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Short communication

Inhibition of the vascular-endothelial growth factor-induced intracellular signaling and mitogenesis of human endothelial cells by epigallocatechin-3 gallate

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Abstract

Galloyl group-containing catechins, such as epigallocatechin-3 gallate, inhibit receptor tyrosine kinase activity of several growth factor receptors. This study investigated the effects of epigallocatechin-3 gallate, as compared to epicatechin, on vascular endothelial growth factor-induced intracellular signaling and mitogenesis of human umbilical endothelial cells. Epigallocatechin-3 gallate concentration-dependently inhibited vascular endothelial growth factor-induced DNA synthesis, cell proliferation, autophosphorylation of vascular endothelial growth factor receptors-1 and -2, phosphorylation of extracellular signal-regulated kinases-1 and -2, and mRNA expression of the early growth response factor-1. In contrast, epicatechin was not effective. Thus, epigallocatechin-3 gallate may be an attractive candidate drug to inhibit tumour angiogenesis.

Keywords: Catechin; Egpigallocatechin-3 gallate; VEGF (vascular endothelial growth factor); Endothelial cell; Cell proliferation

1. Introduction

Angiogenesis plays a pivotal role in different physiological and pathophysiological processes like wound healing, menstrual cycle, diabetic retinopathy, or rheumatoid arthritis (Adamis et al., 1994; Folkman and Shing, 1992; Peacock et al., 1992). There is a large body of evidence supporting a central role of angiogenesis in tumour growth and metastasis (Fidler and Ellis, 1994; Folkman, 1995; O'Reilly et al., 1994; Poste and Fidler, 1980). Accordingly, the expression of vascular endothelial cell growth factor (VEGF), the most potent angiogenic stimulus known, and of its receptors was found to be up-regulated in different tumours (Marme, 1996; Samoto et al., 1995; Takahashi et al., 1995, Weidner et al., 1993). Based on these data, inhibitors of VEGF or its receptors are under development (Ferrara and Alitalo, 1999; Fong et al., 1999), some of them being in phase I–III clinical trials (Drevs et al., 2002).

As a result of large epidemiological studies, some natural compounds with anti-tumour activity were identified in parallel. One of these substances is the polyphenol epigallocatechin-3 gallate (EGCG), the major component of green tea (Jankun et al., 1997). EGCG has been shown to inhibit growth and to induce apoptosis in different human cancer cell lines (Bushman, 1998; Yang et al., 2000). In subsequent studies, it has been proposed that an anti-angiogenic property of EGCG may be one of the mechanisms leading to the inhibition of carcinogenesis (Cao and Cao, 1999; Jung and Ellis, 2001; Tosetti et al., 2002). We and others have demonstrated inhibitory effects of tumour growth by catechins containing a galloyl group in the 3-position of the catechin structure, such as EGCG, on the receptor tyrosine kinase activity of the endothelial cell growth factor (ECGF) receptor, the platelet-derived growth factor (PDGF) receptor, and the VEGF receptor-2 (VEGF-R2) (Ahn et al., 1999; Lamy et al., 2002; Liang et al., 1997).

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Based on these findings, the aim of this study was to elucidate in detail the effects of EGCG, as compared to epicatechin (EC), on VEGF-induced intracellular signaling and mitogenesis of human umbilical arterial endothelial cells (HUAEC).

2. Materials and methods

2.1. Materials

Fetal calf serum and dispase II were from Roche Diagnostics (Mannheim, Germany). EGCG, EC, agarose-coupled anti-phosphotyrosine antibodies, collagenase I, and tri reagent were from Sigma (Deisenhofen, Germany). Moloney murine leukemia virus reverse transcriptase was from Life Technologies (Karlsruhe, Germany). Taq polymerase, random primers, and dNTP were obtained from Perkin Elmer (Weiterstadt, Germany). Human VEGF was obtained from TEBU, (Frankfurt/AM, Germany). RNA-Guard and all chemicals for oligonucleotide synthesis were from Pharmacia (Freiburg, Germany). Phospho-specific extracellular signal-regulated kinases-1 and -2 antibodies were from Cell Signaling Technology (Beverly, MA). Anti-VEGF receptor-1, -2, and anti-phosphotyrosine antibodies were from Santa Cruz (Heidelberg, Germany). The enhanced chemiluminescence detection system was from Amersham (Little Chalfont, UK).

