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EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells ☆

Masahito Shimizu [a,b,*], Yohei Shirakami [a], Hiroyasu Sakai [a], Hideharu Tatebe [a], Takayuki Nakagawa [a], Yukihiko Hara [c], I. Bernard Weinstein [b], Hisataka Moriwaki [a]

[a] *Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan*
[b] *Herbert Irving Comprehensive Cancer Center and Department of Medicine, Columbia University Medical Center, New York, NY 10032, USA*
[c] *Polyphenon Division, Mitsui Norin Co. Ltd., Tokyo 160-8381, Japan*

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Abstract

The receptor tyrosine kinase (RTK) insulin like growth factor-1 (IGF-1)/IGF-1 receptor (IGF-1R) axis plays an important role in the development of hepatocellular carcinoma (HCC). EGCG inhibits activation of the various types of RTKs and that this is associated with inhibition of multiple downstream signaling pathways. In this study we examined the effects of EGCG on activity of the IGF/IGF-1R axis in HepG2 human HCC cells which express constitutive activation of this axis. The level of phosphorylated (*i.e.* activated) form of the IGF-1R protein (p-IGF-1R) was increased in a series of human HCC cell lines when compared with the Hc normal human hepatocytes. EGCG preferentially inhibited growth of HepG2 cells when compared with Hc cells. Treatment of HepG2 cells with EGCG induced apoptosis and caused a decrease in the p-IGF-1R protein and its downstream signaling molecules including the p-ERK, p-Akt, p-Stat-3, and p-GSK-3β proteins, both in the absence or presence of ligand stimulation. EGCG also decreased the levels of both IGF-1 and IGF-2 proteins and mRNAs, but increased the levels of the IGFBP-3 protein. These findings suggest that EGCG can overcome the stimulatory effects of IGFs on the IGF-1R dependent signaling pathway, thus expanding the roles of EGCG as an inhibitor of critical RTKs involved in HCC cell proliferation. These results provide further evidence that EGCG may be useful in the chemoprevention or treatment of liver cancer.

Abbreviations: IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; IGFBP, IGF binding protein; RTK, receptor tyrosine kinase; EGCG, (−)-epigallocatechin-3-gallate; EGF, epidermal growth factor; EGFR, EGF receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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* Corresponding author. Address: Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Tel.: +81 58 230 6308; fax: +81 58 230 6310.
E-mail address: shimim-gif@umin.ac.jp (M. Shimizu).

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1. Introduction

Tea is one of the most popular beverages consumed worldwide. Numerous epidemiologic and experimental studies provide evidence that green tea can inhibit both the development and growth of various types of human malignancies [1–4]. One of the anticancer mechanisms of green tea or its constituents is explained by their inhibitory effects on the activation of specific receptor tyrosine kinases (RTKs) and related downstream pathways of signal transduction [3,4]. Thus, in previous studies we found that EGCG, a major biologically active component of green tea, inhibits growth and induces apoptosis by inhibiting the activation of EGFR (erbB1), HER2 (neu/erbB2), and also HER3 (neu/erbB3), which belong to subclass I of the RTK superfamily, and multiple downstream signaling pathways, in human colon cancer cells [5,6]. We also found that in colon cancer cells EGCG can inhibit activation of IGF-1R, which belongs to a separate family of RTKs [7].

The IGF-1R and its ligands, IGF-1 and IGF-2, play essential roles in cell growth and development [8,9]. The binding of these ligands to their receptors results in the activation of the intrinsic tyrosine kinase domain, thus initiating various signaling pathways, including the PI3K/Akt pathway and the Ras/MAPK pathway, which result either in cellular proliferation or in a particular differentiated function [10–12]. Besides regulation of normal cell growth, it is widely appreciated that a relationship exists between the IGF/IGF-1R system and development of various types of cancer, including hepatocellular carcinoma (HCC) [10–13]. Thus, IGF-2 and IGF-1R, which are expressed at low levels in normal hepatocytes, are overexpressed in human HCC tissue as well as HCC cell lines [14]. High focal expression of IGF-2 is also detected in hepatocytes within the cirrhotic liver induced by persistent infection with hepatitis B and C virus [15,16]. The expression levels of IGFBP-3, which regulates the activity and function of IGFs [10–12], are decreased in human HCC samples when compared to non-neoplastic liver tissue [17], and the decreased expression of this protein is significantly associated with poor survival of HCC patients [18]. Blockade of the IGF/IGF-1R axis by a IGF-1R tyrosine kinase inhibitor induces growth inhibition, apoptosis, and cell cycle arrest in human HCC cell lines [19]. Therefore, the IGF/IGF-1R signaling pathway appears to be a critical molecular target with respect to prevention and treatment of HCC.

