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Epigallocathechin-3 Gallate Selectively Inhibits the PDGF-BB–induced Intracellular Signaling Transduction Pathway in Vascular Smooth Muscle Cells and Inhibits Transformation of *sis*-transfected NIH 3T3 Fibroblasts and Human Glioblastoma Cells (A172)

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Enhanced activity of receptor tyrosine kinases such as the PDGF β-receptor and EGF receptor has been implicated as a contributing factor in the development of malignant and nonmalignant proliferative diseases such as cancer and atherosclerosis. Several epidemiological studies suggest that green tea may prevent the development of cancer and atherosclerosis. One of the major constituents of green tea is the polyphenol epigallocathechin-3 gallate (EGCG). In an attempt to offer a possible explanation for the anti-cancer and anti-atherosclerotic activity of EGCG, we examined the effect of EGCG on the PDGF-BB–, EGF-, angiotensin II-, and FCS-induced activation of the 44 kDa and 42 kDa mitogen-activated protein (MAP) kinase isoforms ($p44^{mapk}$/$p42^{mapk}$) in cultured vascular smooth muscle cells (VSMCs) from rat aorta. VSMCs were treated with EGCG (1–100 μM) for 24 h and stimulated with the above mentioned agonists for different time periods. Stimulation of the $p44^{mapk}$/$p42^{mapk}$ was detected by the enhanced Western blotting method using phospho-specific MAP kinase antibodies that recognized the Tyr204-phosphorylated (active) isoforms. Treatment of VSMCs with 10 and 50 μM EGCG resulted in an 80% and a complete inhibition of the PDGF-BB–induced activation of MAP kinase isoforms, respectively. In striking contrast, EGCG (1–100 μM) did not influence MAP kinase activation by EGF, angiotensin II, and FCS. Similarly, the maximal effect of PDGF-BB on the c-fos and egr-1 mRNA expression as well as on intracellular free Ca^{2+} concentration was completely inhibited in EGCG-treated VSMCs, whereas the effect of EGF was not affected. Quantification of the immunoprecipitated tyrosine-phosphorylated PDGF-Rβ, phosphatidylinositol 3′-kinase, and phospholipase C-γ1 by the enhanced Western blotting method revealed that EGCG treatment effectively inhibits tyrosine phosphorylation of these kinases in VSMCs. Furthermore, we show that spheroid formation of human glioblastoma cells (A172) and colony formation of *sis*-transfected NIH 3T3 cells in semisolid agar are completely inhibited by 20–50 μM EGCG. Our findings demonstrate that EGCG is a selective inhibitor of the tyrosine phosphorylation of PDGF-Rβ and its downstream signaling pathway. The present findings may partly explain the anti-cancer and anti-atherosclerotic activity of green tea.

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INTRODUCTION

Classic growth factors such as PDGF-BB and EGF propagate their mitogenic signals through autophosphorylation of their respective PDGF β-receptor (PDGF-Rβ) and EGF receptor (EGF-R) 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). Substrate proteins carry Src homology region 2 domains that are capable of binding to specific regions of the phosphorylated PDGF-Rβ (Kaplan *et al.*, 1990; Rönnstrand *et al.*, 1992). Activation of PLC-γ1 results in an elevation of inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol (Sachinidis *et al.*, 1990). It is assumed that $InsP_3$ mobilizes Ca^{2+} from intracellular stores (Berridge and Irvine, 1989). Activation of mitogen-activated protein (MAP) kinase pathway is discussed as being critical for the expression of nuclear transcriptional factors such as c-fos and non-nuclear protein kinases such as $p90^{rsk}$, which is involved in the regulation of cell growth (Pelech and Sanghera, 1992; Blenis, 1993). Activation of the MAP kinase pathway by PDGF-BB and EGF is initiated after binding of the adapter proteins Grb2/Sos to the their respective receptor, resulting in an activation of $p21^{ras}$. Sequential phosphorylation results in activation of the Raf-1 kinase, MAP kinase kinase, and $p44^{mapk}$/$p42^{mapk}$ (also known as extracellular response kinases 1 and 2). Grb2 is activated by the Src homology region 2 adaptor protein Shc, which is activated by tyrosine phosphorylation in response to the growth factors (Kaplan *et al.*, 1990; Pelech and Sanghera, 1992; Rönnstrand *et al.*, 1992; Blenis, 1993). After binding to the angiotensin II (Ang II) type 1 receptor (AT1), Ang II stimulates the phosphoinositide signaling system, protein kinase C, and MAP kinase via a Raf-1 kinase-independent pathway (Duan-Fang *et al.*, 1996).

Under physiological conditions the phosphorylated state of the receptor tyrosine kinases such as the PDGF-Rβ and EGF-R is at an equilibrium with the unphosphorylated inactive and the active phosphorylated state. Because enhanced activity of the receptor tyrosine kinases has been implicated in the pathogenesis of many cancers and other nonmalignant proliferative diseases such as atherosclerosis, inhibition of the intracellular signaling pathway of growth factors is crucial for preventing development of cancer and cardiovascular disease (Levitzki and Gazit, 1995). One prominent feature of the atherosclerotic lesions includes the proliferation of vascular smooth muscle cells (VSMCs) (Ross, 1993). It is widely believed that growth factors such as PDGF, EGF, and Ang II play a pivotal role in the development of hypertension and atherosclerosis by promoting VSMC growth (Daemen *et al.*, 1991; Ross, 1993).

During the last decade, green tea has been receiving strong attention as a preventing agent against cancer (Dreosti *et al.*, 1997) and cardiovascular disease (Tijburg *et al.*, 1997). Green tea consists mainly of polyphenols (also known as catechins), including epigallocathechin-3 gallate (EGCG), epigallocathechin (EGC), and epicathechin-3 gallate (ECG); however, up to now little is known about the molecular mechanisms explaining the anti-cancer and anti-atherosclerotic effects of green tea. We examine the hypothesis that the anti-cancer and anti-atherosclerotic effects of green tea can be attributed to the efficacy of EGCG to inhibit the intracellular signaling transduction pathway of growth factors. Therefore, we examined the effect of EGCG (the major constituent of the catechins) on the early intracellular transduction pathway of PDGF-BB, EGF, Ang II, and FCS in VSMCs and VSMC growth. The ability of the cells to grow in an anchorage-independent manner is considered to be the classic predictor of tumorigenicity (Freedman and Shin, 1974). Therefore, we examined the effect of EGCG on the anchorage-independent growth of A172 cells (Vassbotn *et al.*, 1994) and *sis*-transfected NIH 3T3 fibroblasts (Devare *et al.*, 1982; Beckman *et al.*, 1988) in semisolid agar.

MATERIALS AND METHODS

Materials

Ang II and EGF were obtained from Sigma Chemical (Deisenhofen, Germany) and Boehringer Mannheim (Mannheim, Germany), respectively. PDGF-BB was a gift from Professor Dr. Jürgen Hoppe (Physiological Chemistry, University of Würzburg, Germany) and was prepared as described (Hoppe *et al.*, 1989). Tyrphostin AG1296 was obtained from Calbiochem (Bad Soden, Germany). Antibodies were obtained from Transduction Laboratories (Lexington, KY). DMEM, Ham's F-10, Dulbecco's PBS, agar, and MEM were obtained from Life Technologies (Gaithersburg, MD). Hybond N+ membranes and ECL Western blotting detection system were obtained from Amersham (Little Chalfont, England). PhosphoPlus MAPK Antibody Kit was obtained from New England Biolabs (Beverly, MA). EGCG (purity >90%) with a molecular weight (M_r) of 458 was obtained from Wako Pure Chemical (Osaka, Japan). cDNA probes were obtained from Dianova-Oncor (Hamburg, Germany). A172 cells from human (male, 53 years old) were obtained from Interlab Cell Line Collection (Genoa, Italy).

Isolation and Culture of VSMCs

Rat aortic VSMCs were isolated from thoracic aorta of 6- to 8-wk-old Wistar–Kyoto rats (Charles River Wiga GmbH, Sulzfeld, Germany) by enzymatic dispersion using a slight modification of the method of Chamley *et al.* (1979) as described previously (Sachinidis *et al.*, 1995). Cells were cultured in DMEM supplemented with 10% fetal calf serum, nonessential amino acids, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in the Steri-cult incubator from Forma Scientific (Göttingen, Germany) in a humidified atmosphere of 95% air and 5% CO_2. The purity of VSMC cultures was confirmed by immunocytochemical localization of α–smooth-muscle actin.