2.2. Culture and stimulation of human umbilical arterial endothelial cells

Endothelial cells were isolated from human umbilical cord arteries, cultured on human fibronectin-coated culture dishes in medium 199 supplemented with 20% v/v fetal calf serum, 10 μg/ml heparin, and 30 μg/ml crude endothelial cell growth factor, and characterized as described in Ko et al. (1995). For gene-expression studies, subconfluent HUAEC of the third or fourth passage were washed twice with phosphate-buffered saline and then (for the purpose of starving) exposed for 4 h to medium 199 without serum or growth factor substitution. For Western blotting, HUAEC of the third passage were transferred into 6-well plates, grown until subconfluence and then exposed to starvation medium for 4 h. Subsequently, the cells were stimulated with VEGF (50 ng/ml). Catechins were incubated for 4 h and medium was exchanged prior to cell stimulation.

2.3. Reverse transcription/polymerase chain reaction

Total RNA was extracted from cells with tri reagent according to the manufacturer's protocol. Early growth response factor-1 (Egr-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were detected by reverse transcription/polymerase chain reaction as previously described (Ko et al., 1995, 1999).

2.4. Immunoprecipitation and Western blotting

After stimulation, cells were lyzed and phosphorylated extracellular signal-regulated kinases-1 and -2 (ERK1/2) were detected by Western blotting as previously described (Sachinidis et al., 2002). VEGF-R1 and -R2 were immunoprecipitated and tyrosine-phosphorylated receptors were detected by Western blotting as previously described (Sachinidis et al., 2002).

2.5. Determination of cell mitogenesis

DNA synthesis was assessed by determination of [^{3}H]thymidine incorporation, and cell proliferation was measured using the CASY-1 system (Schärfe, Reutlingen, Germany) as described previously (Sachinidis et al, 1999).

2.6. Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance followed by the Bonferroni's multiple comparisons test. P levels of <0.05 were considered significant.

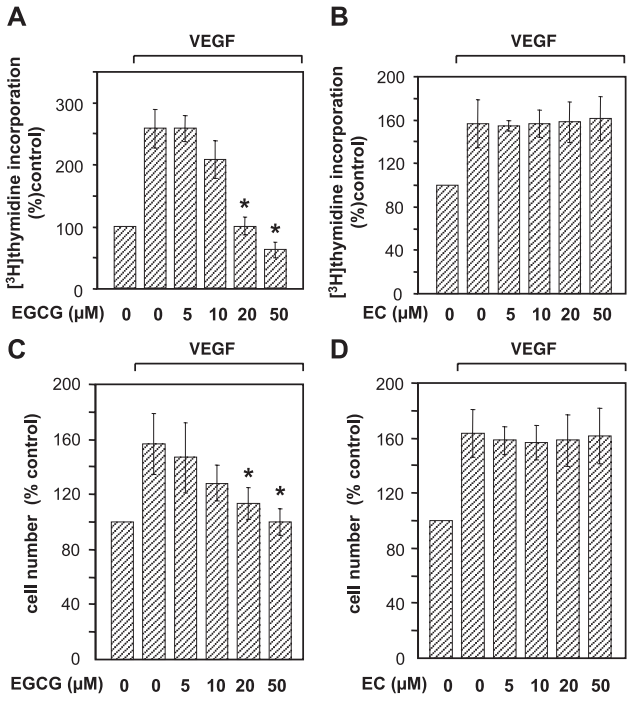

Fig. 1. Effects of epigallocatechi-3 gallate (EGCG) as compared to epicatechin (EC) on vascular endothelial growth factor (VEGF) (50 ng/ml) induced mitogenesis human umbilical arterial endothelial cells. Cells were pretreated for 4 h with EGCG or EC. DNA synthesis was assessed by the determination of [^{3}H] incorporation (A and B). Cell proliferation was measured by cell counting (C and D). Data are means ± standard deviation, $n=3$, $^*P<0.05$ versus vascular endothelial growth factor (VEGF) stimulation (unstimulated control = 100%).

