Effect of curcumin on vascular endothelial growth factor in hypoxic HepG2 cells via the insulin-like growth factor 1 receptor signaling pathway

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Abstract. To investigate the anti-angiogenic effect and underlying molecular mechanisms of curcumin on HepG2 cells under hypoxic conditions, insulin-like growth factor 1 receptor (IGF-1R) knockout HepG2 cells were constructed using a clustered regularly interspaced short palindromic repeats/Cas9 genome-editing system. Hypoxic conditions were generated using cobalt chloride (CoCl2). An MTT assay was performed to measure the effects of curcumin on cell viability in hypoxia-induced IGF-1R knockout HepG2 cells, while western blot analysis was used to detect the expression of IGF-1R, phosphorylated (p)-protein kinase B (Akt), p-extracellular signal-regulated kinases (Erk)1/2, hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF). The results revealed that CoCl2 at low concentrations (50 and 100 µM) had no significant inhibitory effects on IGF-1R knockout HepG2 cells. However, with increasing concentrations of CoCl2 and treatment time, cell viability decreased and was significantly reduced at 150, 200 and 400 µM compared with the control group (P<0.05). The expression of HIF-1α and VEGF were significantly increased when the cells were treated with 150 or 200 µM CoCl2, compared with the control (P<0.05). With the increase of CoCl2 concentration or the treatment time, the expression of HIF-1α and VEGF were upregulated gradually. Additionally, curcumin significantly inhibited the expression of p-Akt, p-Erk1/2, HIF-1α and VEGF in hypoxia-induced IGF-1R knockout HepG2 cells. In conclusion, the findings of the present study suggest that curcumin may serve a pivotal role in tumor suppression via the inhibition of IGF-1R-mediated angiogenesis under hypoxic conditions.

Introduction

Primary liver cancer (PLC) comprises hepatocellular carcinoma (HCC), hepatoblastoma, intrahepatic cholangiocarcinoma and other rarer tumors (1). Hepatoblastoma is the most common type of primary liver tumor in children <5 years old (2). The incidence rate of PLC is increasing in Australia, Central Europe, the United Kingdom, Japan and North America, and this increase will likely continue due to infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) (3). For patients with localized HCC, liver transplantation and resection are viable, useful treatments option (4). However, curative resection or transplantation is only possible in 30% of patients, which means that the overall survival rate of HCC remains poor (5). Transcatheter arterial chemoembolization (TACE) has been demonstrated to have survival benefits in patients with intermediate and advanced HCC and hepatoblastoma (6-8). TACE may reduce tumor burden and significantly improve the prognosis and survival of patients (6). However, TACE may also cause local hypoxia in the tumor environment, resulting in the expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF), which promote neovascularization (9). It is therefore necessary to study the mechanism of hypoxia-induced angiogenesis to identify novel ways of inhibiting the growth of PLC.

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-di-one] is a polyphenolic compound isolated from the rhizomes of Curcuma longa, which has been revealed to have anti-inflammatory, antioxidant, anti-angiogenesis and antitumor activities (10,11). Curcumin has also been reported to protect the liver against the toxic effects of agents, including galactosamine (12), C-C motif chemokine 4 (13) and paracetamol (14). It has previously been reported that curcumin inhibits human breast carcinoma MCF-7 cell proliferation by inhibiting the insulin-like growth factor (IGF)-1 axis (15). Patel et al (16) demonstrated that the inclusion of curcumin to continued folinic acid, fluorouracil and oxaliplatin treatment...
reduced the survival of colon cancer cells and concurrently reduced the activation of endothelial growth factor receptor, v-erb-b2 erythroblast leukemia viral oncogene homolog 2 (HER-2), IGF-1 receptor (IGF-1R) and protein kinase B (Akt). However, to the best of our knowledge, there has been little investigation into whether curcumin affects the angiogenesis of HCC via the IGF-1R signaling pathway.

Previous studies have revealed that IGF-1R is overexpressed in the majority of PLC tissues (17,18). The interaction between IGF-1R and its ligands serves a key role in regulating the expression of VEGF (17,19). Therefore, IGF-1R knockout HepG2 cells were constructed to investigate whether curcumin regulates the expression of HIF-1α and VEGF via the IGF-1R signaling pathway.

Materials and methods

Cell culture and chemicals. The human hepatoblastoma cell line HepG2, was obtained from the American Type Culture Collection (Manassas, VA, USA). IGF-1R knockout HepG2 cells were constructed and conserved within the laboratory. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere containing 5% CO2. Curcumin and CoCl2 were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Curcumin was dissolved in dimethylsulfoxide and CoCl2 was dissolved in DMEM.

