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(–)-Epigallocatechin gallate suppresses the growth of human hepatocellular carcinoma cells by inhibiting activation of the vascular endothelial growth factor–vascular endothelial growth factor receptor axis

Yohei Shirakami,[1] Masahito Shimizu,[1,3] Seiji Adachi,[1] Hiroyasu Sakai,[1] Takayuki Nakagawa,[1] Yoichi Yasuda,[1] Hisashi Tsurumi,[1] Yukihiko Hara[2] and Hisataka Moriwaki[1]

[1]Department of Internal Medicine, Gifu University Graduate School of Medicine, Gifu; [2]Tea Solution, Hara Office, Tokyo, Japan

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The receptor tyrosine kinase vascular endothelial growth factor (VEGF) receptor (VEGFR) plays an important role in tumor angiogenesis of hepatocellular carcinoma (HCC). (–)-Epigallocatechin gallate (EGCG), the major biologically active component of green tea, inhibits growth in a variety of human cancer cells by inhibiting the activation of several types of receptor tyrosine kinases. In this study, we examined the effects of EGCG on the activity of the VEGF–VEGFR axis in human HCC cells. The levels of total and phosphorylated (i.e. activated) form of VEGFR-2 protein (p-VEGFR-2) were observed to increase in a series of human HCC cell lines in comparison to the Hc normal human hepatocytes. EGCG preferentially inhibited the growth of HuH7 HCC cells, which express constitutive activation of the VEGF–VEGFR axis, in comparison to Hc cells. Treatment of HuH7 cells with EGCG caused a time- and dose-dependent decrease in the expression of VEGFR-2 and p-VEGFR-2 proteins. The production of VEGF from HuH7 cells was reduced by treatment with EGCG. Drinking of EGCG significantly inhibited the growth of HuH7 xenografts in nude mice and this was associated with inhibition of the activation of VEGFR-2 and its related downstream signaling molecules, including ERK and Akt. EGCG drinking also decreased the expression of Bcl-x_L protein and VEGF mRNA in the xenografts. These findings suggest that EGCG can exert, at least in part, its growth-inhibitive effect on HCC cells by inhibiting the VEGF–VEGFR axis. EGCG might therefore be useful in the treatment of HCC. (*Cancer Sci* 2009; 100: 1957–1962)

HCC, which commonly arises in the liver with chronic inflammation and cirrhosis, is a major health care problem worldwide.[1] Because the prognosis of patients with HCC is poor, there is a critical need to develop more effective strategies for the therapy and prevention of this malignancy. Recent studies have revealed that the aberrant activation of several RTK and related downstream pathways of signal transduction play a critical role in the development of HCC and thus might be promising targets for the treatment of this cancer.[2–4] For instance, sorafenib, a multikinase inhibitor that targets the serine-threonine kinases Raf-1 and B-Raf and the RTK activity of VEGFR-1, VEGFR-2, and VEGFR-3 and platelet-derived growth factor receptor-β, has shown a survival benefit in patients with HCC.[5] In preclinical experiments, sorafenib exerted antiproliferative effects on the HCC-derived cell lines and reduced tumor growth by inhibiting angiogenesis in a mouse xenograft model of human HCC.[6]

It is widely accepted that neovascularization and angiogenesis play a key role in the growth of solid tumor.[7,8] VEGF, which binds to and activates VEGFR, is important in pathological angiogenesis, and the VEGF–VEGFR axis is therefore closely associated with tumor growth.[7,8] In particular, HCC is a well-known hypervascular tumor and a close relationship has been demonstrated between VEGF expression and either angiogenic activity or tumor progression in HCC.[9,10] Overexpression of VEGFR is also observed in human HCC and this has been shown to correlate with a poor prognosis.[11,12] In addition, several HCC cell lines express VEGFR and VEGF may act as an autocrine growth factor in stimulating the proliferation of these cells.[13] These findings suggest that inhibition of the VEGF–VEGFR axis can theoretically reduce angiogenesis and tumor growth in HCC, and several agents that target this axis have been developed for the treatment of HCC.[2–5] In a recent phase II study, bevacizumab, a humanized anti-VEGF monoclonal antibody, alone or in combination with cytotoxic agents was used as treatment for patients with HCC and they showed a moderate antitumor activity.[14,15] In HCC cells, RTK787, a tyrosine kinase inhibitor of VEGFR, inhibited tumor cell proliferation and induced apoptosis both *in vivo* and *in vitro*.[13]