3. Results

3.1. Effects of catechins on the VEGF-induced mitogenesis of human umbilical arterial endothelial cells

HUAEC, pretreated with EGCG, showed a concentration-dependent reduction of VEGF-induced DNA synthesis. A complete inhibition was observed at 50 μM EGCG (Fig. 1A). In contrast, EC did not inhibit VEGF-induced DNA synthesis (Fig. 1B). Similar results were obtained when cell proliferation was measured. Again, EGCG concentration-dependently reduced the VEGF-induced increase of cell proliferation, while EC was not effective (Fig. 1C and D).

3.2. Effect of catechins on VEGF-induced phosphorylation of extracellular signal-regulated kinases-1 and -2

In order to study the mechanisms for the observed inhibitory effects of EGCG on human umbilical arterial endothelial cell mitogenesis, phosphorylation of ERK1/2 was determined after pretreatment of the cells with different concentrations of EGCG or EC. As shown in Fig. 2A, stimulation of untreated cells with 50 ng/ml VEGF induced a time-dependent tyrosine phosphorylation of ERK1/2 with a maximal stimulation at 5 min. Pretreatment of the cells with EGCG caused a concentration-dependent inhibition of VEGF-induced ERK1/2 phosphorylation (Fig. 2B). In contrast, pretreatment of the HUAEC

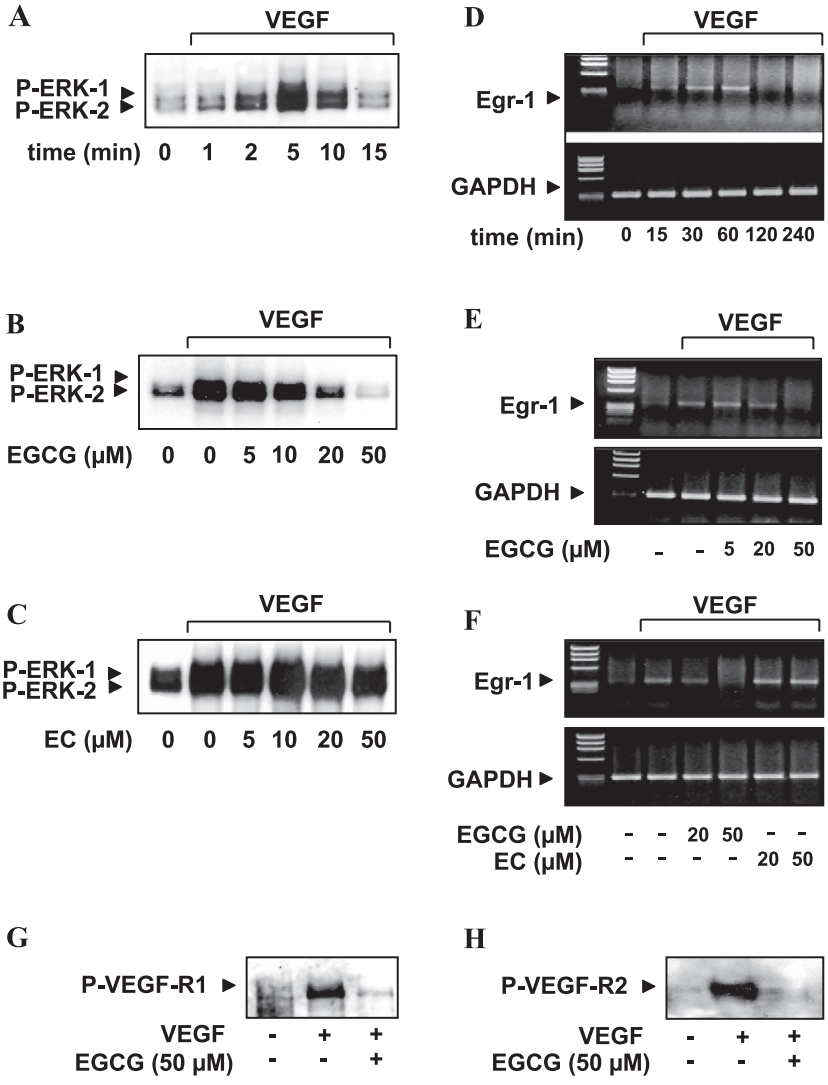

Fig. 2. Effects of EGCG (EGCG) as compared to epicatechin (EC) on vascular endothelial growth factor (50 ng/ml) induced signaling in human umbilical arterial endothelial cells. Cells were pretreated for 4 h with EGCG or EC. Phosphorylation of the extracellular signal-regulated kinases-1 and -2 (P-ERK-1/-2) was determined by Western blotting (A–C). Expression of early growth response factor-1 (Egr-1) as compared to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured by reverse transcription/polymerase chain reaction (D–F). Vascular endothelial growth factor receptor autophosphorylation (P-VEGF-R1/2) was detected by immunoprecipitation/Western blotting (G and H). The gels shown are representative of $n = 3$ independent experiments.

with EC did not influence VEGF-induced ERK1/2 phosphorylation (Fig. 2C).