In the present study we examined in detail the effects of EGCG on activation of IGF-1R and its downstream signaling pathways in HCC cells stimulated with IGFs. We also examined whether EGCG alters the production of IGF-1, IGF-2, and IGFBP-3 by HCC cells because autocrine and/or paracrine loops between these molecules play a critical role in the course of hepatocarcinogenesis and in the proliferation of HCC cells [13,17,18,20].

2. Materials and methods

2.1. Chemicals

EGCG was provided by Mitsui Norin Co., LTD (Tokyo, Japan). Recombinant human IGF-1 and IGF-2 were purchased from R&D systems (Minneapolis, MN).

2.2. Cell lines and cell culture

The HuH7, PLC/PRF/5, HLF, HLE, HepG2, and Hep3B human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). All of HCC cell lines were maintained in DF10 medium containing DMEM (Invitrogen, San Diego, CA) supplemented with 10% FBS (Invitrogen). Normal human hepatocytes, Hc cells, were purchased from Applied Cell Biology Research Institute (Kirkland, WA) and were maintained in the attached CS-C complete media. Cells were cultured in an incubator with humidified air at 37 °C with 5% CO_2.

2.3. Cell viability assays

Cell viability assays were examined using the MTT cell proliferation kit I (Roche Diagnostics Co., Indianapolis, IN), according to the manufacturer's instructions, as described previously [5]. The HepG2 HCC cells and the Hc normal hepatocytes were plated onto 96-well plates (3.0×10^3 cells/well). Twenty-four hours later, the cells were treated with the indicated concentrations (0–75 μg/ml) of EGCG for 48 h in DF10 medium, and cell viability assays were then done using the MTT system. All assays were done in triplicate.

2.4. TUNEL assays

The HepG2 cells were treated with 20 or 40 μg/ml of EGCG for 48 h on cover slips. The cells were then fixed with 3.7% formaldehyde at room temperature for 10 min, permeabilized with 0.3% Triton X-100 in TBS (pH 7.4), and stained with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) methods using *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics Co), as described previously [21].

2.5. IGF-1, IGF-2, and IGFBP-3 production assays

The HepG2 cells were plated into 35-mm dishes and grown to 60% confluence. The cells were then treated with 20 μg/ml of EGCG in serum minus medium for 72 h. The cell free medium was then collected and the amounts of IGF-1, IGF-2, and IGFBP-3 secreted by cells into medium were measured using ELISA kit. The kit for measurement of IGF-1 (DG100) and IGFBP-3 (DGB300) were purchased from R&D Systems and the kit for IGF-2 (10-2600) was from DSL (Webster, TX).

2.6. Protein extraction and Western blot analysis

Total cellular protein was extracted and equivalent amounts of protein were examined by Western blot analysis, as previously described [5–7]. Cell lysates were separated by SDS–PAGE using 7.5–15% polyacrylamide gels and transferred onto Immobilon-P transfer membranes (Millipore Co., Bedford, MA). The primary antibodies for IGF-1Rβ, p-IGF-1R, ERK, p-ERK, Akt, and p-Akt were described previously [5,7]. The primary antibodies for Stat3, p-Stat3, and p-GSK-3β were purchased from Cell Signaling Technology (Beverly, MA). The primary antibody for GSK-3β was purchased from BD Transduction Laboratories (San Jose, CA). An antibody to GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. Each membrane was developed using an ECL-enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

2.7. RNA extraction and semiquantitative RT-PCR analysis

RNA extraction and semiquantitative RT-PCR analysis were performed as described previously [7]. Total RNA was isolated from HepG2 cells using Trizol reagent (Invitrogen) as recommended by the manufacturer. The cDNA was amplified from 1 μg of total RNA using SuperScript one-step RT-PCR with the platinum Taq system (Invitrogen). The primer used for amplification of *IGF-1* specific gene is described previously [7]. The sequences for IGF-2 specific primer, F2IGF2 (5′-GGT GCT TCT CAC CTT CTT GG-3′) and R2IGF2 (5′-CTT GGG TGG GTA GAG CAA TC), were designed using published sequence [22]. The amplified products obtained with GAPDH-specific primers [21] served as internal control. By using a thermal controller (Programmable Thermal Controller; MJ Research Inc., Watertown, MA), 35-cycle rounds of PCR were chosen for data analysis of expression of IGF-1 and IGF-2 mRNAs, respectively, since a semiquantitative assessment indicated that the reaction had not reached a plateau and were still in the log phase. The intensities of the PCR products stained with ethidium bromide were quantified with NIH Image software version 1.62.