Preparation of IGF-1R knockout cells. The candidate 20-base guide (g)RNA sequences were derived from the IGF-1R genome sequence (gene ID: 3480). The cDNA sequence was obtained from Han's lab (http://hanlab.xmu.edu.cn/cdna/Query.aspx, No. 25207). The clustered regularly interspersed short palindromic repeats (CRISPR)/Cas9 design tool from Zhang's lab (crispr.mit.edu) was used to search the functional gRNA sequence (5'-GAG AAC TGC ACG GTG ATC GAG GG-3'), which contain the downstream 3'protospacer-adjacent motif with GG dinucleotide (N20-NGG). According to the functional gRNA sequence, a pair of oligo DNA were designed (hIGF1R- seq-F: 5'-GGT TAC CCT CTT GTC TCC CT-3' and hIGF1R- seq-R: 5'-CGTAATGACCCTGTAAGTG-3'). PCR was performed using the following thermocycling conditions: 95°C for 10 sec, 60°C for 10 sec, 72°C for 20 sec for 30 cycles. Amplicons were sent to the Beijing Genomics Institute (Beijing, China) for deep sequencing.

Hypoxia and CoCl2 incubation. HepG2 cells were plated in 96-well plates (5,000 cells/well) or 6-well plates (3x10^5 cells/well) in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere containing 5% CO2. Following 24 h, the medium was changed to fresh serum-free DMEM with different concentrations of CoCl2 (0, 50, 100, 150, 200 and 400 µM) for different time periods (1, 2, 4, 6 and 8 h).

Cell survival assay. HepG2 cells were plated in a 96-well plate in 100 µl medium at a density of 5,000 cells/well and incubated using standard culture conditions overnight. The medium was removed carefully and the purple formazan was dissolved with 150 µl dimethyl sulfoxide. Cell viability was determined by an MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. The optical density was determined at 570 nm by SpectraMax M2 (Molecular Devices, LLC, Sunnyvale, CA, USA).

Western blot analysis. Total protein was isolated from cells using a radioimmunoprecipitation buffer reagent (cat. no. P0013B, Beyotime Institute of Biotechnology, Shanghai, China). The lysates were ultrasonicated and centrifuged at 12,000 x g and at 4°C for 10 min. Supernatants were collected and stored at -70°C. Protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Protein (50 µg per lane) was separated by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (TBS)/5% nonfat dry milk at room temperature for 2 h and the membranes were subsequently incubated with primary antibodies overnight at 4°C. Following three washes with TBS+Tween-20 (TBST), the membranes were incubated with goat polyclonal immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-2004; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 45 min at room temperature. The membranes were washed with TBST three times prior to visualization using a Western Blotting Luminol Reagent (cat. no. sc-2048, Santa Cruz Biotechnology, Inc.). Image J 1.6 software (National Institutes of Health, Bethesda, MD, USA) was used to detect and analyze the relative densitometry. The primary antibodies used in the experiment were as follows: Rabbit anti-IGF-1R (cat. no. 9750), rabbit anti-HIF-1α (cat. no. 3716), rabbit anti-VEGF (cat. no. 2463), rabbit anti-Akt (cat. no. 9272), rabbit anti-phosphorylated (p)-Akt (cat. no. 5012), rabbit anti-extracellular signal-regulated kinases (Erk1/2; cat. no. 4695), rabbit anti-p-Erk1/2 (cat. no. 4377) (all 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA).
and rabbit anti-β-actin (cat. no. sc-7210; 1:4,000; Santa Cruz Biotechnology, Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation and were analyzed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Differences between the groups at each time point were examined for statistical significance using one-way analysis of variance and a least significant difference post-hoc comparison. The correlation between HIF-1α and VEGF protein levels was evaluated statistically using Pearson's correlation coefficient analysis if the quantitative data had a normal distribution; if the data was not normally distributed, the Spearman rank correlation coefficient analysis was used. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of hypoxia on the viability of IGF-1R knockout HepG2 cells. An IGF-1R knockout HepG2 cell line was successfully constructed using a CRISPR/Cas9 genome-editing system. To investigate the effect of hypoxia on the IGF-1R knockout HepG2 cell line, cells were treated with different concentrations of CoCl₂ for 1-8 h and the viability was subsequently analyzed using an MTT assay. No significant differences were observed between the control cells and those treated with low CoCl₂ doses (50-100 µM; Fig. 1). However, the viability of IGF-1R knockout HepG2 cells was significantly reduced in the 150, 200 and 400 µM CoCl₂ treated groups compared with the control (no CoCl₂ treatment) at the same time points (P<0.05; Fig. 1).