Gel Electrophoresis and Immunostaining

Confluent cells in 3-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 EGCG for 24 h. VSMCs were then stimulated for different time periods with PDGF-BB. After removal of the medium, cells were lyzed with SDS sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS (wt/vol), 10% glycerol, and 50 mM dithiothreitol. Aliquots were used for protein determinations using the Bio-Rad (Bio-Rad, Richmond, CA) protein assay according to the method of Bradford (1976). Protein (10 μg) was analyzed with SDS polyacrylamide gel (SDS-PAGE) in a 12.5% acrylamide using the Mini Gel Protean system (Bio-Rad). Proteins were transferred to a polyvinylidene difluoride membrane overnight at 100 mA with a buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol, pH 8.3. The protein transfer was checked using Ponseau S staining. MAP kinase protein analysis was performed with the chemiluminescence Western blotting method as described in the instructions of the PhosphoPlus MAPK Antibody Kit (New England Biolabs) using a phospho-specific mapk rabbit polyclonal IgG primary antibody and the alkaline phosphatase-conjugated anti-rabbit secondary antibody. The primary antibody recognized $p42^{mapk}$ and $p44^{mapk}$ only when catalytically activated by phosphorylation at Tyr204 (Marshall, 1995).

Immunoprecipitation of PDGF-Rβ, PLC-γ1, and PI 3′-K was performed using Sepharose-coupled anti-phosphotyrosine antibodies. Briefly, confluent cells in 3-cm (diameter) culture dishes were incubated in serum-free medium in the presence and absence of EGCG for 24 h. VSMCs were then stimulated with PDGF-BB for 5 min. After removal of the medium, cells were lysed with 1 ml of buffer containing 137 mM NaCl, 20 mM Tris-HCl, pH 6.7, 2% SDS, 2% mercaptoethanol, 1 mM sodium orthovanadate. After 10 min at 0°C, cell lysates were centrifuged at 14,000 $\times$ *g* for 2 min. Then cell lysates were mixed with 80 μl of Sepharose-coupled anti-phosphotyrosine antibody to immunoprecipitate PI 3′-K, PLC-γ1, and PDGF-Rβ. Tyrosine-phosphorylated proteins were eluted with 100 μl of the lysis buffer containing 5 mM phenylphosphate. Twenty microliters were mixed with sample buffer and heated for 5 min at 95°C. After separation of proteins (5 μg) in a 7.5% SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane overnight by 100 mA with a buffer containing 25 mM Tris-base, 192 mM glycine, and 20% methanol, pH 8.3. The protein transfer was checked using Ponseau S. Enhanced chemiluminescence detection of PI 3′-K and PLC-γ1 was performed as described previously using monoclonal mouse anti-PI 3′-K (1:5000), mouse anti-phospholipase Cγ IgG (1:1000), and polyclonal rabbit anti–PDGF-Rβ IgG (1:500) and monoclonal mouse anti-horse radish peroxidase-labeled anti-mouse IgG.

Measurement of $[Ca^{2+}]_i$

VSMCs were cultured on round glass microscope slides (diameter 12 mm) under normal tissue culture conditions until confluence. Then medium was replaced with serum-free medium, and the cells were incubated in the presence and absence of EGCG for 24 h. Medium was then replaced with HEPES buffer (in mM: 20 HEPES, 16 glucose, 130 NaCl, 1 $MgSO_4$, 7 H_2O, 0.5 $CaCl_2$, Tris-base, pH 7.4) containing 2 μM fura-2 pentaacetoxymethyl ester and 1% BSA (wt/vol). Measurements were performed in HEPES buffer containing 1 mM $CaCl_2$. The Ca^{2+}–fura-2 fluorescence was measured at 37°C in a Perkin Elmer-Cetus (Norwalk, CT) LS50 fluorescence spectrofluorometer at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm (Grynkiewicz *et al.*, 1985). After calibration of fluorescence signals, $[Ca^{2+}]_i$ was calculated using the following equation: $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times (S_{f2}/S_{b2})$. K_d for the fura-2/Ca^{2+} complex at 37°C is assumed to be 224 nM. S_{f2} is the 380 nm-exited fluorescence in the absence of Ca^{2+} (EGTA added), and S_{b2} is the 380 nm-excited fluorescence in the presence of a saturating Ca^{2+} concentration (1 mM Ca^{2+}).

RNA Extraction and Analysis

The expression of c-fos and egr-1 mRNA was studied after preincubation of the cells for 24 h in serum-free medium (75-cm^2 culture flasks) in the presence and absence of EGCG. Then the VSMCs were stimulated with PDGF-BB for 30 min. VSMCs were lysed with 1 ml of TRI reagent (Sigma), and total RNA was extracted according to the manufacturer's protocol. Northern blotting was performed as described previously (Sambrook *et al.*, 1989). Ten micrograms of total RNA were separated by electrophoresis in a 6% formaldehyde/1.2% agarose gel, blotted on Hybond N+ membranes (Amersham), washed at room temperature in 5$\times$ SSC (1$\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 5 min, and fixed with UV irradiation. After fixing, the blots were washed at 60°C in 0.1$\times$ SSC, 0.1% SDS for 5 min. Prehybridization and hybridization were performed overnight at 60°C in 5$\times$ SSC, 0.2% SDS, 50 mM sodium phosphate, 10$\times$ Denhardt's solution (Sigma Chemical), and 200 μg/ml salmon sperm DNA. The DNA probes were labeled with ^{32}P-deoxycytidine triphosphate by random oligonucleotide priming to a specific activity of 2–4 $\times$ 10^9 dpm/μg DNA (Amersham Buchler, Braunschweig, Germany). The stringency of the final wash was 0.2$\times$ SSC containing 0.1% SDS at 65°C, two times for 45 min. A ^{32}P-labeled 1.0-kb v-fos cDNA fragment and a 2.1-kb egr-1 cDNA fragment were used as probes. Blots were exposed to Kodak films (Kodak X-OMAT, 8 $\times$ 10 inches; Kodak, Rochester, NY) for 3–7 d at −70°C. Blots were standardized using a 0.77-kb cDNA probe for β-actin (Dianova-Oncor). The size in kilobases of the detected mRNA was calculated by the 18S (1.8 kb) and 28S (4.6 kb) rRNA migration from the gel wells.

Determination of the Cell Counts

For cell counting, VSMCs were seeded in 24-well culture plates (5 $\times$ 10^4 cells/well; well diameter 12 mm) and cultured at 37°C for 24 h. Under these conditions, a cell confluence of ~70% was reached. The medium was then replaced by serum-free medium consisting of DMEM and Ham's F-10 (1:1, vol/vol) and EGCG. After 24 h medium was replaced with serum-free medium, and cells were stimulated with 50 ng/ml PDGF-BB. After 24 h the cells were trypsinized, and cell counting as well as determination of cell diameter was performed using the CASY-1 system based on the Coulter counter principle (Schärfe, Reutlingen, Germany).

Determination of the DNA Synthesis in VSMCs

The effect of PDGF-BB on [^{3}H]thymidine incorporation into cell DNA was assessed as performed previously (Sachinidis *et al.*, 1995). VSMCs were seeded in 24-well culture plates and grown to 70% confluence. The medium was then replaced by serum-free medium consisting of DMEM and Ham's F-10 (1:1, vol/vol) and EGCG. Cultures were then exposed to 50 ng/ml PDGF-BB for 20 h before 3 μCi/ml [^{3}H]thymidine were added to the serum-free medium. Four hours later, experiments were terminated by aspirating the medium and subjecting the cultures to sequential washes with PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% trichloroacetic acid, and ethanol:ether (2:1, vol/vol). Acid-insoluble [^{3}H]thymidine was extracted into 250 μl/dish 0.5 M NaOH, and 100 μl of this solution were mixed with 5 ml scintillant (Ultimagold; Packard, Meriden, CT) and quantified using a liquid scintillation counter, model Beckman LS 3801 (Düsseldorf, Germany). Fifty microliters of the residual solution were prepared for the determination of protein using the Bio-Rad protein assay as described previously (Bradford, 1976).