Numerous epidemiological and experimental studies suggest that green tea catechins have both anticancer and cancer chemopreventive effects at various organ sites.[16–18] One of the anticancer mechanisms of green tea or its constituents is explained by their inhibitory effect on angiogenensis. Namely, EGCG, the major biologically active component of green tea, induces potent inhibition of VEGF-dependent tyrosine phosphorylation of VEGFR-2 and this is associated with the suppression of *in vitro* angiogenesis.[19] The production of VEGF by cancer cells decreases after treatment with EGCG, thus contributing to its potent antiangiogenic activity.[20,21] Green tea extract and EGCG also caused a decrease in VEGF production by HCC cells;[22] however, whether EGCG can inhibit activation of the VEGF–VEGFR axis, thus inducing growth inhibition of HCC tumor, has not yet been examined. In the present study we investigated in detail the effects of EGCG on activation of the VEGF–VEGFR axis and the growth of HCC cells using *in vitro* and *in vivo* models.

Materials and Methods

Chemicals. EGCG was obtained from Mitsui Norin Co. (Tokyo, Japan).

Cell lines and cell culture conditions. Six human HCC cell lines, HLF, PLC/PRF/5, HepG2, HuH7, HLE, and Hep3B, were obtained from the Japanese Cancer Research Resources Bank

[3]To whom correspondence should be addressed. E-mail: shimim-gif@umin.ac.jp

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(Tokyo, Japan) and maintained in DF10 medium containing DMEM (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen). The Hc human normal hepatocyte cell line was purchased from Applied Cell Biology Research Institute (Kirkland, WA, USA) and maintained in CS-C complete medium (Cell Systems Biotechnologie Vertrieb, St Katharinen, Germany). The cells were cultured in an incubator with humidified air with 5% CO_2 at 37°C.

Cell viability assays. Cell viability assays were conducted using the MTT cell proliferation kit I (Roche Diagnosis Co., Indianapolis, IN, USA), according to the manufacturer's instructions, as described previously.[23] Three thousand HuH7 or Hc cells were seeded into 96-well plates. Twenty-four hours later, the cells were treated with the indicated concentrations of EGCG (0–100 μg/mL) for 48 h in DF10 medium, and cell viability was examined. All assays were carried out in triplicate.

VEGF production assays. HuH7 cells were plated into six-well plates and grown to 70% confluence. After washing with PBS, the cells were treated with the indicated concentrations of EGCG (0–100 μg/mL) in serum-minus medium for 24 h. The cell-free medium was then collected and the amounts of VEGF secreted by the cells into the medium were measured using a VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Protein extraction and western blot analysis. Total protein was extracted from the cell lines or xenografts of HuH7 cells and equivalent amounts of protein (20 μg/lane) were subjected to a western blot analysis, as described previously.[24,25] The primary antibodies for ERK, p-ERK, Akt, and p-Akt were described previously.[23] The primary antibodies for VEGFR-2 (#2479), p-VEGFR-2 (#2478), Bcl-x_L (#2762), and GAPDH (#2118) were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody to GAPDH served as a loading control.

RNA extraction and semiquantitative RT-PCR analysis. A semiquantitative RT-PCR analysis was carried out, as described previously.[26] Total RNA was isolated from the xenopgrafts of HuH7 cells using ISOGEN reagent (Nippon Gene Co., Tokyo, Japan), according to the manufacturer's instructions. The cDNA was amplified from 1 μg of total RNA using SuperScript one-step RT-PCR with the platinum *Taq* system (Invitrogen). The primers used for amplification of *VEGF* and *GAPDH* specific genes were as follows: *VEGF* forward, 5′-CTA CCT CCA CCA TGC CAA GT-3′; *VEGF* reverse, 5′-AAA TGC TTT CTC CGC TCT GA-3′; *GAPDH* forward, 5′-CGA GAT CCC TCC AAA ATC AA-3′; and *GAPDH* reverse, 5′-TTC AGC TCA GGG ATG ACC TT-3′. Using a thermal controller (Programmable Thermal Controller; MJ Research, Watertown, MA, USA), 35-cycle rounds of PCR were chosen for data analysis of the mRNA expression as a semiquantitative assessment indicated that the reaction had not reached a plateau and was still in log phase. The amplified products obtained with *GAPDH*-specific primers served as an internal control. The intensities of the PCR products stained with ethidium bromide were quantified using the NIH Image software program version 1.62 (URL: http://rsb.info.nih.gov/nih-image/index.html).