Effect of CoCl₂-induced hypoxia on HIF-1α, IGF-1R and VEGF expression in IGF-1R knockout HepG2 cells. Western blot analysis was performed to examine the effects of CoCl₂-induced hypoxia on HIF-1α, IGF-1R and VEGF protein expression in IGF-1R knockout HepG2 cells. The cells were treated with 0, 50, 100, 150, 200 or 400 µM CoCl₂ for 6 h and the expression of HIF-1α, IGF-1R and VEGF was determined by western blotting. The protein expression of HIF-1α and VEGF was significantly higher in cells treated with 150 or 200 µM CoCl₂ compared with the control group (P<0.05; Fig. 2A and B). Correlation analysis revealed that the expression of the HIF-1α was positively correlated with the expression of VEGF (r=0.85, P<0.05) in a dose-dependent manner (data not shown).

The IGF-1R knockout HepG2 cells were treated with 150 µM CoCl₂ for the indicated time periods and the protein expression of HIF-1α, IGF-1R and VEGF was determined by western blotting (Fig. 2C). The relative expression of each protein was calculated and it was observed that the protein expression of HIF-1α and VEGF was significantly increased at 2-8 h compared with the control group (P<0.05; Fig. 2D). In addition, HIF-1α expression was positively correlated with the expression of VEGF (r=0.71, P<0.05) in a time-dependent manner (data not shown).

Effect of curcumin on IGF-1R knockout HepG2 cell viability under CoCl₂-induced hypoxia. IGF-1R knockout HepG2 cells were incubated with increasing concentrations of curcumin (5, 10, 20, 30 and 40 µM) in the presence of 150 µM CoCl₂. It was observed that the cell survival rate decreased as the incubation time or dose increased, and all doses of curcumin caused a reduction in cell survival (Fig. 3). The incubation of cell cultures with different concentrations of curcumin at each time point (8, 12, 24 and 48 h) had significant inhibitory effects on the survival of IGF-1R knockout HepG2 cells compared with the control group (P<0.05; Fig. 3).

Effect of curcumin on IGF-1R, p-Akt, p-Erk1/2, HIF-1α and VEGF protein expression in IGF-1R knockout HepG2 cells in the presence of CoCl₂. HepG2 cells and IGF-1R knockout HepG2 cells were treated with or without curcumin (20 µM) under CoCl₂-induced hypoxic conditions. Following 24 h of treatment, total cell protein was extracted and analyzed using western blotting (Fig. 4A). The expression of IGF-1R, p-Akt, p-Erk1/2, HIF-1α and VEGF proteins was significantly reduced in HepG2 cells treated with curcumin compared with untreated cells (P<0.05; Fig. 4B-F). IGF-1R knockout cells treated with curcumin also exhibited markedly reduced p-Akt, p-Erk1/2, HIF-1α and VEGF protein expression compared with the IGF-1R knock out cells not treated with curcumin (P<0.05). Knockdown IGF-1R also inhibited the expression of HIF-1α, VEGF, p-Akt and p-Erk proteins in CoCl₂-induced HepG2 cells (P<0.05, Fig. 4C-F). Additionally, the difference between control group (lane A) and HepG2 cells with CoCl₂ plus curcumin (lane B) seems greater than the difference between IGF-1R knockout HepG2 cells with CoCl₂ (lane C) and IGF-1R knockout HepG2 cells with CoCl₂ plus curcumin (lane D).