Soft Agar Assay

The soft agar assay was performed as described previously (Freedman and Shin, 1974). Briefly, 35-mm Petri dishes were underlaid with 1 ml MEM supplemented with 0.7% agar, 10% FCS, and EGCG. After trypsinazation, 5 $\times$ 10^4 A172 cells or *sis*-transfected NIH 3T3 fibroblasts

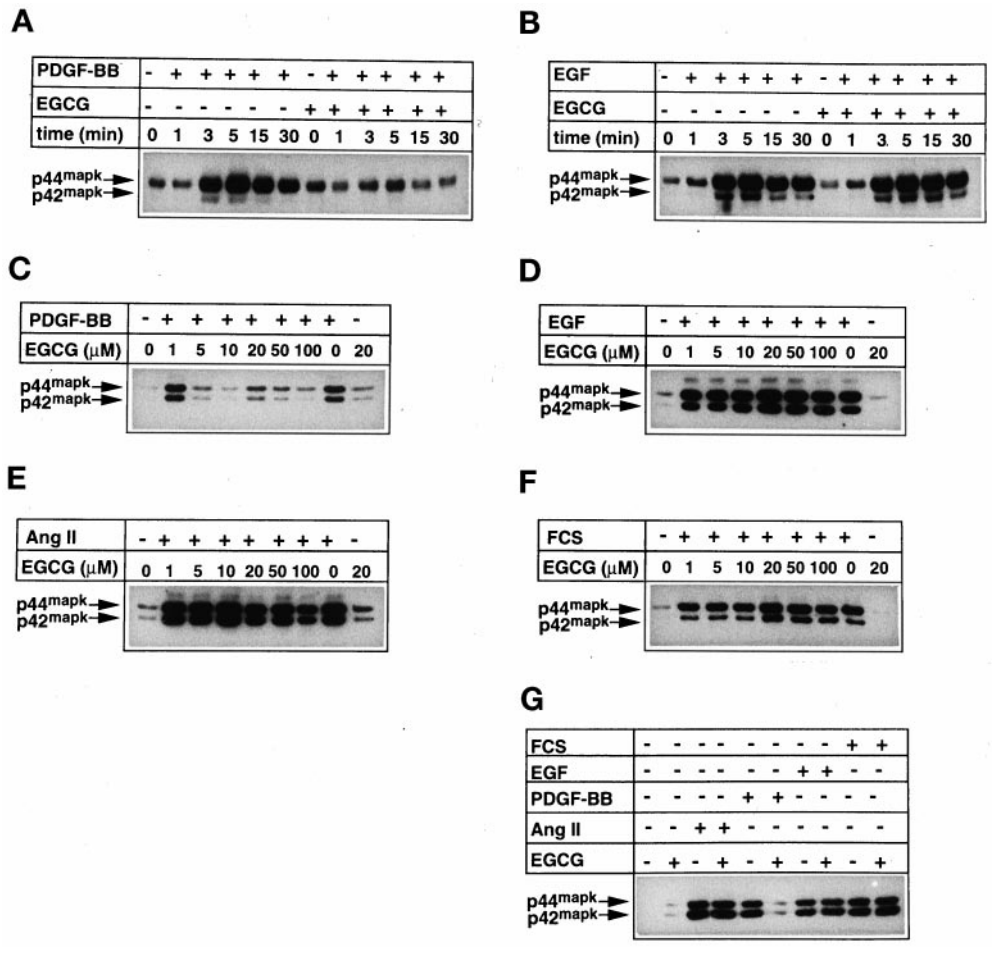


Figure 1. Effect of growth factors on the phosphorylation of the $p44^{mapk}/p42^{mapk}$ at Tyr 204 in EGCG pretreated VSMCs. (A) VSMCs were cultured in dishes (diameter: 3 cm) and cultivated until confluence. Then the medium was replaced by serum-free medium containing 50 μM EGCG. After 24 h the medium was replaced by serum-free medium without EGCG, and VSMCs were stimulated with PDGF-BB (A) and EGF (B) for different time periods. VSMCs were pretreated with different concentrations of EGCG and then stimulated for 5 min with 50 ng/ml PDGF-BB (C), 100 nM Ang II (E), 50 ng/ml EGF (D), and 5% FCS (F). EGCG-treated VSMCs were stimulated with 50 ng/ml PDGF-BB, 100 nM Ang II, and 50 ng/ml EGF for 5 min. (G) Cells were treated with 50 μM EGCG for 24 h and then stimulated with 50 ng/ml PDGF-BB, 50 ng/ml EGF, 100 nM Ang II, and 5% FCS for 5 min. Cells were lysed, and 20 μg of protein were analyzed with SDS-PAGE. MAP kinase was detected after blotting on polyvinylidene difluoride membranes by a specific MAP kinase antibody that recognizes the catalytically activated $p44^{mapk}/p42^{mapk}$. Cells were stimulated with 50 ng/ml PDGF-BB, 100 nM Ang II, and 50 ng/ml EGF for 5 min.

were suspended in 1.5 ml MEM supplemented with 0.35% agar, 10% FCS, and 20 or 50 μM EGCG and plated on the 0.7% agar underlay. Cells were fed once per week with 2 ml of MEM supplemented with 10% FCS and 20 or 50 μM EGCG. Cells were photographed by phase-contrast light microscope after 1 h and 2–3 wk.

Statistics

Values are expressed as means ± SE. Statistical analysis of the data was performed using the Mann–Whitney *U* test. Each experiment was performed independently a minimum of three times. Data presented are from representative experiments unless indicated otherwise. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of EGCG on PDGF-BB–induced MAP Kinase Activation

Stimulation of the cells with 50 ng/ml PDGF-BB resulted in a time-dependent increase of $p44^{mapk}/p42^{mapk}$ detected with the phospho-specific MAP kinase antibodies that recognized the Tyr204-phosphorylated isoforms showing a maximum at 5 min (Figure 1A). Remarkably, PDGF-BB failed to stimulate the MAP kinase isoforms in EGCG-treated VSMCs (Figure 1A). As shown in Figure 1B, EGF also stimulated phosphorylation of $p42^{mapk}/p44^{mapk}$ with a maximum at 5 min. In striking contrast, the effect of EGF was not affected in EGCG-treated VSMCs. Moreover, as shown in Figure 1C, EGCG at a concentration higher than 1 μM inhibited the maximal phosphorylation of the MAP kinase isoforms at 5 min in a dose-dependent manner. On the other hand, treatment of VSMCs with EGCG (1–100 μM) did not influence the maximal effect of EGF (Figure 1D), Ang II (Figure 1E), and FCS (Figure 1F), which occurs at 5 min. Figure 1G shows the effect of 50 μM EGCG on the agonist-induced phosphorylation of $p44^{mapk}/p42^{mapk}$ after stimulation of VSMCs for 5 min. Again, 50 μM EGCG selectively inhibited the PDGF-BB–induced phosphorylation of the MAP kinase isoforms without influencing the effect of Ang II, EGF, and FCS. Statistical analysis of the band densities by laser densitometry obtained by separate experiments revealed that EGCG

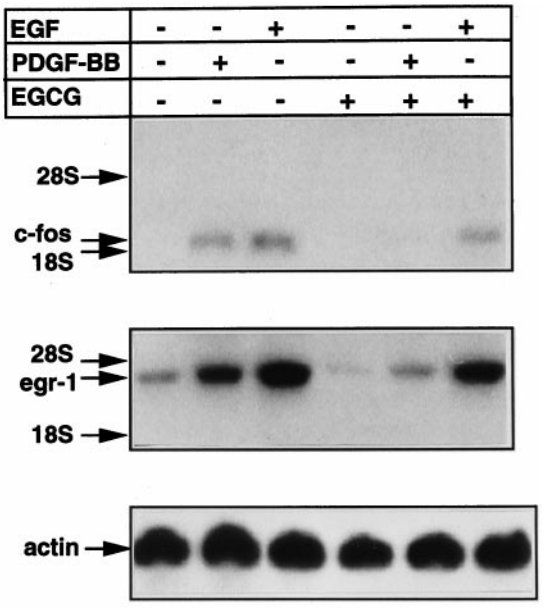


Figure 2. Effect of PDGF-BB and EGF on the expression of c-fos and egr-1 mRNA in EGCG-treated VSMCs. Confluent cells in 75-cm^2 flasks were precultured in the presence and absence of 50 μM EGCG in serum-free medium for 24 h. Then medium was replaced with serum-free medium without EGCG, and VSMCs were stimulated with 50 ng/ml PDGF-BB and 50 ng/ml EGF for 30 min. Ten micrograms of total RNA were electrophoresed on formaldehyde–agarose gels, blotted onto Hybond N+ membranes, and probed with a ^{32}P-labeled 1.0-kb v-fos cDNA probe that hybridized to the 2.2-kb mRNA of c-fos. The same blot was rehybridized with a ^{32}P-labeled 2.1-kb egr-1 cDNA probe that hybridized to the 3.4-kb egr-1 mRNA and with a 0.77-kb cDNA probe for β-actin mRNA. Arrows show the 28S (4.6 kb), the 18S rRNA (1.8 kb), the 2.2-kb c-fos mRNA, and the 2.0-kb β-actin mRNA.

at 10, 20, and 50 μM caused a 79 ± 16, 90 ± 6, and 95 ± 2% inhibition of the maximal PDGF-BB–induced phosphorylation of $p44^{mapk}$/$p42^{mapk}$ (=100%), respectively.