***In vivo* experimental protocol.** Twenty-four male BALB/c nude mice (5 weeks of age) were obtained from Charles River Japan (Tokyo, Japan). All mice were maintained at Gifu University Life Science Research Center, according to the Institutional Animal Care Guidelines, and were housed in plastic cages with free access to drinking water (tap water supplemented with or without EGCG) and the pelleted basal diet CRF-1 (Oriental Yeast Co., Tokyo, Japan). Xenograft tumors were made by subcutaneous injection of HuH7 cells, at a concentration of 5×10^6 cells per 200 μL, into the flanks of these mice. One week after tumor cell injection, the mice were randomly divided into three groups (eight mice per group) and then treated with (groups 2 and 3) or without (group 1) EGCG for 5 weeks. The mice in groups 2 and 3 were given tap water containing 0.01 or 0.1% EGCG, respectively.

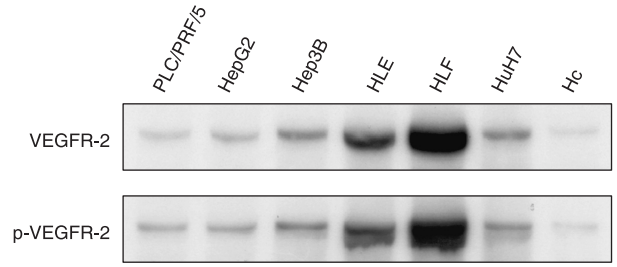

Fig. 1. The expression levels of total vascular endothelial growth factor receptor (VEGFR)-2 and phosphorylated vascular endothelial growth factor receptor (p-VEGFR)-2 proteins in human hepatocellular carcinoma cell lines and Hc normal hepatocytes. Total protein extracts were prepared from 70% confluent cultures of the indicated cell lines and equivalent amounts of protein (20 μg/lane) were examined by western blot analysis using appropriate antibodies. Repeat western blots gave similar results.

The concentrations of EGCG (0.01 and 0.1%) were established according to the findings of previous reports[27,28] because these doses could exert anticancer properties without causing various side effects in any organs. The mice in group 1 were given tap water and served as an untreated control. A freshly prepared solution of EGCG in tap water was supplied to the experimental mice three times a week. The tumor size and bodyweight were measured once a week and the tumor volume was calculated using the formula: largest diameter × (smallest diameter)^2 × 0.5.

Statistical analysis. The data were expressed as the mean ± SD. The statistical significance of the difference in mean values was tested using a one-way analysis of variance (ANOVA) and the unpaired *t*-test. Significance was defined as a *P*-value less than 0.05. All analyses were carried out using the StatView ver. 5.0 software program (SAS Institute, Cary, NC, USA).

Results

Expression of VEGFR-2 and p-VEGFR-2 proteins in human HCC cell lines and Hc normal hepatocytes. Among the VEGFR, VEGFR-2 is considered to be the major mediator of the mitogenic and angiogenic effects of VEGF.[7,8] We therefore initially examined whether VEGFR-2 protein is overexpressed and constitutively activated in HLF, PLC/PRF/5, HepG2, HuH7, HLE, and Hep3B human HCC cell lines and in Hc human normal hepatocytes using western blot analysis. Among these HCC cell lines, the level of VEGFR-2 protein was observed to markedly increase in the HLF and HLE cells, whereas it was moderately expressed in Hep3B and HuH7 cells (Fig. 1). The level of phosphorylated (i.e. activated) VEGFR-2 protein (p-VEGFR-2) also increased in these four cell lines, thus indicating the constitutive activation of this receptor (Fig. 1). Moreover, all HCC cell lines that were examined in this experiment significantly expressed VEGFR-2 and p-VEGFR-2 proteins in comparison to the Hc normal human hepatocytes (Fig. 1).

