Epigallocatechin-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 epigallocatechin-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 (p44mapk/p42mapk) 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 p44mapk/p42mapk 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 Ca2+ 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), p21ras 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 (InsP₃) and diacylglycerol (Sachinidis et al., 1990). It is assumed that InsP₃ mobilizes Ca²⁺ 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 p90rsk, 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 p21ras. Sequential phosphorylation results in activation of the Raf-1 kinase, MAP kinase kinase, and p44mapk/p42mapk (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 preventive 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 epigallocatechin-3 gallate (EGCG), epigallocatechin (EGC), and epicatechin-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 si-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). Hybrid 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.W.) 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-week-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% CO₂. 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 lysed 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 Protein 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 PhosphoFlus 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 p42mapk and p44mapk 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 for different time periods 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 × 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 C γ1 (1:1000), and polyclonal rabbit anti–PDGF-Rβ IgG (1:500) and monoclonal mouse anti-horse radish peroxidase-labeled anti-mouse IgG.

**Measurement of [Ca2+]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, 12 mm) under normal tissue culture conditions until confluence. VSMCs were then stimulated for different time periods with PDGF-BB. After removal of the medium, cells were lysed 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 Protein 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 PhosphoFlus 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 p42mapk and p44mapk 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 for different time periods 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 × 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 C γ1 (1:1000), and polyclonal rabbit anti–PDGF-Rβ IgG (1:500) and monoclonal mouse anti-horse radish peroxidase-labeled anti-mouse IgG.

**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-cm² 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 Hybrid N+ membranes (Amersham), washed at room temperature in 5× SSC (1× 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× SSC, 0.1% SDS for 5 min. Prehybridization and hybridization were performed overnight at 60°C in 5× SSC containing 0.2% SDS, 50 mM sodium phosphate, 10× Denhardt’s solution (Sigma Chemical), and 200 μg/ml salmon sperm DNA. The DNA probes were labeled with 32P-deoxyctydine triphosphate by random oligonucleotide priming to a specific activity of 2–4 × 109 dpm/μg DNA (Amersham Buchler, Braunschweig, Germany). The stringency of the final wash was 0.2× SSC containing 0.1% SDS at 65°C, two times for 45 min. A 32P-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 × 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 × 104 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 [3H]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. After 4 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).

**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 trypsinization, 5 × 104 A172 cells or sis-transfected NIH 3T3 fibroblasts
were suspended in 1.5 ml MEM supplemented with 0.33% agar, 10% FCS, and 20 or 50 \(\mu M\) 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 \(\mu M\) 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\(\text{mapk}\)/p42\(\text{mapk}\) detected with the phospho-specific MAP kinase antibodies that recognized the Tyrr204-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 p44\(\text{mapk}\)/p44\(\text{mapk}\) 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 \(\mu M\) 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 \(\mu M\)) 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 \(\mu M\) EGCG on the agonist-induced phosphorylation of p44\(\text{mapk}\)/p44\(\text{mapk}\) after stimulation of VSMCs for 5 min. Again, 50 \(\mu M\) 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...
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\textsuperscript{mapk}/p42\textsuperscript{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-R\textsubscript{β}, 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-R\textsubscript{β} (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-R\textsubscript{β} 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-R\textsubscript{β} in untreated cells (=100%). The IC\textsubscript{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-R\textsubscript{β}, p44\textsuperscript{mapk}/p42\textsuperscript{mapk} and PI 3'-K
PDGF-BB (1–50 ng/ml) caused a dose-dependent increase of tyrosine phosphorylation of PDGF-R\textsubscript{β} with maximal stimulation at a concentration of 50 ng/ml PDGF-BB (Figure 4). Remarkably, maximal phosphorylation of p44\textsuperscript{mapk}/p42\textsuperscript{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\textsuperscript{mapk}/p42\textsuperscript{mapk} occurs at a relatively low concentration of PDGF-BB.