Effect of EGCG on the PDGF-BB– and EGF-induced Expression of c-fos and egr-1 mRNA

Stimulation of VSMCs with 50 ng/ml PDGF-BB and 50 ng/ml EGF for 30 min resulted in a marked expression of c-fos and egr-1 mRNA (Figure 2). In EGCG-treated VSMCs, PDGF-BB failed to stimulate expression of c-fos and egr-1 mRNA. In striking contrast, EGCG treatment of VSMCs did not influence the EGF-induced expression of c-fos and egr-1 mRNA.

Effect of EGCG on the PDGF-BB–induced Tyrosine Phosphorylation of PDGF-Rβ, PI 3′ K, and PLC-γ1

After preincubation of VSMCs with various concentrations of EGCG for 24 h, VSMCs were stimulated for 5 min with PDGF-BB. After immunoprecipitation of tyrosine-phosphorylated proteins with anti-tyrosine Sepharose, specific proteins were detected by enhanced Western blotting analysis using the appropriate antibodies. PDGF-BB caused a marked phosphorylation of PDGF-Rβ (Figure 3A), PI 3′-K (Figure 3B), and PLC-γ1 (Figure 3C) in untreated VSMCs at 5 min. Treatment of the VSMCs with EGCG resulted in a dose-dependent inhibition of the tyrosine-phosphorylated proteins. Laser densitometric analysis of the band densities of the tyrosine-phosphorylated PDGF-Rβ obtained by three separate experiments is presented in Figure 3D. Treatment of VSMCs with 50 μM EGCG resulted in a 75 ± 15% inhibition of the PDGF-BB–induced tyrosine autophosphorylation of the PDGF-Rβ in untreated cells (=100%). The IC_{50} value was calculated to be 20 μM (Figure 3D). In 50 μM EGCG-treated VSMCs, the effect of PDGF-BB on the phosphorylation of PI 3′-K and PLC-γ1 was inhibited by 70 ± 10% (three separate experiments).

Effect of Different Concentrations of PDGF-BB on Tyrosine Phosphorylation of PDGF-Rβ, $p44^{mapk}$/$p42^{mapk}$ and PI 3′-K

PDGF-BB (1–50 ng/ml) caused a dose-dependent increase of tyrosine phosphorylation of PDGF-Rβ with maximal stimulation at a concentration of 50 ng/ml PDGF-BB (Figure 4). Remarkably, maximal phosphorylation of $p44^{mapk}$/$p42^{mapk}$ and PI 3′-K occurred at a concentration of 3 and 10 ng/ml PDGF-BB, respectively. These results demonstrate that maximal stimulation of $p44^{mapk}$/$p42^{mapk}$ occurs at a relatively low concentration of PDGF-BB.

Effect of EGCG on PDGF-BB–induced Increase in $[Ca^{2+}]_i$

PDGF-BB (50 ng/ml) induced a maximal increase in $[Ca^{2+}]_i$ from 70 to 250 nM within 40 s (representative tracing from four independent experiments) (Figure 5a). As shown in Figure 5b, PDGF-BB failed to stimulate increase in $[Ca^{2+}]_i$ in EGCG-treated VSMCs.

Effect of EGCG on the Total PDGF-Rβ Amount

To show that treatment of VSMCs with EGCG does not lead to a downregulation of the PDGF-Rβ number, we quantified the total amount of PDGF-Rβ in EGCG-treated VSMCs by enhanced Western blotting analysis. Statistical analysis of the band densities by laser densitometry revealed that treatment of VSMCs with EGCG for 24 h did not influence the total number of PDGF-Rβ (Figure 6). The amount of the PDGF-Rβ in EGCG-treated VSMCs was 87 ± 8% of that in untreated VSMCs (= 100 ± 16, $p > 0.05$ for PDGF-Rβ vs. PDGF-Rβ in EGCG-treated VSMCs).

Effect of EGCG on the Cell Number

Stimulation of untreated VSMCs (control) with 50 ng/ml PDGF-BB resulted in an increase of cell number

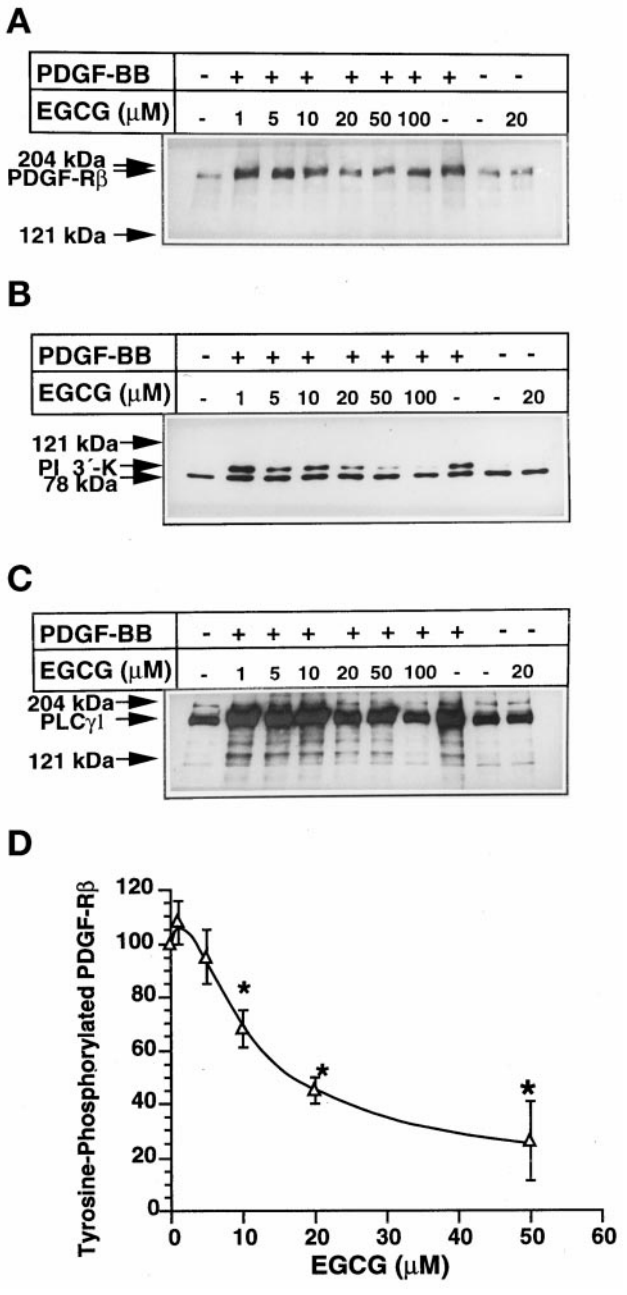


Figure 3. Effect of PDGF-BB on tyrosine phosphorylation of PDGF-Rβ, PI 3′-K, and PLC-γ1 in EGCG-treated VSMCs. Confluent cells in 75-cm^2 flasks were preincubated in serum-free medium in the presence and absence of different concentrations of EGCG. Then the medium was replaced with serum-free medium without EGCG, and VSMCs were stimulated with 50 ng/ml PDGF-BB for 5 min. Then the cells were lysed, and tyrosine-phosphorylated proteins were immunoprecipitated using an anti-phosphotyrosine antibody coupled to Sepharose. Proteins (5 μg) were analyzed by 7.5% SDS-PAGE. Tyrosine-phosphorylated PDGF-Rβ (A), PI 3′-K (B), and PLC-γ1 (C) were detected on the same blot by the enhanced chemiluminescence method using the respective monoclonal antibodies. (D) Laser densitometric analysis of the band densities obtained by three separate experiments showing the effect of PDGF-BB on tyrosine phosphorylation of PDGF-Rβ in VSMCs after treatment with various concentrations of EGCG.

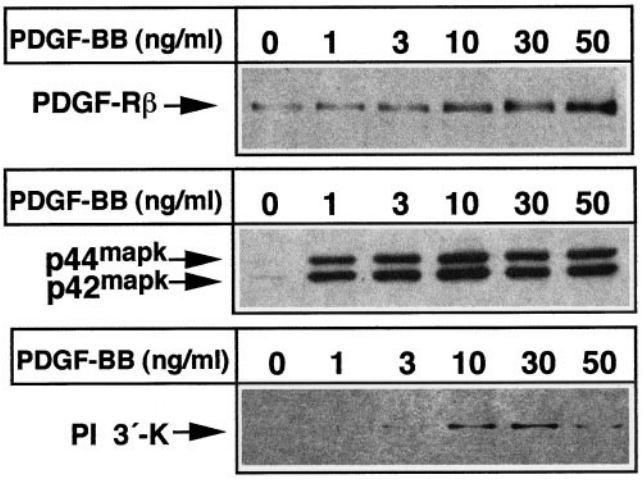


Figure 4. Effect of different concentrations of PDGF-BB on tyrosine phosphorylation of PDGF-Rβ, p44^{mapk}/p42^{mapk}, and PI 3′-K. Confluent cells in 75-cm^2 flasks were preincubated in serum-free medium for 24 h. Then VSMCs were stimulated with 1–50 ng/ml PDGF-BB for 5 min. Then the cells were lysed, and tyrosine-phosphorylated proteins were immunoprecipitated using an anti-phosphotyrosine antibody coupled to Sepharose. Proteins (5 μg) were analyzed by 7.5% SDS-PAGE. Tyrosine-phosphorylated PDGF-Rβ, p44^{mapk}/p42^{mapk}, and PI 3′-K were detected by the enhanced chemiluminescence method using the respective monoclonal antibodies.