Effects of EGCG on the growth of HuH7 and Hc cells. We then examined the growth-inhibitory effects of EGCG on HuH7 and Hc cell lines using MTT assays. As shown in Figure 2, EGCG inhibited the growth of HuH7 cells with an IC_{50} value of approximately 25 μg/mL. However, the Hc cells were more resistant to EGCG because the IC_{50} value with this agent was approximately 84 μg/mL (Fig. 2). These findings suggest that EGCG preferentially inhibits the growth of HuH7 HCC cells, which express higher levels of the VEGFR-2 and p-VEGFR-2 proteins, when compared with Hc cells that do not express these proteins (Fig. 1).

Effects of EGCG on the expression and activation of VEGFR-2 in HuH7 cells. We next examined whether EGCG alters the expression and activation of VEGFR-2 in HuH7 cells. A time-course study indicated that when the cells were treated with 25 μg/mL EGCG, which is the same as the IC_{50} concentration determined by the

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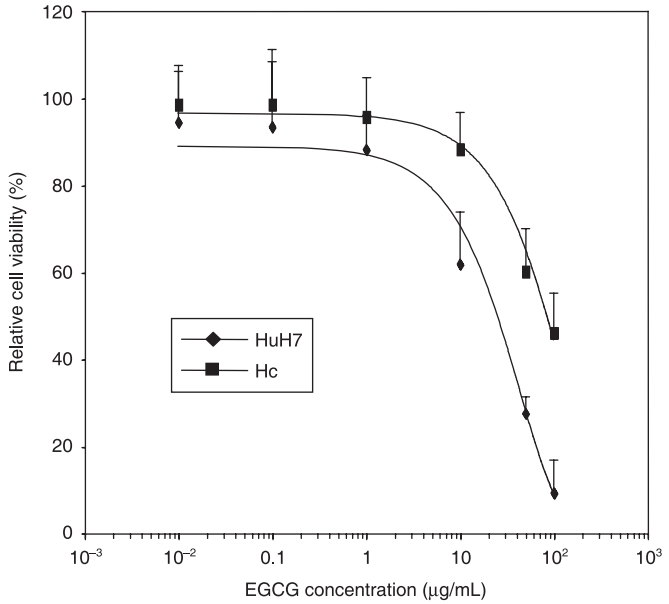

Fig. 2. Inhibition of cell growth by (–)-epigallocatechin gallate (EGCG) in HuH7 human hepatocellular carcinoma cells and Hc normal hepatocytes. These cells were treated with the indicated concentrations of EGCG or DMSO for 48 h and cell viability assays were conducted using the MTT system. Results are expressed as a percentage of growth with 100% representing control cells treated with DMSO alone. Bars, SD of triplicate assays.

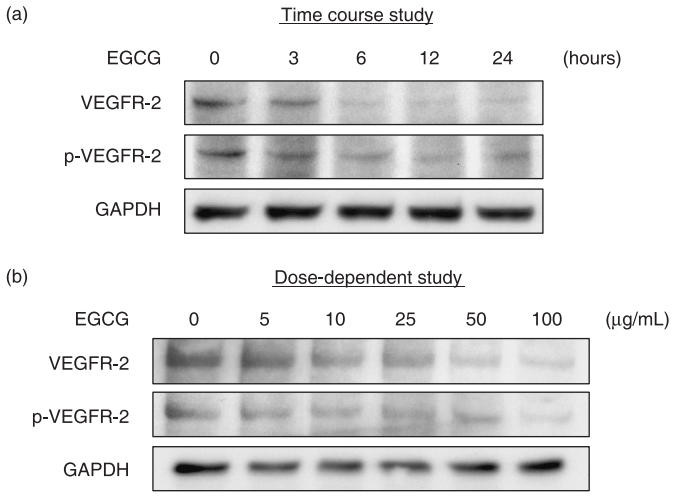

Fig. 3. Effects of (–)-epigallocatechin gallate (EGCG) on expression levels of total vascular endothelial growth factor receptor (VEGFR)-2 and phosphorylated vascular endothelial growth factor receptor (p-VEGFR)-2 proteins in HuH7. The cells were treated with (a) 25 μg/mL EGCG for the indicated times (0, 3, 6, 12, and 24 h, time course study) or (b) the indicated concentration of EGCG (0, 5, 10, 25, 50, and 100 μg/mL, dose-dependence study) for 6 h, and the cell extracts were then examined by western blot analysis using the respective antibodies. An antibody to GAPDH served as a loading control. Similar results were obtained in a repeat experiment.