Discussion

Angiogenesis serves a crucial role in tumor growth, invasion and metastasis (20). Tumor neovascularization is a complex process that is regulated by angiogenic activators and inhibitors (20). Hypoxia is an important stimulus and key regulator of angiogenesis in cancer, and tumor hypoxia may occur due to increased metabolic activity and oxygen consumption by rapidly proliferating tumor cells (19). Hypoxia may be
induced by TACE, transarterial embolization, portal venous embolizations or other treatments for PLC (21). TACE may reduce the tumor burden and improve the prognosis and survival rate of patients with PLC; however, TACE also induces hypoxic conditions, which promotes the secretion of HIF-1α and VEGF and leads to tumor angiogenesis, recurrence and metastasis (8). Several previous studies have confirmed that VEGF is one of the most reliable prognostic markers of HCC (22-24). VEGF may be useful in monitoring the response to treatment in patients with HCC undergoing TACE, and angiogenesis in these patients may be a target for adjuvant treatments with drugs interfering with the process (25). Liapi and Geschwind (26) reviewed several anti-angiogenic agents combined with TACE for the treatment of HCC, including sorafenib, bevacizumab. Sorafenib as a complementary treatment acting on VEGF improved overall survival, time to progression and objective response rate when administered prior to or in conjunction with TACE (27). Sorafenib was also reported to inhibit CoCl2-induced HIF-1α and VEGFA expression in hepatoma cells (28). However, the incidence of adverse reactions was higher in the TACE + sorafenib group compared with the TACE only group; these adverse reactions included hepatotoxicity, hypertension and abdominal pain (27,29). These results suggest that sorafenib may reduce the overexpression of VEGF when combined with TACE, although it cause liver injury. Curcumin was reported to have protective effects on the liver and anticancer effects (30), whereas its effects on angiogenesis under hypoxic conditions are poorly understood.

HIF-1α is the major transcription factor specifically activated during hypoxia (19). It regulates the expression of molecules associated with glucose transport, glycolysis, erythropoiesis, iron transport and angiogenesis (31). In the present study, CoCl2 was utilized to mimic hypoxia as it enhances the stability of HIF-1α. It was observed that CoCl2 at a concentration of 150, 200 and 400 µM suppressed the survival ability of IGF-1R knockout HepG2 cells; this effect may be due to its cytotoxicity at high concentrations. Additionally, the expression of HIF-1α and VEGF was increased in a time- and dose-dependent manner and correlation analysis revealed that the expression of HIF-1α was positively correlated with the expression of VEGF. These results are consistent with a previous study by the authors (32), which concluded that hypoxia induced the accumulation of IGF-1R and HIF-1α and regulated the expression of VEGF in HepG2 cells. In the present study, IGF-1R knockout HepG2 cells were constructed...
and it was revealed that curcumin inhibits the IGF-1R signaling pathway under hypoxic conditions.

The IGF system has emerged as an important pathway in the development and progression of hepatoblastoma and represents a potential therapeutic target (33,34). The anti-IGF-1R antibody has been reported to be effective in reducing cell proliferation and promoting apoptosis, as well as having a synergistic effect with doxorubicin (35). CoCl$_2$ is a chemical inducer of HIF-1 and active HIF-1 induces the expression of VEGF (36). In the present study, IGF-1R knockdown inhibited the expression of HIF-1$\alpha$, VEGF p-Akt and p-Erk proteins. Curcumin also significantly suppressed the expression of IGF-1R, HIF-1$\alpha$, VEGF p-Akt and p-Erk proteins. The IGF-1R signaling pathway consists of IGF-1, IGF-1R and its downstream signaling targets (37). Akt and mitogen-activated protein kinase are two main downstream signaling targets of the IGF-1R signaling pathway (37). In the present study, the addition of curcumin reduced the expression of HIF-1$\alpha$, VEGF, p-Akt and p-Erk, which indicates that curcumin may inhibit HIF-1$\alpha$ and VEGF in an IGF-1R-independent manner. These results suggest that curcumin may suppress the expression of HIF-1$\alpha$ and VEGF by inhibiting IGF-1R or its downstream signaling pathways (Fig. 5).

The present study was not without limitations. Firstly, CoCl$_2$-induced hypoxia primarily depends on the regulation of HIF-1$\alpha$ and there are HIF-independent pathways that are capable of inducing angiogenesis in response to hypoxia (38). In future studies it would be preferable to study cells under natural hypoxic conditions. Secondly, the HepG2 cell line has been reported to be misidentified; it was originally thought to be an HCC cell line but was later revealed to be derived from a hepatoblastoma cell line (39). As ~90% of PLCs are HCC, experiments on HCC cell lines are required to validate the results of the present study. Thirdly, intact curcumin was used in the present cell culture study and it has been reported that, within an in vivo system, the majority of curcumin is converted to curcumin.
glucuronide, the effects of which are weaker than those of curcumin (40). Whether curcumin would have the same effects in vivo as in the cultured cells is not known. Further studies are required to investigate the effects of curcumin on the IGF-1R signaling pathway in vivo.

In conclusion, the present study suggests that IGF-1R and its downstream signals may be targets for anti-angiogenesis treatment of hepatoblastomas. Curcumin in combination with TACE may be an effective treatment for liver cancer, considering its liver protective, pro-apoptosis and anti-angiogenesis effects.

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