Effect of EGCG on PDGF-BB–induced Increase in [Ca\textsuperscript{2+}]\textsubscript{i}
PDGF-BB (50 ng/ml) induced a maximal increase in [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{i} in EGCG-treated VSMCs.

Effect of EGCG on the Total PDGF-R\textsubscript{β} Amount
To show that treatment of VSMCs with EGCG does not lead to a downregulation of the PDGF-R\textsubscript{β} number, we quantified the total amount of PDGF-R\textsubscript{β} 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-R\textsubscript{β} (Figure 6). The amount of the PDGF-R\textsubscript{β} in EGCG-treated VSMCs was 87 ± 8% of that in untreated VSMCs (=100 ± 16, p > 0.05 for PDGF-R\textsubscript{β} vs. PDGF-R\textsubscript{β} 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 4. Effect of different concentrations of PDGF-BB on tyrosine phosphorylation of PDGF-Rβ, p44mapk/p42mapk, and PI 3'-K. Confluent cells in 75-cm² 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, p44mapk/p42mapk, and PI 3'-K were detected by the enhanced chemiluminescence method using the respective monoclonal antibodies.

Figure 5. Effect of PDGF-BB on \([\text{Ca}^{2+}]_i\) 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 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 \([\text{Ca}^{2+}]_i\).
from $3.8 \times 10^5$ to $5.8 \times 10^5$ 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 $3.85 \times 10^5$ (control = untreated cells) to $2.87 \times 10^5$ and $2.41 \times 10^5$ 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 $3.33 \times 10^5$, $2.87 \times 10^5$, and $2.41 \times 10^5$ cells/ml to $4.71 \times 10^5$, $3.89 \times 10^5$, and $2.68 \times 10^5$ 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 [3H]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 [3H]thymidine incorporation with an IC50 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-transformed 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-transformed 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 auto-phosphorylation 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 p44mapk/p42mapk 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
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, PI3-kinase, and PLC-γ1 was quantified. We found that tyrosine phosphorylation of the PDGF-Rβ was inhibited in EGCG-treated cells with an IC50 value of 20 μM. In concordance with this finding, tyrosine phosphorylation of PI3-kinase 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 p44/42 MAPK phosphorylation by EGCG occurs at a concentration of 5 μM, whereas inhibition of tyrosine phosphorylation of PLC-γ1 and PI3-kinase occurs less efficiently with 5 μM of EGCG and requires a higher concentration of EGCG (IC50 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 p44MAPK/p42MAPK phosphorylation by EGCG occurs at a concentration of 5 μM, whereas inhibition of tyrosine phosphorylation of PLC-γ1 and PI3-kinase occurs less efficiently with 5 μM of EGCG and requires a higher concentration of EGCG (IC50 value: 20–50 μM). This phenomenon has been described for other PDGF-BB–induced early intracellular events including DNA synthesis; e.g., maximal elevation of [Ca2+]i by PDGF-BB occurs at a concentration of 5 ng/ml, whereas maximal stimulation of InsP3 and DNA synthesis by EGF occurs at a concentration of 5 ng/ml. Therefore, the difference in the concentration response for PDGF-BB–induced early intracellular events and DNA synthesis may result from the difference in the concentration response for PI3-kinase and MAPK phosphorylation.

**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. After 24 h, 3 μCi/ml of [3H]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.

**Figure 8.** Effect of PDGF-BB+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 [3H]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.
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\(^{\text{mapk}}\)/p42\(^{\text{mapk}}\); 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\(^{2+}\)]. In concordance

**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.
The text discusses the effects of EGCG, a polyphenol from green tea, on cell proliferation and the PDGF-BB receptor. It mentions that EGCG inhibits the proliferation of NIH 3T3 cells in the presence and absence of EGCG or tyrphostin AG1296. The text notes that EGCG may contribute to the development of atherosclerosis and cancer, suggesting that green tea could be protective against these diseases.

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 × 103) in 1.5 ml of MEM supplemented with 0.35% agar, 10% FCS, and 20 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 and 2 wk by phase-contrast light microscopy. 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).
oration 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-Rβ 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), 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