MTT assays (Fig. 2), a marked decrease was observed in the expression levels of both VEGFR-2 and p-VEGFR-2 proteins within 6 h of the addition of this agent (Fig. 3a). When the cells were treated with the indicated concentrations of EGCG (0–100 μg/mL) for 6 h, the expression levels of VEGFR-2 as well as p-VEGFR-2 proteins were also inhibited in a dose-dependent manner (Fig. 3b).

Effects of EGCG on VEGF production by HuH7 cells. VEGF, which is produced by cancer cells, has been reported to play a critical

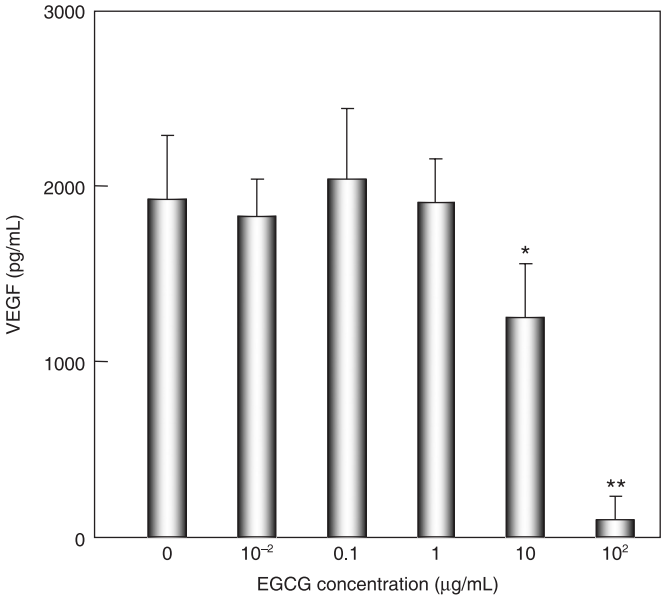

Fig. 4. Effects of (–)-epigallocatechin gallate (EGCG) on production of vascular endothelial growth factor (VEGF) by HuH7 cells. The cells were treated with the indicated concentration of EGCG (0, 0.01, 0.1, 1.0, 10, and 100 μg/mL) in serum-free medium for 24 h. The medium was then collected and assayed for VEGF using an ELISA kit. Bars, SD of triplicate assays. $*P < 0.05$, $**P < 0.01$: significant differences obtained by comparison with EGCG-untreated control group.

role in tumor angiogenesis.[7,8] We therefore next examined the effects of EGCG on production of VEGF by HuH7 cells using an ELISA system. As shown in Figure 4, HuH7 cells secreted an abundant amount of VEGF into the growth medium when the cells were cultured in serum-free medium for 24 h and, interestingly, a low (10 μg/mL, less than IC_{50} value) and high (100 μg/mL) concentration of EGCG significantly reduced the production of VEGF from these cancer cells.

Effects of EGCG on the growth of HCC xenografts in nude mice. We next examined whether the growth inhibition of the treatment with EGCG in HuH7 cells was also observed *in vivo* using a nude mouse xenograft model. Figure 5 shows that drinking water with not only a high concentration (0.1%), but also a low concentration (0.01%) of EGCG strongly inhibited the growth of the HuH7 xenograft during treatment with this agent (5 weeks). The tumor volume of the mice treated with 0.1% EGCG was less than that of tumors in 0.01% EGCG-drinking mice, but the difference was not significant (Fig. 5). All of the treatments were well tolerated and the bodyweights remained stable in all groups during the experiment (data not shown). There were no pathological alterations suggesting toxicity of EGCG in the liver, spleen, and kidneys of mice (data not shown).