2.8. IGF stimulation assays

After 50% confluent HepG2 cells were incubated in DMEM minus serum for 24 h, the cells were incubated in the presence or absence of 20 μg/ml of EGCG in serum minus medium for an additional 24 h and then harvested. One half of these cultures were also stimulated with 15 μM of IGF-1 or IGF-2 for 3 h, just prior to harvesting. Proteins and mRNAs were extracted, and Western blot and RT-PCR analyses were then performed as described above.

2.9. Statistical analysis

Values were expressed as mean and standard deviation. Statistical significance of the difference in mean values was tested by one-way analysis of variance (ANOVA) followed by Scheffe's *t*-test. Significance was declared with P value less than 0.05. All analyses were performed by StatView ver. 5.0 (SAS Institute, Cary, NC).

3. Results

3.1. Expression of IGF-1R and p-IGF-1R proteins in HCC cell Lines and Hc normal hepatocytes

In our initial study we examined the expression levels of IGF-1R and p-IGF-1R proteins in six human HCC cell lines and in Hc normal human hepatocytes by Western blot analysis (Fig. 1). We detected the IGF-1Rβ protein in all six HCC cell lines and in Hc cells, with a very high level in the HepG2, HuH7, and Hep3B cells. We also detected the p-IGF-1R protein, which indicates constitutive activation of this receptor, in the HCC cell lines, except for HLF cells. The highest levels of p-IGF-1R were again found in the HepG2, HuH7, and Hep3B cells. However, the p-IGF-1R protein was not detected in Hc cells (Fig. 1).

3.2. Effects of EGCG on cell growth in HepG2 and Hc cells

We then examined growth inhibitory effects of EGCG on the HepG2 human HCC cells and the Hc normal hepatocytes, using MTT assays. As shown in Fig. 2, EGCG

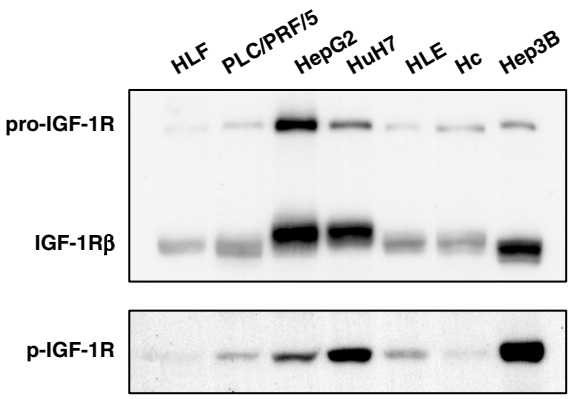

Fig. 1. The expression levels of IGF-1β and p-IGF-1R proteins in HCC cell lines and Hc normal hepatocytes. Total protein extracts were prepared from 70% confluent cultures of the indicated cell lines and equivalent amounts of protein (60 μg/lane) were examined by Western blot analysis using the appropriate antibodies, as described in Section 2. Repeat Western blots gave similar results.

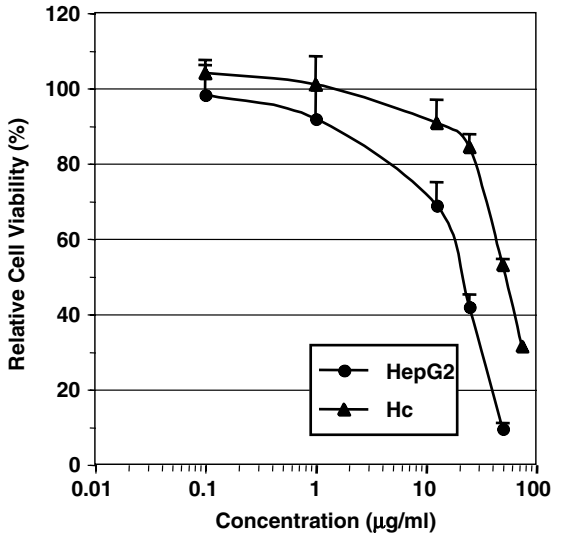

Fig. 2. Inhibition of cell growth by EGCG in HepG2 human HCC cells and in Hc normal hepatocytes. These cells were treated with the indicated concentrations of EGCG or DMSO for 48 h and the cell viability assays were examined using the MTT system. Results are expressed as percentage of growth with 100% representing control cells treated with DMSO alone. *Bars,* SD of triplicate assays.