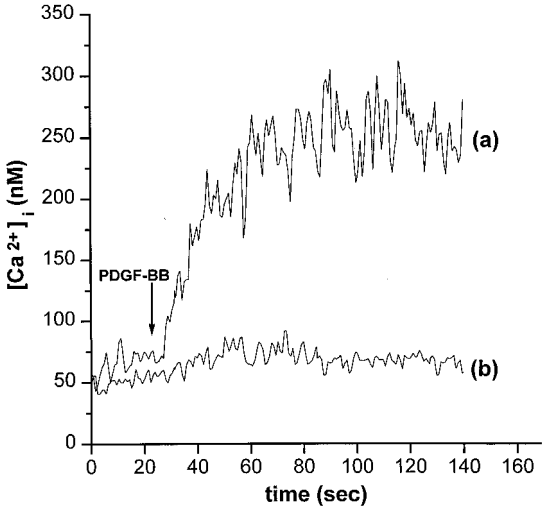


Figure 5. Effect of PDGF-BB on $[Ca^{2+}]_i$ in EGCG-treated VSMCs. Confluent VSMCs on slides were precultured for 24 h in serum-free medium in the presence and absence of 50 μM EGCG for 24 h. After loading of the cells with fura-2, PDGF-BB (50 ng/ml) was applied to VSMCs, and changes in fluorescence were monitored. After subtraction of autofluorescence, changes in 340/380 nm excitation wavelength ratio by the emission wavelength of 505 nm were converted into corresponding levels of $[Ca^{2+}]_i$.

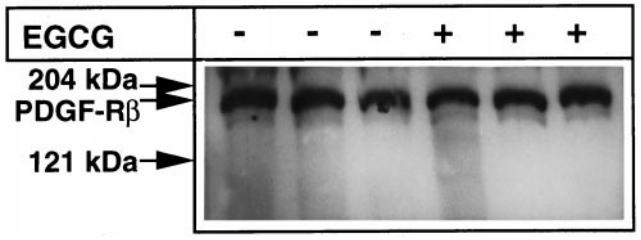


Figure 6. Effect of EGCG on the PDGF-Rβ amount in VSMCs. VSMCs were cultured in dishes (diameter: 3 cm) and cultivated until confluence. Then the medium was replaced by serum-free medium, and VSMCs were incubated in the presence and absence of 50 μM EGCG for 24 h. VSMCs were then lysed, and 20 μg of protein were analyzed with SDS-PAGE. PDGF-Rβ was detected by enhanced chemiluminescence Western blotting using anti–PDGF-Rβ antibodies.

from 3.8×10^5 to 5.8×10^5 cells/ml (Figure 7). Treatment of VSMCs with 20 and 50 μM EGCG for 24 h resulted in an attenuation of the cell number from 3.85×10^5 (control = untreated cells) to 2.87×10^5 and 2.41×10^5 cells/ml. Stimulation of the 10, 20, and 50 μM EGCG-treated VSMCs with 50 ng/ml PDGF-BB resulted in an increase of cell number from 3.53×10^5, 2.87×10^5, and 2.41×10^5 cells/ml to 4.71×10^5, 3.89×10^5 and 2.68×10^5 cell/ml, respectively. To compare the inhibitory potency of EGCG on the PDGF-BB, FCS, and EGF effect on cell number, VSMCs were treated with 50 μM EGCG and then stimulated with 5% FCS and 50 ng/ml EGF. The percentage increase of the cell number is shown in Figure 7. These results show that PDGF-BB caused a 51% increase of cell number. Stimulation of the 10, 20, and 50 μM EGCG-treated VSMCs with PDGF-BB caused a 33, 36, and 11% increase of the cell number. Stimulation of untreated VSMCs with 5% FCS and 50 ng/ml EGF induced a 65 and 76% increase in cell number, respectively. Stimulation of 50 μM EGCG-treated VSMCs with FCS and EGF resulted in a 57 and 48% increase in cell number. These findings suggest that the proliferative effect of PDGF-BB is inhibited by 80% in 50 μM EGCG-treated VSMCs. In contrast, the proliferative effect of FCS and EGF is inhibited by 12 and 37% in the 50 μM EGCG-treated VSMCs.

Effect of EGCG on the PDGF-BB–induced DNA Synthesis

As demonstrated in Figure 8, stimulation of VSMCs with 50 ng/ml PDGF-BB caused an increase of [^{3}H]thymidine incorporation from 118 $\pm$ 5 to 2175 cpm/μg protein. Treatment of the cells with EGCG resulted in a dose-dependent inhibition of the PDGF-BB–induced [^{3}H]thymidine incorporation with an IC_{50} value of 18 μM.

Effect of EGCG on A172 Multicellular Spheroid Formation

Multicellular spheroids of A172 cells were obtained in 0.35% semisolid agar (Figure 9). A172 spheroid formation was completely inhibited in the presence of 50 μM EGCG (Figure 9C). EGCG at a concentration of 20 μM was less effective.

Effect of EGCG on sis-NIH 3T3 Multicellular Colony Formation

EGCG at a concentration of 20 and 50 μM completely inhibited the colony formation of the *sis*-transformed NIH 3T3 cells in semisolid agar (Figure 10, A and B). Control experiments were performed using tyrphostin AG1296, which is known to be a potent inhibitor of the *sis*-transfected NIH 3T3 colony formation (Kovalenko *et al.*, 1994). As indicated in Figure 10A, in the presence of 20 and 50 μM EGCG and 25 μl of tyrphostin AG1296, a complete inhibition of the colony formation of the *sis*-transfected NIH 3T3 fibroblasts was achieved.

DISCUSSION

In the past decade many efforts were made to develop drugs that inhibit the tyrosine kinase receptors and their intracellular signaling transduction pathway (Levitzki and Gazit, 1995). In this context, several selective inhibitors of receptor tyrosine kinases have been developed, i.e., tyrphostin 1296, which is a selective inhibitor of the PDGF-Rβ, and tyrphostin 1478, a selective inhibitor of the EGF-R (Levitzki and Gazit, 1995). Recently, Kovalenko *et al.* (1997) demonstrated that tyrphostin AG1296 neither interferes with PDGF-BB binding to the PDGF-Rβ nor has any effect on receptor dimerization (the first step by the autophosphorylation of the PDGF-Rβ). Instead, they propose a mechanism of action that implicates conformational changes at the ATP-binding site (Kovalenko *et al.*, 1997). The biological anti-proliferative activities of the tyrphostin analogues have been studied extensively in tissue culture systems of transformed cells in vivo (Levitzki and Gazit, 1995).

In this study, we present findings demonstrating that EGCG, a natural substance isolated from green tea, is a selective inhibitor of the tyrosine phosphorylation of the PDGF-Rβ and its signaling transduction cascade. Our conclusion is well documented by the use of PDGF-BB and EGF, two classic growth factors acting through tyrosine kinase receptors, and Ang II, acting through a G-coupled receptor. We found that EGCG selectively inhibited the PDGF-BB–induced $p44^{mapk}/p42^{mapk}$ phosphorylation. In concordance with this finding, we also observed a selective inhibition of the PDGF-BB–induced expression of the transcriptional factors c-fos and egr-1 mRNA. To demonstrate whether the inhibitory effects of EGCG on

