubated in serum-free medium consisting of DMEM in the presence and absence of 50 μM of each catechin compound or 25 μM Tyrphostin 1296 for 24 h. Then the medium was replaced with serum-free medium without catechins and A172 glioblastoma were stimulated with 50 ng/ml PDGF-BB for 5 min. Cell lysates were mixed with 80 μl Sepharose-coupled anti-phosphotyrosine antibody to immunoprecipitate PDGF-Rβ. Tyrosine phosphorylated PDGF-Rβ was eluted with lysis buffer containing 5 mM phenylphosphate. Tyrosine phosphorylated PDGF-Rβ (180 kDa) was detected by the enhanced chemiluminescence Western blotting method using polyclonal rabbit anti-PDGF-Rβ IgG (1:500) and secondary horseradish peroxidase-labeled anti-rabbit IgG (1:5000).

NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF for 5 min at 4°C. Lysates (500 μg protein) were incubated for 2 h with 2 μl polyclonal anti-CL100 antibody. Immunocomplexes were adsorbed to protein A-Sepharose and washed three times with lysis buffer. Proteins were resolubilized by addition of an equal volume of 2× sample buffer. Then SDS–PAGE was performed using 4% and 10% acrylamide for stacking and resolving gels, respectively. Protein was transferred to nitrocellulose and probed with polyclonal antibodies against CL100. Detection of the MKP-1 was performed using horseradish peroxidase-conjugated protein A and the ECL chemiluminescence system from Amersham.

3. Results and discussion

Treatment of A172 glioblastoma cells with CG, ECG, EGCG at a concentration of 50 μM and with 25 μM Tyrphostin 1296 resulted in a remarkable inhibition of the PDGF-BB-induced tyrosine phosphorylation of PDGF-Rβ (Fig. 1A–C). CG, ECG and EGCG at a concentration of 50 μM as well as 25 μM Tyrphostin 1296 had no effect on the basal tyrosine phosphorylation of PDGF-Rβ. Laser densitometric analysis of the band densities of the tyrosine phosphorylated PDGF-Rβ obtained by three separate experiments showed that treatment of the A172 cells with 50 μM CG, ECG, EGCG and 25 μM Tyrphostin 1296 resulted in a 82 ± 17%, 77 ± 21%, 75 ± 8% and 55 ± 11%, respectively, (mean ± S.D., $n = 3$), inhibition of the PDGF-BB-induced tyrosine phosphorylation of the PDGF-Rβ in untreated cells (= 100%). Catechin and EC had no effect on the PDGF-BB-induced tyrosine phosphorylation of PDGF-Rβ (Fig. 1D). Again, treatment of the cells with 50 μM EC or ECG caused a remarkable decrease of the PDGF-BB-induced tyrosine phosphorylation of PDGF-Rβ (Fig. 1D). In order to demonstrate that inhibition of the PDGF-BB-induced tyrosine phosphorylation of PDGF-Rβ by effective catechins also results in an inhibition of the downstream intracellular signalling transduction pathway of PDGF-BB, parallel determination of the tyrosine phosphorylated PDGF-Rβ, PLC-γl and PI 3′-K in CG-treated cells was performed. Again, as demonstrated in Fig. 2, treatment of the cells with 50 μM CG resulted in a marked decrease of the PDGF-BB-induced tyrosine phosphorylation of PDGF-Rβ, PLC-γl and PI 3′-K. Results from three independent experiments demonstrated that treatment of the cells with 1 to 20 μM CG, ECG and EGCG had no statistically significant effect on tyrosine phosphorylation of PDGF-Rβ, PLC-γl and PI 3′-K.

In order to demonstrate whether there is a correlation between the inhibitory effect of CG, ECG, EGCG or Tyrphostin 1296 on tyrosine phosphorylation of PDGF-Rβ and the transformation of A172 cells, spheroid formation of these cells in soft agar in the presence and absence of CG, ECG, EGCG or Tyrphostin 1296 was examined. Spheroid formation was almost completely inhibited by 50 μM ECG, CG and EGCG and by 25 μM Tyrphostin 1296 (Fig. 3). CG, ECG and EGCG at a concentration of 10 μM had no effect on spheroid formation (Fig. 3). Catechin and EC at a concentration of 50 μM had no effect on spheroid formation (data not shown). The present study gives evidence that not only EGCG but also catechins that possess the gallate group (Fig. 4) are able to suppress the tyrosine kinase activity of the PDGF-Rβ. However, one may question whether concentrations of green tea catechins are sufficiently high for inhibition of the kinase activity of PDGF-Rβ. Green tea contains significant