Effects of EGCG on the activation of VEGFR-2 and its downstream signaling molecules and on the cellular levels of Bcl-x_L in xenografts of HuH7 cells. We next examined whether treatment with EGCG inhibits the activation of VEGFR-2 and its multiple downstream signaling pathways in the HuH7 xenografts. Drinking both 0.01 and 0.1% EGCG decreased the total levels of VEGFR-2 and Akt proteins in these xenografts (Fig. 6a). There was also a marked decrease in the levels of p-VEGFR-2, p-ERK, and p-Akt proteins by treatment with both concentrations of EGCG (Fig. 6a). In addition, EGCG caused a decrease in the levels of Bcl-x_L, an anti-apoptotic Bcl-2 family member, in HuH7 xenogrfts (Fig. 6a).

Effects of EGCG on the expression of VEGF mRNA in xenografts of HuH7 cells. A semiquantitative RT-PCR study showed that there was a significant decrease in the level of VEGF mRNA in the

xenografts of mice treated with 0.1% EGCG compared to that of the tumors in control mice (Fig. 6b). Therefore, the inhibitory effect of EGCG on the production of VEGF was not only observed *in vitro* (Fig. 4) but also *in vivo*.

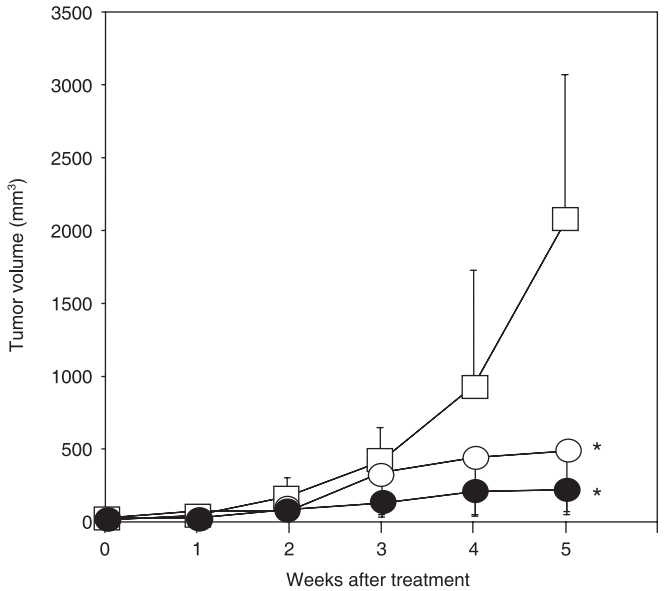

Fig. 5. Effects of (–)-epigallocatechin gallate (EGCG) on the growth of HuH7 xenografts in nude mice. Male BALB/c nude mice were injected subcutaneously with 5×10^6 HuH7 cells. One week after the injection, the mice were divided into three groups and treated with following conditions for 5 weeks: group 1, control group (tap water drinking group, □); group 2, 0.01% EGCG-drinking group (○); and group 3, 0.1% EGCG-drinking group (●). The growth curve of HuH7 tumors in each group are represented. Bars, SD. $*P < 0.05$: significant differences obtained by comparison with EGCG-untreated control group.

Discussion

The results of the present study clearly indicate that EGCG effectively suppresses the growth of HuH7 human HCC cells both *in vitro* (Fig. 2) and *in vivo* (Fig. 5) and this was associated with inhibition of the VEGF–VEGFR axis (Figs 3,6a). It should be particularly emphasized that not only a high concentration (0.1%), but also a low concentration (0.01%) of EGCG similarly inhibited the growth of HCC xenografts by blocking the VEGF–VEGFR axis to the same extent (Figs 5,6a). This is the first report indicating a low dose of EGCG (0.01%) to be sufficient to reduce HCC tumor growth, although the feeding protocol of EGCG at a high dose (0.1%), which mimics an approximate consumption of six cups of green tea per day by an average adult human, has been used in mice in many prior chemopreventive studies.[16,29] These findings, together with the result of a previous study reporting that drinking 0.05% EGCG also significantly repressed the tumor growth of highly angiogenic sarcoma xenografts by inhibiting angiogenesis *in vivo*,[30] might thus be preferable when considering the clinical use of this agent because a lower dose is more acceptable for administration to patients.