3.3. Effects of catechins on VEGF-induced early growth response factor-1 mRNA expression

To study whether the observed inhibition of extracellular signal-regulated kinases-1 and -2 would also result in an inhibition of the expression of immediate early genes, the effect of EGCG and EC on the expression of Egr-1 mRNA was investigated. Stimulation of HUAEC with 50 ng/ml VEGF resulted in a time-dependent increase in Egr-1 mRNA with a maximum between 30 and 60 min (Fig. 2D). Pretreatment of the cells with EGCG resulted in concentration-dependent inhibition of VEGF-induced expression of Egr-1 mRNA (Fig. 2E). Again, pretreatment of the cells with EC had no effects on Egr-1 mRNA levels (Fig. 2F).

3.4. Effect of catechins on VEGF-induced tyrosine phosphorylation of VEGF receptor-1 and -2

EGCG (50 μM) almost completely inhibited VEGF-induced tyrosine phosphorylation of VEGF-R1 and -R2 (Fig. 2G and H), while EC (50 μM) was not effective (data not shown).

4. Discussion

VEGF is a mitogen for endothelial cells that is often associated with tumour-induced angiogenesis (Ferrara, 2001); VEGF binds to VEGF receptor-1 and -2, the latter being responsible for most of its mitogenic and chemotactic effects (Ferrara, 2001). VEGF is involved in the angiogenesis of many solid tumours including breast cancer (Kurebayashi et al., 1999), colon cancer (Takahashi et al., 1995), hepatoma (Yoshiji et al., 1999), bladder cancer (Droller, 1998), brain tumours (Samoto et al., 1995), and prostate cancer (Weidner et al., 1993). Moreover, there is increasing evidence that neovascularization is one characteristic feature of atherosclerotic plaques (Kumamoto et al., 1995; Chen et al., 1999; Couffinhal et al., 1997).

The two VEGF receptors form a dimer to activate autophosphorylation of tyrosine residues on the cytoplasmic domain (for a review, see Ferrara, 2001). In the present study, we have shown that treatment with EGCG resulted in an inhibition of human umbilical arterial endothelial cell mitogenesis (DNA synthesis and cell proliferation). In addition, the signal transduction pathways of VEGF in HUAEC, including autophosphorylation of VEGF-R1 and -R2, phosphorylation of ERK1/2, as well as the expression of Egr-1 mRNA, were also inhibited in EGCG-pretreated cells. In agreement with our previous findings in vascular smooth muscle cells, fibroblasts, and glioblastoma cells, EC did not affect signaling or mitogenesis (Ahn et al., 1999; Sachinidis et al., 2000, 2002).

Although several possible inhibitory mechanisms of action of catechins can be discussed, we favour the hypothesis that EGCG, enriched in the plasma membrane, may prevent the binding of growth factors to their respective receptors thereby suppressing tyrosine phosphorylation of these receptor types. Direct interactions between soluble catechins with VEGF are not a likely mechanism to explain the inhibitory effects described in this study because the cells were enriched with the catechins for 4 h and stimulation was performed in the absence of the catechins in the medium. In addition, it is well established that catechins are able to interact with several proliferation-related proteins (Jankun et al., 1997; Kazi et al., 2002; Nam et al., 2001). Thus, the inhibition of growth factor binding may represent a general principle in modulation of growth factor activity by EGCG.

Taken together, the inhibition of VEGF-induced endothelial cell mitogenesis by EGCG, which persists after removal of the compound—in addition to possible direct binding of growth factors—would result in a sustained inhibition of endothelial cell proliferation even at low plasma concentrations between doses. Thus, EGCG or other galloyl group-containing plant-derived catechins are attractive candidates for prevention and/or treatment of proliferative diseases.

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