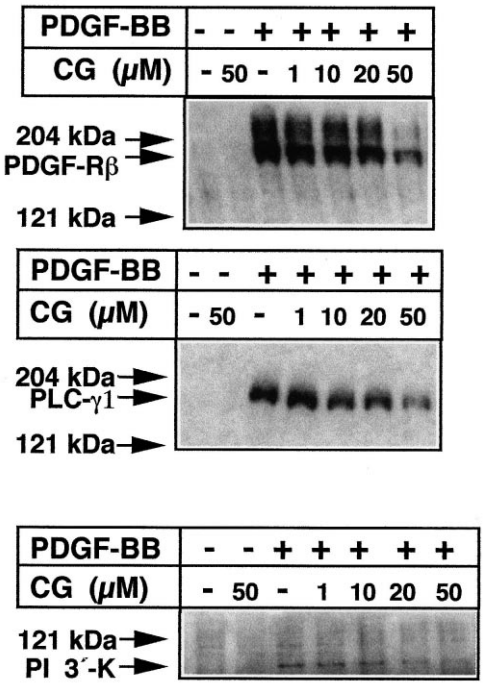

Fig. 2. Effect of PDGF-BB on tyrosine phosphorylation of PDGF-Rβ, PLC-γl and PI 3′-K in A172 glioblastoma treated with different concentrations of (−)-CG. Confluent cells in 75 cm^2 were preincubated in serum-free medium consisting of DMEM in the presence and absence of different concentrations of (−)-CG for 24 h. Then the medium was replaced with serum-free medium without (−)-CG and A172 glioblastoma were stimulated with 50 ng/ml PDGF-BB for 5 min. Cell lysates were mixed with 80 μl Sepharose-coupled anti-phosphotyrosine antibody to immunoprecipitate tyrosine phosphorylated PDGF-Rβ (180 kDa), PLC-γl (150 kDa) and PI 3′-K (85 kDa). Tyrosine phosphorylated proteins were detected by the enhanced chemiluminescence Western blotting method using primary polyclonal rabbit anti-PDGF-Rβ IgG (1:500), monoclonal anti-PI 3′-K (1:5000) and monoclonal anti-PLC-γl (1:1000) IgGs. Detection of tyrosine phosphorylated PDGF-Rβ was performed using secondary horseradish peroxidase-labeled anti-rabbit IgG (1:5000). Detection of PI 3′-K and PLC-γl was performed using secondary horseradish peroxidase-labeled anti-mouse IgG (1:5000).

amounts of catechins. Approximately 30–42% of the dry weight of the green tea are catechins [19]. Furthermore, a cup of green tea contains approximately 200 mg EGCG and after drinking of two to three cups of tea the concentration of EGCG in the plasma was 0.3 μM [4,7,20]. However, it is possible that catechins are able to accumulate in tissues thereby resulting in locally and temporally high concentrations of catechins. Since EGCG and probably other catechins bind strongly to many molecules and affect a variety of enzyme activities and signal transduction pathways at μM concentrations [20] it can not be concluded that the anticancer activity of the catechins is only related to the inhibition of the kinase activity of PDGF-Rβ. Indeed, catechins may also exert anticancer activities via inhibition of other kinases. On the other hand it is well established that the PDGF-Rβ tyrosine kinase of the A172 cells is chronically activated by endogenous PDGF-BB [16]. Autocrine activation of the PDGF-Rβ seems to be the initial cause of the development of A172 glioblastoma [15,16]. In this context, we demonstrated that several catechins prevented multicellular spheroid formation of human A172 cells in semisolid agar. This finding suggests that EGCG