inhibited growth of HepG2 cells with an IC_{50} value of about 20 μg/ml. However, the Hc cells were more resistant to EGCG because the IC_{50} value with this chemical was about 55 μg/ml (Fig. 2). Theses findings suggest that EGCG preferentially inhibits the growth of HepG2 HCC cells which express high levels of the p-IGF-1R protein, when compared with Hc normal human hepatocytes which do not express this protein.

3.3. Effects of EGCG on induction of apoptosis in HepG2 cells

To determine whether the growth inhibition we observed with HepG2 cells (Fig. 2) was associated with induction of apoptosis we next carried out TUNEL assays (Fig. 3). We found that the treatment of HepG2 cells with either 20 or 40 μg/ml of EGCG for 48 h significantly induced TUNEL-positive cells in approximately 10–15% of the total remaining cells, respectively (Fig. 3).

3.4. Effects of EGCG on production of IGF-1, IGF-2, and IGFBP-3 by HepG2 cells

Recent studies indicate that alterations in autocrine/paracrine loops involving IGFs and IGFBP-3 are associated with the proliferation of HCC cells [13,17,18,20]. Therefore, we next examined whether EGCG has effects on the production of IGF-1, IGF-2, and IGFBP-3 by HepG2 cells, using ELISA system (Fig. 4). We found that HepG2 cells secreted significant amounts of IGF-1 and IGF-2 into the growth medium when the cells were cultured in serum free medium for 72 h. We also found that treatment of these cells with 20 μg/ml of EGCG, approximately the IC_{50} concentration determined by MTT assays (Fig. 2), caused about a 35% decrease of IGF-1, and about a 70% decrease of IGF-2 in the growth medium (Fig. 4A and B). On the other hand, EGCG caused about a two-fold increase in the production of IGFBP-3 (Fig. 4C). Therefore, EGCG exerts two potent effects that cause inhibition of the activation of the IGF/IGF-1R axis; one is direct inhibition of secretion of IGFs (Fig. 4A and B) and the other is production of IGFBP-3 (Fig. 4C) since this protein binds to IGF-1 and IGF-2 and inhibits their bioavailability [10–12].

3.5. Effects of EGCG on activation of IGF-1R and its downstream signaling pathways stimulated by IGFs in HepG2 cells

We then examined whether EGCG inhibits activation of IGF-1R and its multiple downstream signaling pathways under the stimulation of IGFs in HepG2 cells (Fig. 5) because IGFs support the growth of HCC cells in an autocrine and paracrine manner [13,17,18,20] and, as shown in Fig. 4, EGCG can inhibit the bioavailability of IGFs. We found that exogenous IGF-1 and IGF-2 increased the levels of p-IGF-1R and its downstream signaling molecules p-GSK-3β, p-ERK, p-Akt, and p-Stat3 proteins in HepG2 cells. These increases were stronger with IGF-2 than with IGF-1. Moreover, both in the presence and absence of IGF-1 and IGF-2 stimulation, treatment of these cells with 20 μg/ml of EGCG for 24 h markedly decreased the levels of the p-IGF-1R protein. There was also a marked decrease in the levels of p-GSK-3β, p-ERK, p-Akt, and p-Stat3 proteins after the