One of the main mechanisms of how EGCG can block the VEGF–VEGFR axis in cancer cells is explained by its efficacy in reducing VEGF secretion from these cells.[20–22] The expression of VEGF is regulated by micro-environmental alterations, such as hypoxia, and recent studies have revealed that HIF-1 strongly activates transcription of the *VEGF* gene by phosphorylating the ERK and Akt proteins.[7,31–33] The increased expression of VEGFR and HIF-1α, the regulated subunit of HIF-1, is considered to play a role in the progression of HCC.[34] Hepatitis C virus infection leads to the stabilization of HIF-1α via activation of the MAPK–ERK and PI3K–Akt signaling pathways, thus leading to neovascularization.[35] In the present study we demonstrated that EGCG reduces the expression of VEGF mRNA and production of this growth factor by inhibiting the activation of ERK and Akt proteins in HuH7 cells (Figs 4,6b). These findings are

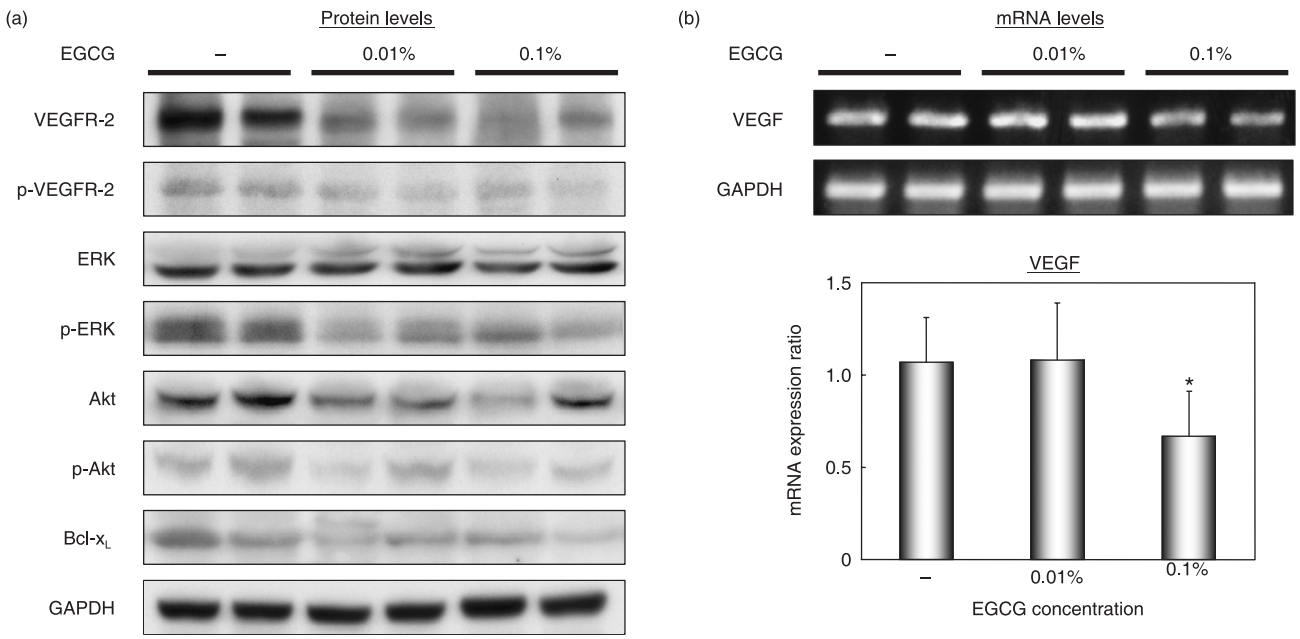

Fig. 6. Effects of (–)-epigallocatechin gallate (EGCG) on activation of vascular endothelial growth factor receptor (VEGFR)-2, its related downstream signaling pathways, and on the cellular levels of Bcl-xL proteins and vascular endothelial growth factor (VEGF) mRNA in HuH7 xenografts. The xenografts were excised from each animal at the termination of the experiment and tumor extracts were examined by (a) western blot analysis using the respective antibodies or (b) a semiquantitative RT-PCR analysis using *VEGF*-specific primers. An antibody to GAPDH served as a loading control (A). Amplified PCR products obtained with *GAPDH*-specific primers served as internal controls. (b) The results obtained from RT-PCR analysis were quantified by densitometry and are displayed in the lower panel. Bars, SD of triplicate assays. $*P < 0.05$: significant differences obtained by comparison with EGCG-untreated control group. p-, phosphorylated.