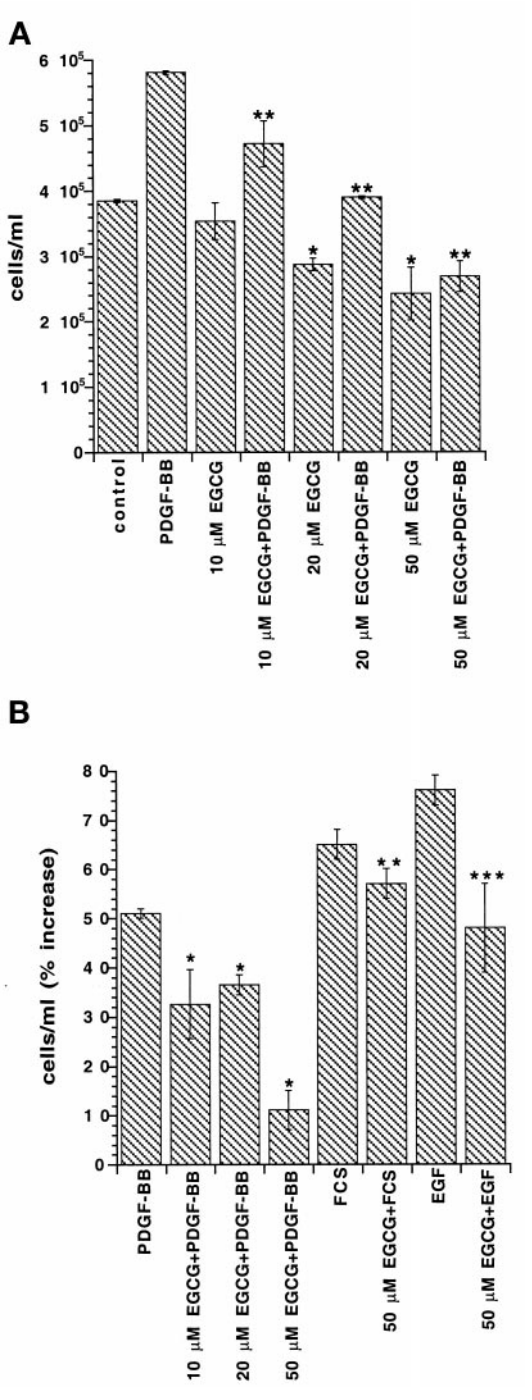


Figure 7. Effect of PDGF-BB, FCS, and EGF on cell number. VSMCs in 24-well plates were precultured in serum-free medium in the presence and absence of EGCG for 24 h. Then the medium was replaced with serum-free medium without EGCG, and VSMCs were stimulated with 50 ng/ml PDGF-BB, 5% FCS, and 50 ng/ml EGF. After 24 h cells were trypsinized, and cell counts were determined with the cell counter system CASY-1 (Schärfe System). (A) Effect of different concentrations of EGCG on the PDGF-BB–induced increase of cell number (mean ± SD, n = 3; *$p < 0.05$ for EGCG-treated vs. untreated [control] VSMCs, **$p < 0.05$ for EGCG+PDGF-BB versus PDGF-BB effect). (B) Percentage increase of cell number after stimulation of the 50 μM EGCG-treated VSMCs with PDGF-BB, FCS, and EGF (mean ± SD; *$p < 0.05$ for EGCG+PDGF-BB vs. PDGF-BB, **$p < 0.05$ for FCS+EGCG vs. FCS effect, ***$p < 0.05$ for EGF+EGCG vs. EGF effect).

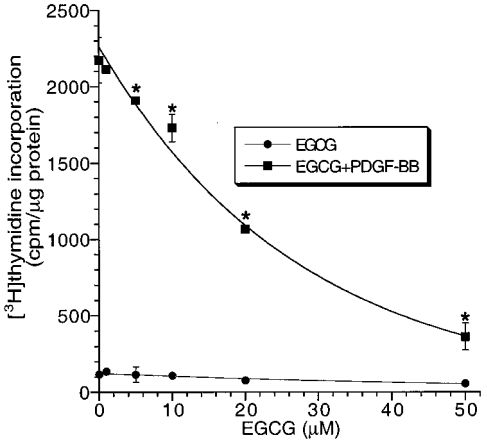


Figure 8. Effect of EGCG on the PDGF-BB–induced DNA synthesis. VSMCs in 24-well plates were precultured in serum-free medium in the presence and absence of different concentrations of EGCG for 24 h. Then the medium was replaced with serum-free medium without EGCG, and VSMCs were stimulated with 50 ng/ml PDGF-BB. After 20 h, 3 μCi/ml of [^{3}H]thymidine were added to the serum-free medium. Four hours later, experiments were terminated. *$p < 0.05$ for EGCG+PDGF-BB vs. PDGF-BB effect.

tyrosine phosphorylation occurs at the PDGF-Rβ receptor level, tyrosine-phosphorylated proteins were immunoprecipitated by Sepharose-coupled anti-phosphotyrosine antibodies, and then the amount of the phosphorylated PDGF-Rβ, PI 3′-K, and PLC-γ1 was quantified. We found that tyrosine phosphorylation of the PDGF-Rβ was inhibited in EGCG-treated cells with an IC_{50} value of 20 μM. In concordance with this finding, tyrosine phosphorylation of PI 3′-K and PLC-γ1 was almost completely inhibited in VSMCs that have been treated with 50 μM EGCG for 24 h. One remarkable finding was that inhibition of $p44^{mapk}$/$p42^{mapk}$ phosphorylation by EGCG occurs at a concentration of 5 μM, whereas inhibition of tyrosine phosphorylation of PLCγ1 and PI 3′-K occurs less efficiently with 5 μM of EGCG and requires a higher concentration of EGCG (IC_{50} value: 20–50 μM). This discrepancy may be explained by the observation that a concentration of 3–10 ng/ml PDGF-BB induces maximal stimulation of $p44^{mapk}$/$p42^{mapk}$, whereas maximal tyrosine phosphorylation of PDGF-Rβ occurs by 50 ng/ml. This phenomenon has been described for other PDGF-BB–induced early intracellular events including DNA synthesis; e.g., maximal elevation of $[Ca^{2+}]_i$ by PDGF-BB occurs at a concentration of 5 ng/ml, whereas maximal stimulation of $InsP_3$ and DNA