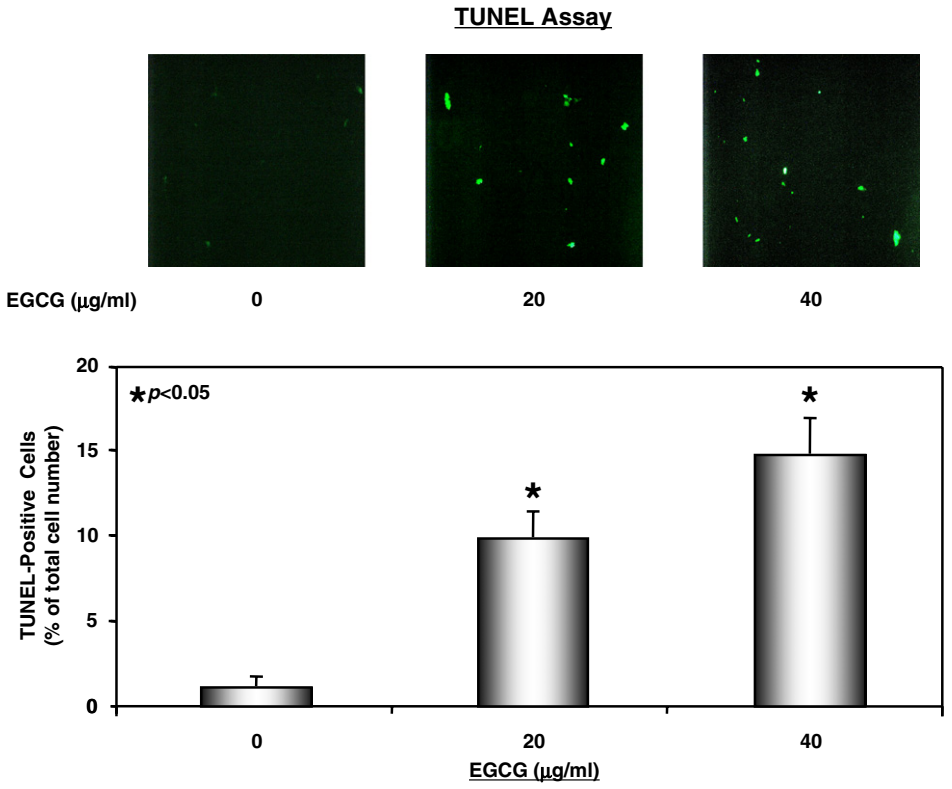

Fig. 3. Effects of EGCG on the induction of apoptosis in HepG2 cells. The cells were treated with DMSO, 20 μg/ml of EGCG, or 40 μg/ml of EGCG for 48 h. The cells were then stained using TUNEL method. TUNEL positive cells were counted and expressed as the percentage of total cell number (500 cells were counted in each flask). Values are the means ± SD. Asterisks indicate a significant difference ($p < 0.05$) between the control DMSO-treated cells and the EGCG-treated cells. *Bars,* SD of triplicate assays. Representative results from three independent experiments with similar results are shown.

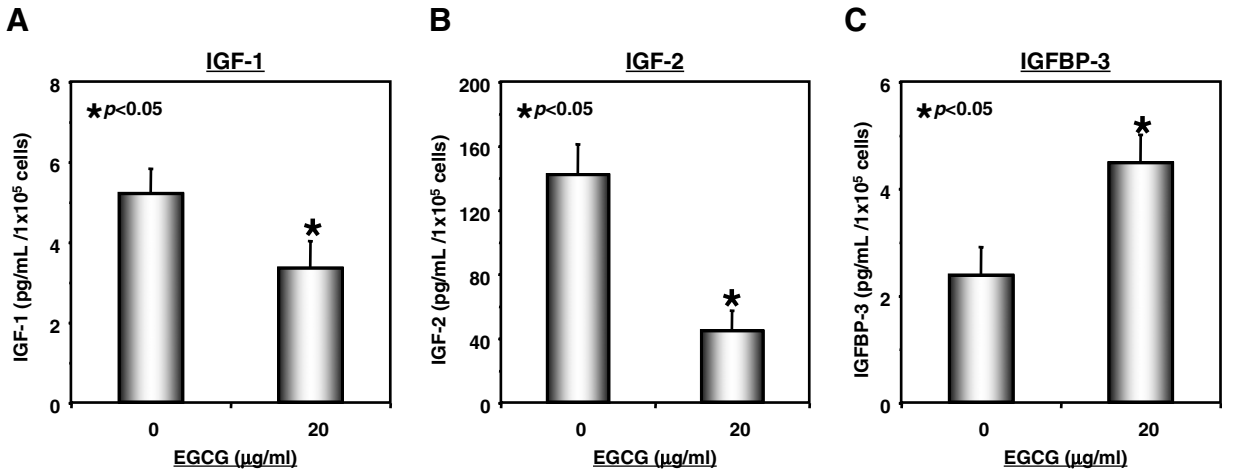

Fig. 4. Effects of EGCG on secretion of IGF-1, IGF-2, and IGFBP-3 by HepG2 cells. The cells were treated with 20 μg/ml of EGCG or DMSO (control) in serum minus medium for 72 h. The cell free medium was then collected and assayed for secreted IGF-1 (A), IGF-2 (B), and IGFBP-3 (C) using ELISA kits. Asterisks indicate a significant difference ($p < 0.05$) between the control DMSO-treated cells and the EGCG-treated cells. *Bars,* SD of triplicate assays.