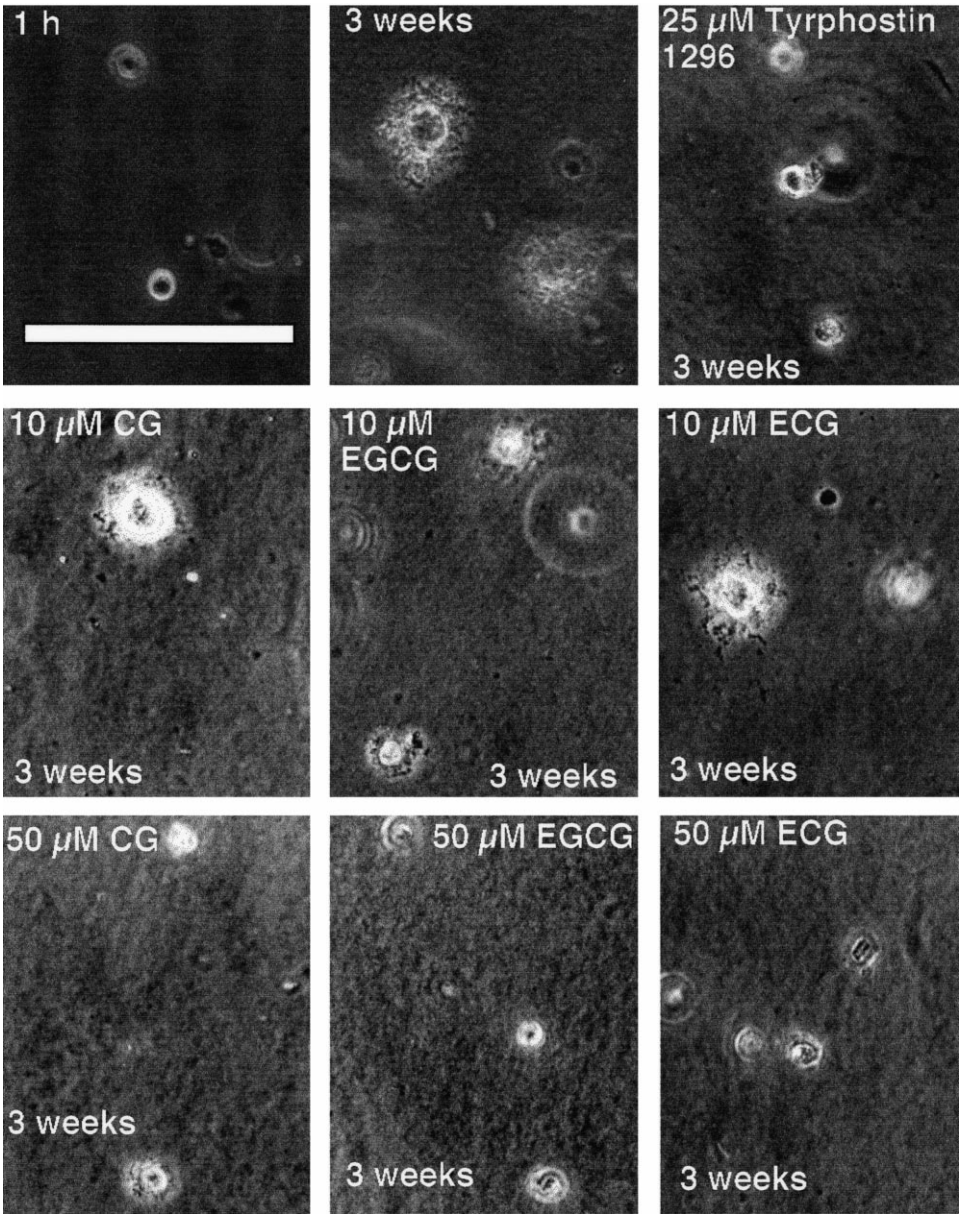

Fig. 3. Anchorage-independent growth of A172 glioblastoma cells in the presence and absence of the catechins. Petri dishes (35 mm) were underlain with 1 ml modified Eagle's medium (MEM) supplemented with 0.7% agar, 10% fetal calf serum (FCS) and catechins. After trypsinization 5×10^4 A172 cells were suspended in 1.5 ml MEM supplemented with 0.35% agar, 10% FCS and 10 to 50 μM of each catechin compound and plated on the 0.7% agar underlay. Representative fields were photographed after 1 h and 3 weeks by phase-contrast light microscope. Calibration bar represents 250 μm.

might reduce cancer diseases in which activation of PDGF-Rβ is causatively involved.

In order to explain the anticarcinogenic effects of green tea observed in several epidemiological studies [3] it has been also postulated that EGCG acts as a scavenger of reactive oxygen species (ROS) thereby preventing carcinogenesis [3,21]. It is widely believed that ROS are involved in the initiation of carcinogenesis. Besides the proposed mechanism that inhibition of tumor growth by green tea might be due to EGCG mediated suppression angiogenesis [7] or due to the antioxidant potency of EGCG protecting DNA from oxidative damage, the mechanism we suggest might partly explain the anticancer activity of several catechins found in green tea. Indeed, consumption of green tea may act protectively against cancer diseases by several mechanisms.