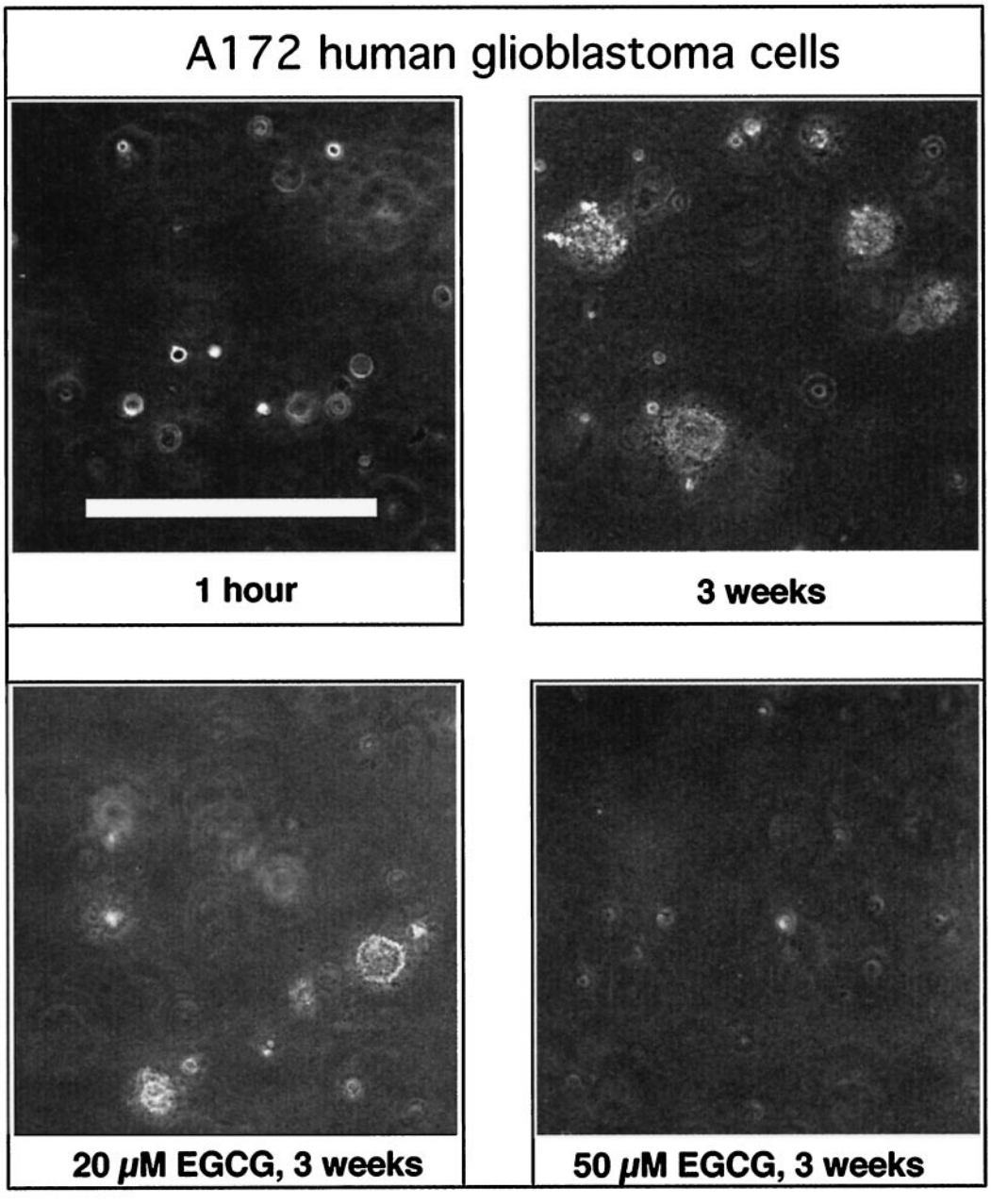
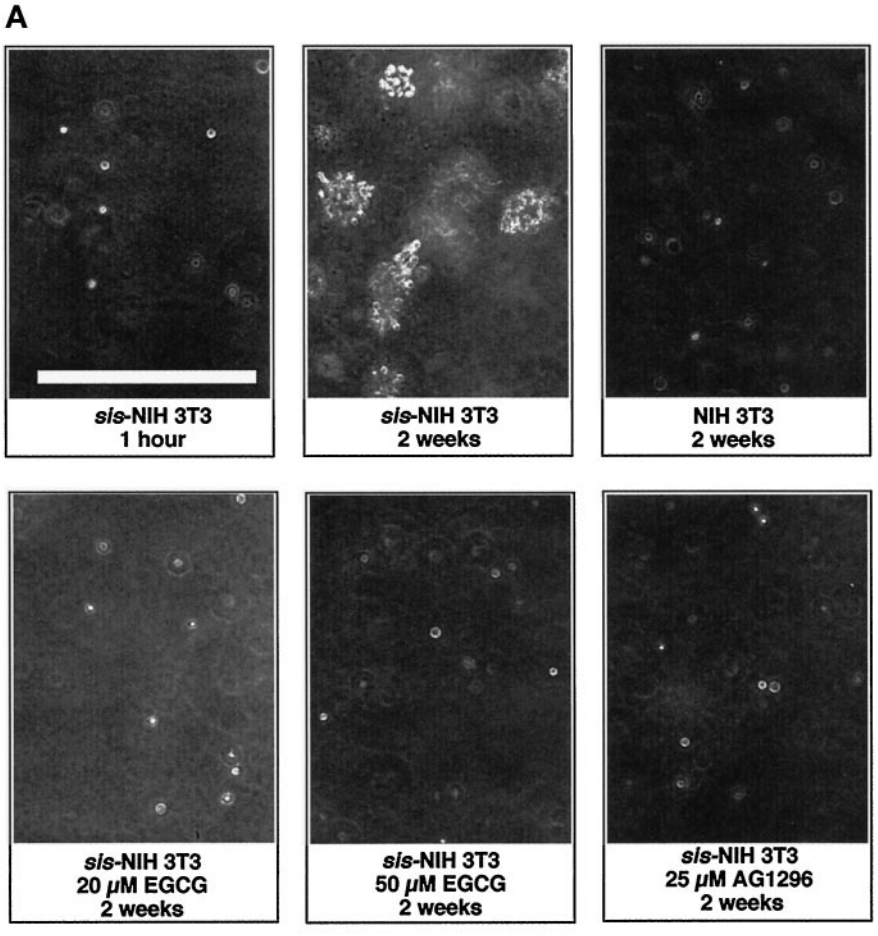


Figure 9. Anchorage-independent growth of A172 cells in the presence and absence of EGCG; 5×10^4 single cells in 1.5 ml MEM supplemented with 0.35% agar, 10% FCS, and 20 μM or 50 μM EGCG were plated on a layer of 1 ml of 0.7% agar containing MEM supplemented with 10% FCS and 20 μM or 50 μM EGCG. Representative fields were photographed after 1 h and after 3 wk by phase-contrast light microscope. Bar, 250 μm.

synthesis occurs at 30 ng/ml (Sachinidis *et al.*, 1990). Moreover, 3–10 ng/ml PDGF-BB induce only a 15–30% of the maximal tyrosine phosphorylation of PDGF-Rβ obtained by 50 ng/ml (Figure 4). Therefore, compared with tyrosine phosphorylation of PDGF-Rβ, PI 3′-K, and PLCγ, a concentration of 5 μM EGCG is sufficient to induce complete inhibition of $p44^{mapk}/p42^{mapk}$; however, these findings demonstrate the complex action mechanisms of EGCG, and further efforts might be necessary to dissect its complex mechanisms of action.

Because activation of PLC-γ1 results in an elevation of $InsP_3$ that mobilizes Ca^{2+} from intracellular stores, we further examined whether EGCG was able to block the PDGF-BB–induced increase in $[Ca^{2+}]_i$. In concordance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50 μM EGCG
2 weeks
sis-NIH 3T3
25 μM AG1296
2 weeks
B
sis-NIH 3T3 fibroblasts
2 weeks
20 μM EGCG
2 weeks
50 μM EGCG
2 weeks
25 μM tyrphostinAG12962
2 weeks

with the above finding, a complete blockade of the PDGF-BB–induced increase in $[Ca^{2+}]_i$ in the EGCG-treated VSMCs was observed. The possibility that the inhibition of the early mitogenic signals by EGCG may be due to a downregulation of the PDGF-Rβ could be excluded by the observation that treatment of VSMCs with EGCG did not alter the amount of PDGF-Rβ in EGCG-treated VSMCs; however, it is possible that like tyrphostin AG1296, EGCG may induce conformational changes at the ATP-binding site of the PDGF-Rβ, thereby inhibiting its tyrosine phosphorylation.

We further show that EGCG inhibits the PDGF-BB–induced DNA synthesis with an IC_{50} value of 20 μM. In concordance with the above results, we demonstrated that 80% of the proliferative effect of PDGF-BB was eliminated after treatment of VSMCs with 50 μM EGCG. In striking contrast, only 12% of the proliferative effect of FCS was inhibited; however, although the EGF-induced MAP kinase activation as well as the expression of c-fos and egr-1 mRNA was not significantly inhibited in the EGCG-treated VSMCs, the proliferative effect of EGCG was inhibited by 37%. Also, treatment of VSMCs with 50 μM EGCG caused a 35% inhibition of the EGF-induced [^{3}H]thymidine incorporation (our unpublished observation). From these findings we may conclude that other "side effects" of EGCG are responsible for the inhibitory effects of EGCG on the EGF-induced proliferation. Once again, these findings demonstrate the complexity of the action mechanisms of EGCG.

Epidemiological studies revealed that consumption of green tea might prevent the incidence of various proliferative diseases such as cancer (Dreosti *et al.*, 1997) and atherosclerosis in humans (Tijburg *et al.*, 1997). Also, the anti-cancer activity of EGCG has been repeatedly demonstrated in animal models. Recently, it has been reported that EGCG might inhibit cancer formation by inhibition of the enzyme urokinase, one of the most frequently overexpressed enzymes in human cancers. Therefore, authors suggest that the anti-cancer activity of green tea is related to the inhibition of urokinase (Jankun *et al.*, 1997). Because PDGF and its PDGF-Rβ contribute to the development of the atherosclerotic plaque leading to restenosis (Ross, 1993), we postulate that the anti-atherosclerotic activity of green tea is driven by EGCG, which prevents activation of the PDGF-Rβ receptor. Also, because oxidation of low-density lipoprotein (LDL) is thought to play an important role in the development of atherosclerosis, the ability of polyphenols to prevent oxidation of LDL has been extensively discussed as a potential mechanism for the anti-atherosclerotic effect of green tea (Salah *et al.*, 1995). In this context, it has been proposed that polyphenols of green tea may prevent the oxidation of LDL by their ability to scavenge free radicals (Salah *et al.*, 1995). Extensive human epidemiological studies suggest that green tea consumption (up to 10 cups per day) significantly decreased the LDL cholesterol in serum (Imai and Nakachi, 1995). Therefore, it has been postulated that green tea may be protective against cardiovascular disease by lowering LDL cholesterol (Imai and Nakachi, 1995; Tijburg *et al.*, 1997). Indeed, consumption of green tea may act protectively against cancer and cardiovascular disease by several mechanisms; however, we postulate that EGCG might reduce carcinogenesis or cardiovascular disease by its efficacy to inhibit tyrosine phosphorylation of PDGF-Rβ. A172 cells produce relatively high amounts of PDGF-BB and PDGF-Rβ (Vassbotn *et al.*, 1994; Westermark *et al.*, 1995). It is well established that the PDGF-Rβ tyrosine kinase of the A172 cells is chronically activated by endogenous PDGF-BB (Vassbotn *et al.*, 1994). Autocrine activation of the PDGF-Rβ seems to be the initial cause of the development of A172 glioblastoma (Vassbotn *et al.*, 1994; Westermark *et al.*, 1995). To ensure that EGCG might reduce carcinogenesis, we demonstrated that EGCG prevents multicellular spheroid formation of human A172 cells in semisolid agar. This finding suggests that EGCG might reduce cancer diseases in which activation of PDGFRβ is causatively involved. The oncogene v-*sis* of simian sarcoma virus is homologous to the cellular gene encoding the PDGF-B chain, and transformation of NIH 3T3 fibroblats with v-*sis* leads to their tranformation because of persistent autocrine stimulation of PDGF-Rβ (Devare *et al.*, 1982; Beckman *et al.*, 1988). We show that colony formation of the *sis*-transfected NIH 3T3 cells in semisolid agar was completely inhibited by 20 μM EGCG and tyrphostin AG1296. Tyrphostin AG1296 is known to be a selective inhibitor of the PDGF-Rβ tyrosine phosphorylation (Levitzki and Gazit, 1995) and inhibits colony formation of *sis*-transfected NIH 3T3 cells (Kovalenko *et al.*, 1994); however, although the latest findings further support our conclusions that EGCG acts as a selective inhibitor of tyrosine phosphorylation of PDGF-Rβ, further efforts are necessary to demonstrate selective action of EGCG, i.e., by examining the effect of EGCG on *ras*-induced transformation of the NIH 3T3 cells. Furthermore, PDGF-BB activates PDGF-Rβ and PDGF-Rα, which have separable roles in oncogenesis and devel-