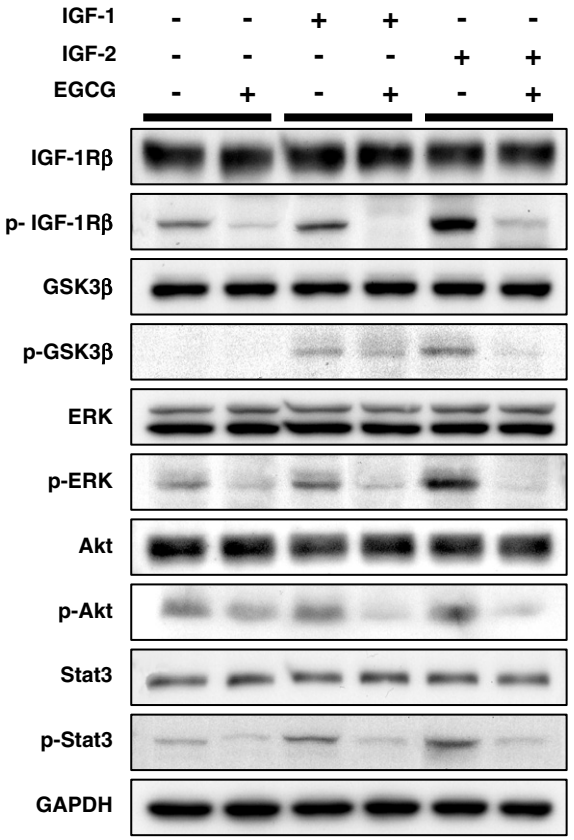

Fig. 5. Effects of EGCG on activation of the IGF-1R and its downstream signaling molecules under the stimulation of IGFs in HepG2 cells. The cells were treated with 20 μg/ml of EGCG or DMSO (control) in serum minus medium for 24 h. Before harvesting, the cells were stimulated with 15 μM of IGF-1 or IGF-2 for 3 h. The cells were then collected and protein extracts were examined by Western blot analyses, as described in Section 2. Repeat Western blots gave similar results.

treatment with EGCG, in the presence or absence of IGFs (Fig. 5). These findings indicate that EGCG can overcome the stimulatory effects of IGFs on activation of the IGF/IGF-1R axis and its downstream signaling pathway, thus inhibiting the growth of HCC cells (Fig. 2).

3.6. Effects of EGCG on expression of IGF-1 and IGF-2 mRNAs in HepG2 cells

We then examined whether EGCG can inhibit the production of IGF-1 and IGF-2 mRNAs by HepG2 cells because these cells secrete significant concentrations of IGFs into the medium (Fig. 4A and B), thus activating the IGF/IGF-1R axis and its downstream signaling molecules in these cells (Fig. 5). Semiquantitative RT-PCR analysis indicated that exogenous IGF-2 caused an increase in the levels of expression of both IGF-1 and IGF-2 mRNAs (Fig. 6). This finding suggests that IGF-2 plays a critical role in activation of the IGF/IGF-1R axis via stimulation of autocrine/paracrine loops. We also found that treatment with EGCG caused a decrease in the levels of IGF-1 and IGF-2 mRNAs in the presence or absence of exogenous IGFs, especially in the presence of exogenous IGF-2 (Fig. 6).