Enhanced activity of receptor tyrosine kinases including the PDGF-Rβ, the EGF-R, VEGF-R has been implicated as a contributing factor for the development of malignant proliferative diseases such as cancer [10,22]. PDGF and its receptors have been implicated in a number of cancer diseases including gliomas [15], sarcomas [15], breast cancer [23], colon cancer [24] and melanoma [25]. Therefore, in the last decade tremendous efforts have been made to develop chemical synthesized tyrosine kinase inhibitors as drug target for therapeutically treatment of cancer diseases [10,26]. In the present study we demonstrate that several natural non-toxic compounds isolated from green tea are also able to act as PDGF-Rβ tyrosine kinase inhibitors thereby preventing spheroid formation of A172 glioblastoma cells. Therefore, we suggest that these green tea compounds may be useful for treatment of cancer diseases in which PDGF-BB and PDGF-Rβ are involved [15,16]. Furthermore, chemical modification of

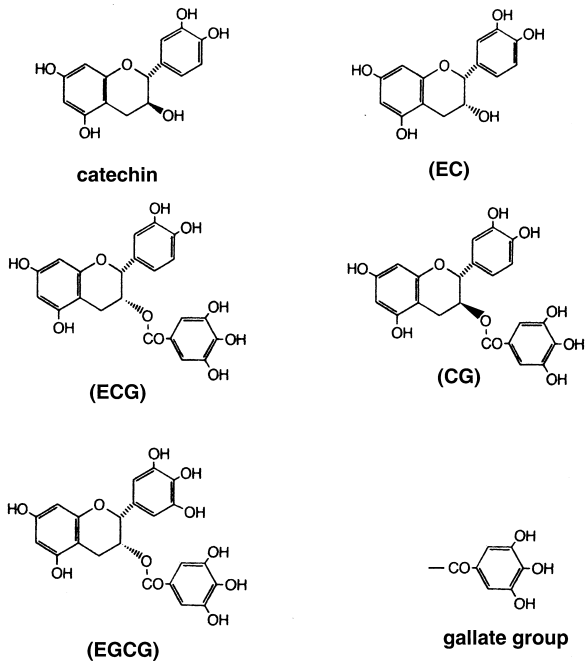

Fig. 4. Chemical structure of the different catechins found in green tea.

the chemical structure of the gallate group may result in much more effective compounds in inhibiting PDGF-related carcinogenesis. However, further clinical studies are necessary to apply green tea compounds for treatment of cancer diseases.

The vaccine H-1 gene product (VH-1) was the first phosphatase shown to effectively hydrolyze both phosphotyrosine and phosphoserine/phosphothreonine [27]. The MKP-1 is a mammalian VH-1-like dual specificity tyrosine phosphatase [28]. The human homologue of MKP-1 is called CL100 (97% identity) [28]. Since it is possible that a decrease of the tyrosine phosphorylation of the PDGF-Rβ could be due to an enhanced phosphatase activity we examined the effect of EGCG on the MKP-1 formation in the A172 cells after stimulation with 50 ng/ml PDGF-BB for 1 h. Furthermore, in order to demonstrate whether catechins specifically inhibit the tyrosine phosphorylation of PDGF-Rβ we examined the effect of EGCG on the tyrosine phosphorylation of the EGF-R after stimulation of the cells with 50 ng/ml EGF for 10 min and immunoprecipitation of tyrosine phosphorylated EGF-R using Sepharose-coupled anti-phosphotyrosine antibody. Pretreatment of the cells for 24 h with 50 μM EGCG had no effect on the PDGF-induced MKP-1 induction or on the EGF-induced increase of the tyrosine phosphorylation of the EGF-R in A172 cells (data not shown). These findings are in good agreement with our previous findings demonstrating that EGCG had no effects on the EGF-induced stimulation of the extracellular signal-regulated kinases (ERK)1/2 in vascular smooth muscle cells from rat aorta [8]. Furthermore, we suggest that effective catechins act via inhibition of the kinase activity of PDGF-Rβ and not via increase of MKP-1.

Since it has been demonstrated that green tea and EGCG inhibit VEGF-induced angiogenesis [7] we assume that EGCG and other effective consumption catechins may act via inhibition of the tyrosine phosphorylation of the VEGF receptors. However, the question whether catechins inhibit tyrosine phosphorylation of FGF or insulin receptors remain to be elucidated.

In summary, we demonstrated that catechins of the green tea possessing the gallate group in their chemical structure act as anticancer agents probably partly via their ability to suppress the tyrosine kinase activity of the PDGF-Rβ. The present findings may be helpful to explain the anticancer activity of green tea observed in several epidemiological studies. Finally, these findings may be important for development of new prophylactic strategies for prevention of carcinogenesis in the general human population.

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