Figure 10 (facing page). Anchorage-independent growth of *sis*-transfected NIH 3T3 cells in the presence and absence of EGCG or tyrphostin AG1296. (A) Single cells (5×10^4) in 1.5 ml of MEM supplemented with 0.35% agar, 10% FCS, and 20 μM or 50 μM EGCG or 25 μM tyrphostin AG1296 were plated in 35-mm Petri dishes on a layer of 1 ml of 0.7% agar containing MEM supplemented with 10% FCS, 20 μM, 50 μM EGCG, or 25 μM tyrphostin AG1296. Representative fields were photographed after 1 h hour and 2 wk by phase-contrast light microscope. Bar, 250 μm. (B) Petri dishes (35-mm diameter) containing the visible colonies of *sis*-NIH 3T3 fibroblasts were scanned with a SnapScan 600 scanner (AGFA, Cologne, Germany) by the inverted modus, and scans were analyzed by the Adobe photoshop software (Adobe Systems, San Jose, CA).

opment of different cells (Westermark *et al.*, 1995). The present work examines the effect of EGCG on the activation of PDGF-Rβ. The effects of EGCG on activated PDGF-Rα remain to be elucidated.

In comparison to the synthetic inhibitors of phosphorylation of PDGF-Rβ such as tyrphostin AG1296, which might have toxic effects in vivo, EGCG is a nontoxic natural substance that can be consumed in high amounts, e.g., one cup of tea contains 250 mg EGCG (M_r 458), and some consumers drink at least 10 cups per day (Yang and Wang, 1993; Imai and Nakachi, 1995; Jankun *et al.*, 1997). In summary, we offer a novel mechanism that partly explains the anti-cancer and anti-atherosclerotic activity of green tea. Moreover, our findings may be helpful for the development of new prophylactic strategies for the prevention of cancer and atherosclerosis using a nontoxic natural substance.

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REFERENCES

Beckman, M.P., Betzholz, C., Heldin, C.-H., Westermark, B., DiMarco, E., DiFiore, P.P., Robbins, K.C., and Aaronson, S.A. (1988). Comparison of biological properties and transforming potential of human PDGF-A and PDGF-B chains. Science *241*, 1346–1349.

Berridge, M.J., and Irvine, R.F. (1989). Inositolphosphates and cell signaling. Nature *341*, 197–205.

Blenis, J. (1993). Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. USA *90*, 5889–5892.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.

Chamley, J.H., Campbell, G.R., and Ross, R. (1979). The smooth muscle cell in culture. Physiol. Rev. *39*, 1–61.

Daemen, M.J.A.P., Lombardi, D.M., Bosman, F.T., and Schwartz, S.M. (1991). Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. Circ. Res. *68*, 450–456.

Devare, S.G., Reddy, E.P., Robbins, K.C., Andersen, P.R., Tronick, S.R., and Aaronson, S.A. (1982). Nucleotide sequence of the transforming gene of simian sarcoma virus. Proc. Natl. Acad. Sci. USA *79*, 3179–3182.

Dreosti, I.E., Wargovich, M.J., and Yang, C.S. (1997). Inhibition of carcinogenesis by tea: the evidence from experimental studies. Crit. Rev. Food Sci. *37*, 761–770.

Duan-Fang, L., Duff, J.L., Daum, G., Pelech, S.L., and Berk, B.C. (1996). Angiotensin II stimulates MAP kinase kinase kinase activity in vascular smooth muscle cells. Circ. Res. *79*, 1007–1014.

Freedman, V.H., and Shin, S.I. (1974). Cellular tumorigenicity in nude mice: correlation with cell growth in semisolid medium. Cell *4*, 355–359.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. *260*, 3440–3450.

Hoppe, J., Weich, H.A., and Eichner, W. (1989). Preparation of biologically active PDGF type BB from a fusion protein expressed in *Escherichia coli*. Biochemistry 28, 2956–2960.

Imai, K., and Nakachi, K. (1995). Cross sectional study of effects of drinking green tea on cardiovascular and liver diseases. Br. Med. J. *310*, 693–696.

Jankun, J., Selman, S.H., and Swiercz, R. (1997). Why drinking green tea could prevent cancer. Nature *387*, 561.

Kaplan, D.R., Morrison, D.K., Wong, G., McCormick, F., and Williams, L. (1990). PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell *61*, 125–133.

Kovalenko, M., Gazit, A., Böhmer, A., Rorsman, C., Rönnstrand, L., Heldin, C.-H., Waltenberger, J., Böhmer, F.-D., and Levitzky, A. (1994). Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. Cancer Res. *54*, 6106–6114.

Kovalenko, M., Rönnstrand, L., Heldin, C.H., Loubtchenkov, M., Gazit, A., Levitzki, A., and Bohmer, F.D. (1997). Phosphorylation site-specific inhibition of platelet-derived growth factor β-receptor autophosphorylation by the receptor blocking tyrphostin AG1296. Biochemistry *36*, 6260–6269.

Levitzki, A., and Gazit, A. (1995). Tyrosine kinase inhibition: an approach to drug development. Science *267*, 1782–1788.

Marshall, C.J. (1995). Specifity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179–185.

Pelech, S.L., and Sanghera, S. (1992). MAP kinases: charting the regulatory pathways. Science *257*, 1355–1356.

Rönnstrand, L., Mori, S., Arridson, A.K., Erikssen, A., Wernstedt, C., Hellman, U., Claesson-Welsh, L., and Heldin, C.H. (1992). Identification of two C-terminal autophosphorylation sites in the PDGF-β-receptor: involvement in the interaction with phospholipase C-γ. EMBO J. *11*, 3911–3919.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature *362*, 801–809.

Sachinidis, A., Flesch, M., Ko, Y., Schrör, K., Böhm, M., Düsing, R., and Vetter, H. (1995). Thromboxane A_2 and vascular smooth muscle cell proliferation. Hypertension *26*, 771–780.

Sachinidis, A., Locher, R., Vetter, W., Tatje, D., and Hoppe, J. (1990). Different effects of PDGF-isoforms on rat vascular smooth muscle cells. J. Biol. Chem. *265*, 10238–10243.

Salah, M., Miller, N.J., Paganga, G., Tijburg, L., Bolwell, G.P., and Rice-Evans, C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Arch. Biochem. Biophys. *322*, 339–346.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 7.39–7.52.

Tijburg, L.B.M., Mattern, T., Folts, J.D., Weigerber, U.M., and Katan, M.B. (1997). Tea flavonoids and cardiovascular diseases. Crit. Rev. Food Sci. *37*, 771–785.

Vassbotn, F.S., Östman, A., Langeland, N., Holmsen, H., Westermark, B., Heldin, C.-H., and Nistér, M. (1994). Activated platelet-derived growth factor autocrine pathway drives the transformed phenotype of a human glioblastoma cell line. J. Cell. Physiol. *158*, 381–389.

Westermark, B., Heldin, C.-H., and Nistér, M. (1995). Platelet-derived growth factor in human glioma. Glia *15*, 257–263.

Yang, C.S., and Wang, Z.Y. (1993). Tea and cancer. J. Natl. Cancer. Inst. *85*, 1038–1049.