4. Discussion

As reviewed in Section 1, there is considerable evidence that the IGF/IGF-1R axis can play a critical role in the development of HCC [10–13]. In the present study we found that cellular levels of the IGF-1R and p-IGF-1R proteins are increased in a series of human HCC cell lines, especially in the HepG2, HuH7, and Hep3B cell lines, when compared with the Hc normal human hepatocyte cell line (Fig. 1). These differences might be associated with preferential growth inhibition by EGCG in HepG2 cells when compared with Hc cells (Fig. 2). Although both exogenous IGF-1 and IGF-2 can activate the IGF-1R, as well as the downstream effectors ERK, Akt, Stat-3, and GSK-3β proteins, these effects are more prominent when the HepG2 cells are stimulated by IGF-2 rather than IGF-1 (Fig. 5). The level of expression of the *IGF-2* gene, which is up-regulated by the MAPK/ERK pathway [23], is also apparently increased by stimulation of the cells with IGF-2 itself (Fig. 6). We also found that both IGF-1 and IGF-2 are secreted by HepG2 cells into the growth medium (Fig. 4A and B). Our findings and previous reports that IGFs, especially IGF-2 produced by HCC cells, induce cell proliferation and DNA synthesis in these cells [17,18,20] suggest that there are autocrine and/or paracrine loops which may contribute to the abnormal proliferation of HCC cells.

In the present study we also provide the first evidence that in HCC cells the green tea catechin EGCG inhibits activation of IGF-1R as well as its downstream effectors, especially when the cells are stimulated by IGF-2 (Fig. 5). EGCG also inhibits the expression of the IGF-1 and IGF-2 mRNAs (Fig. 6) and the production of these two proteins (Fig. 4A and B). These findings suggest that EGCG can overcome the stimulatory effects of IGFs, especially IGF-2, and thereby disrupt the IGF/IGF-1R related autocrine and/or paracrine loops. Furthermore, EGCG preferentially inhibits the growth of HepG2 cells when compared with the Hc normal human hepatocyte cell line (Fig. 2). EGCG also