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consistent with those of a previous report, in which green tea extract and EGCG were observed to cause a drastic decrease in VEGF expression at both the mRNA and protein levels by suppressing the expression of HIF-1α and blocking both the PI3K–Akt and MAPK–ERK signaling pathways in HepG2 human HCC cells.[22] Because several HCC cell lines express the constitutive activation of VEGFR-2 (Fig. 1), our findings, together with those of the previous report,[22] suggest the possibility that EGCG might be able to inhibit cell growth by disrupting the VEGF–VEGFR-related autocrine loop that exists in HCC cells.

The transcription of VEGF mRNA is also induced by the activation of a variety of RTK.[7] For instance, activation of the IGF-1–IGF-1 receptor axis induces expression of the *VEGF* gene via induction of HIF-1α.[31] Both the activation of EGFR and HER2 induce the secretion of VEGF by activating the PI3K–Akt signaling pathway.[32,33] These reports seem to be significant when considering the characteristic effects of EGCG because this agent can inhibit not only VEGFR-2 (Figs 3,6a), but also the activation of several other RTK, including IGF-1R, EGFR, HER2, and HER3, and multiple downstream signaling pathways in human HCC and colon cancer cells.[23–26] One of the possible mechanisms for this remarkable range of effects by EGCG might be associated with its ability to bind directly to all of these receptors, thereby inhibiting their tyrosine kinase activities, perhaps because of sufficient homologies in their kinase domain.[36] This presumption might be supported by the previous report that EGCG can act directly to inhibit the kinase activity of some RTK.[37,38] The recent report describing that EGCG inhibits the binding of VEGF to VEGFR in a concentration-dependent manner[39] also encourages this hypothesis. In addition, the effects of EGCG to decrease the total levels of VEGFR-2 itself contribute to inhibit activation of the VEGF–VEGFR axis in the present study (Figs 3,6a). These findings are consistent with a recent report that green tea extract could decrease the expression of both VEGFR-1 and VEGFR-2 on human umbilical vein endothelial cells.[40]

In addition to the direct effects of EGCG on specific RTK at the cell surface, recent studies revealed that EGCG exerts its effects on RTK indirectly by targeting the lipid organization of the plasma membrane, so-called 'lipid rafts', which are associated with these RTK. Indeed, the inhibitory effect of EGCG on EGF binding to the EGFR and the subsequent dimerization of this receptor is associated with alterations in the lipid rafts of colon cancer cells.[41] EGCG also decreases cell surface-associated EGFR by inducing the internalization of EGFR into endosomal vesicles, thereby inhibiting the activation of this receptor and exerting anticancer effects.[42] Because VEGFR-2 is also localized to lipid rafts,[43] future studies would be required to elucidate whether the inhibitory effect of EGCG on the activation of VEGFR-2 is associated with alterations in membrane lipid order in cancer cells.

Agents that can co-target several different RTK, such as sorafenib, are expected to be promising candidates for the treatment of HCC.[2–6] In conclusion, the ability of EGCG to target both the VEGF–VEGFR axis, as demonstrated in the present study, and other types of RTK that play critical roles in the proliferation of cancer cells,[23–26] is thus considered to provide evidence that this naturally occurring agent may be effective in the chemoprevention and therapy of HCC, and likely of other malignancies as well, that show hypervascularity.

Acknowledgments

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Abbreviations

EGCG	(–)-epigallocatechin gallate
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
HCC	hepatocellular carcinoma
HER	human epidermal growth factor receptor
HIF	hypoxia-inducible factor
IGF	insulin like growth factor
PDGFR	platelet-derived growth factor receptor
RTK	receptor tyrosine kinase
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor.

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