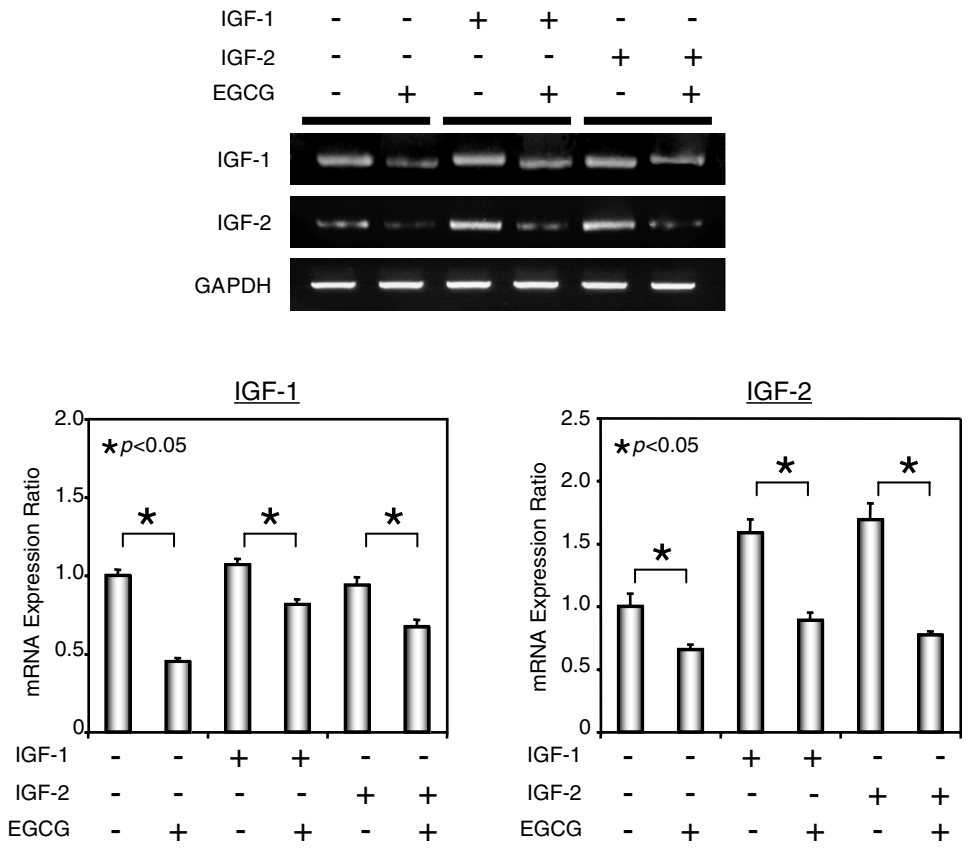

Fig. 6. Effects of EGCG on cellular levels of IGF-1 and IGF-2 mRNAs in HepG2 cells. The cells were treated with 20 μg/ml of EGCG or DMSO (control) in serum minus medium for 24 h. Before the harvesting, the cells were stimulated with 15 μM of IGF-1 or IGF-2 for 3 h. The cells were then collected and mRNA extraction and semiquantitative RT-PCR analyses were performed as described in Section 2. The results obtained from RT-PCR analysis were quantitated by densitometry and are displayed in the lower panels. Asterisks indicate a significant difference ($p < 0.05$) between the control cells and the EGCG-treated cells. *Bars,* SD of triplicate assays. Similar results were obtained in a repeat experiment.

induces apoptosis in these HCC cells (Fig. 3). Therefore, EGCG might be a promising agent for the prevention and/or treatment of HCC.

The growth inhibitory effects of EGCG in various human cancer cell lines are mediated, at least in part, through inhibition of the activity of specific RTKs and related downstream pathways of signal transduction [3,4]. Thus, we previously reported that EGCG inhibits activation of several RTKs including EGFR, HER2, and HER3 [5,6]. There is evidence that EGCG inhibits activation of EGFR by inhibiting the binding of EGF to the EGFR and/or direct inhibition of the tyrosine kinase activity of this receptor [24]. In addition to EGFR, a recent report provides evidence that EGCG directly inhibits IGF-1R kinase activity by competing with ATP for binding to the kinase domain of this receptor [25]. However, the precise mechanism(s) by which EGCG inhibits activation of RTKs remains to be determined. Nevertheless, we found that EGCG inhibits activation of the IGF-1R and related downstream signaling pathways (Fig. 5). Amongst the downstream signaling molecules, recent studies indicates that phosphorylation (*i.e.* inactivation) of GSK-3β, which is mediated by PI3K/Akt, is one of the critical targets in the constitutive activation of the IGF/IGF-1R axis in hepatocarcinogenesis [26,27]. Therefore, our discovery that EGCG can inhibit the phosphorylation of GSK-3β induced by IGFs, thus reactivating this protein (Fig. 5), is also of interest with respect to the preventive effects of EGCG on liver carcinogenesis.

In this study we found that EGCG induces the secretion of IGFBP-3 by HepG2 cells (Fig. 4C). This finding is consistent with studies in a transgenic mouse model of prostate adenocarcinoma in which

oral consumption of green tea polyphenols inhibited the development and progression of prostate cancer and this was associated with induction of increased levels of IGFBP-3 and reduction of biological activity of IGF-1 [28,29]. Treatment of HCC cells with recombinant IGFBP-3 leads to a significant reduction in cell proliferation by inhibiting IGF-1-induced activation of IGF-1R, ERK, and Akt proteins [17]. Transcriptional activation of IGFBP-3 and the resulting neutralization of IGF-2 may represent one of the tumor-suppressive mechanisms of p53 [30]. Therefore, the induction of IGFBP-3 by EGCG in HCC cells may also contribute to the growth inhibitory effects of EGCG in these cells.

HCC is one of the most common malignancies worldwide and thus there is a critical need to develop effective strategies for the prevention and therapy of this disease. Agents which target RTKs are strong candidates for the treatment of HCC. Thus, the selective EGFR tyrosine kinase inhibitor gefitinib (ZD1839) inhibited the development of HCC in diethylnitrosamine-exposed cirrhotic rats [31]. In addition, there is evidence that overexpression of IGF-2 contributes to the development of HCC in this model [31]. There is also an interesting report that IGF-2 stimulates proliferation in HCC cells, at least in part, *via* the autocrine/paracrine release of EGFR ligands, and that gefitinib inhibits ERK phosphorylation and DNA synthesis induced by IGF-2 in these cells [32]. These findings suggest that there is cross-talk between the IGF-1R and EGFR receptors, and that the IGF/IGF-1R survival pathway may contribute to gefitinib resistance in HCC cells [32]. Moreover, co-inhibition of EGFR and IGF-1R exerts synergistic growth-inhibitory and proapoptotic effects in some types of cancer cells, including HCC cells [19,32–34]. Therefore, the ability of EGCG to target both IGF-1R, demonstrated in the present study, and the EGFR family of RTKs [3–7] provides a further rationale for the use of EGCG alone, or in combination with other agent, in the chemoprevention and/or treatment of liver cancer.

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