DEC1 promotes hypoxia-induced epithelial-mesenchymal transition (EMT) in human hepatocellular carcinoma cells

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(Received 13 March 2017; and accepted 29 April 2017)

ABSTRACT

Differentiated embryonic chondrocyte (DEC) 1 has been reported to be involved in cell differentiation, hypoxia response, and cancer progression. Recent studies have demonstrated that hypoxia-inducible factor (HIF)-1α induces epithelial-mesenchymal transition (EMT) in carcinoma cells to facilitate cell invasiveness and metastasis. However, it remains unclear whether DEC1 participates in hypoxia-mediated EMT processes. In the present study, we reported that hypoxia induced DEC1 expression in hepatocellular carcinoma (HCC) HepG2 cells, and DEC1 negatively regulated expression of HIF-1α and E-cadherin in transcriptional/translational levels. Cell morphological changes were evaluated with hematoxylin and eosin (H-E) staining. Exposure to hypoxia caused spindle-like shape in some of the HepG2 cells, and DEC1 overexpression furthered these changes. In conclusion, DEC1 is involved in hypoxia-induced EMT processes via negatively regulating E-cadherin expression in HepG2 cells.

There has been a great discussion about the relation between hypoxia and epithelial-mesenchymal transition (EMT). Intratumoral hypoxia followed by stabilization of hypoxia-inducible factor (HIF)-1α promotes acquisition of EMT-like features in various kinds of carcinomas (2, 9, 11, 18) as well as hematopoietic tumors (1). Several studies have reported that HIF-1α overexpression is positively correlated with EMT induction in hepatocellular carcinoma (HCC) cell lines and surgical resection specimens (7, 8, 20). In addition, hypoxia-induced EMT resulted in multidrug resistance in HCC cells (16), tumor metastasis after transcatheter arterial embolization (TAE) (4), and poor prognosis in HCC patients (6). It has been shown that curcumin, a botanical agent derived from the dried rhizome of Curcuma longa, eliminates the accumulation of HIF-1α to reduce the invasive potential of HepG2 cells (3). These findings indicated that hypoxia-induced EMT may be a significant prognosis marker and a crucial therapy target of HCC patients.

Differentiated embryonic chondrocyte expressed gene (DEC) 1 (BHLHE40/Stra13/Sharp2) has been found as a transcriptional factor promoting the differentiation of chondrocytes (15) and regulating the circadian rhythm via suppressing CLOCK/BMAL1-enhanced promoter activity (5). Recently, DEC1 has been reported to be located on the downstream of HIF-1α pathway and the activation of HIF-1α and DEC1 causes tumor progression, invasion, and metastasis (13). Our previous studies have shown that DEC1 promotes TGF-β-induced EMT of pancreatic adenocarcinoma PANC-1 cells through Smad3 phosphorylation (17). However, the relation between DEC1 and hypoxia-induced EMT has not been reported in any human cancer cell lines. This study was undertaken to examine the relation between DEC1 and hypoxia-induced EMT in human HCC HepG2 cells.
MATERIALS AND METHODS

Cell culture and treatment. Human HCC HepG2 cells were cultured in Dulbecco’s Modified Eagle’s Medium-high glucose (Sigma Chemical, St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO2. Hypoxic exposure (2% O2) was conducted by culturing cells in a multi-gas incubator (MCO-SM; Panasonic Healthcare, Tokyo, Japan) at various periods.

Cell viability assay. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. HepG2 cells cultured in 96-well plates were exposed to the normoxic (20% O2) or hypoxic (2% O2) condition for various periods of time (24, 48, and 72 h). Subsequently, the cells were added along with the CellTitre 96® AQueous One Solution Reagent (Promega, Madison, WI, USA) to each well and were incubated at 37°C for an additional 1 h. Absorbance (OD 490 nm) was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

DEC1 overexpression. Human DEC1 expression plasmid (DEC1 pcDNA) was a kind gift from Dr. Katsumi Fujimoto (Hiroshima University) (5). HepG2 cells were seeded at 5 × 10^4 cells per 35-mm well. DEC1 pcDNA was transiently transfected into HepG2 cells using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). Following transfection, the cells were cultured under normoxic (20% O2) or hypoxic (2% O2) condition for 48 h, and subjected to quantitative real-time PCR or western blotting analyses.

Short interference RNA (siRNA). The sequences for the sense and antisense DEC1 siRNA were 5’-r(CCAAAGUGAUGGACUCUACUA) d (TT)-3’ and 5’-r(UUGAAGCUCAUCACUUUGG) d (GA)-3’. The negative control (scrambled) siRNA sequences were 5’-r(UUUCGGACGUGUCAGCG) d (TT)-3’ and 5’-r(ACGUGACACGUUCCGAGAA) d (TT)-3’. The siRNA against HIF-1α was obtained from Qiagen (SI02664053; Qiagen, Hilden, Germany). For the siRNA transfection experiments, cells were seeded at 5 × 10^4 cells per 35-mm well. The siRNA was transfected into the cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. After transfection, the cells were incubated under normoxic (20% O2) or hypoxic (2% O2) condition for another 2 or 48 h (DEC1 knockdown cells) and 6 h (HIF-1α knockdown cells), and subjected to western blotting analyses.

Western blotting. The cells were lysed using M-PER lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) and the protein concentration was determined using the bicinchoninic acid (BCA) assay. Cell lysates (10 μg) were subjected to SDS-PAGE and the proteins were transferred to PVDF membrane (GE Healthcare, Wauwatosa, WI, USA), followed by immunoblotting utilizing the antibodies specific to HIF-1α (1 : 1,000; Cell Signaling Technology, Danvers, MA, USA), DEC1 (1 : 1,000; Novus Biologicals, Littleton, CO, USA), E-cadherin (1 : 1000; Takara Bio, Shiga, Japan), and actin (1 : 5,000; Sigma-Aldrich; Merck Millipore, St Louis, MO, USA). Can Get Signal immunoreaction enhancer solution (Toyobo, Osaka, Japan) was used to dilute the primary antibodies. After immunoblotting with horseradish peroxidase-conjugated secondary antibody (Immuno-Biological Laboratories, Fujioka, Japan), signals were detected using the ECL-prime or ECL-select Western blotting Detection System (GE Healthcare).

Quantitative real-time PCR. Total RNA was extracted from HepG2 cells using RNeasy Mini kit (Qiagen), and cDNA was synthesized from 1 μg of total RNA using ReverTra Ace (Toyobo). The quantitative real-time PCR was performed using SYBR-Green Master mix (Bio-Rad). The sequences of the primers and products sizes are shown in Table 1.

Hematoxylin and eosin (H-E) staining. The morphological changes of HepG2 cells were evaluated with H-E staining. The cells were transfected with pcDNA (the empty vector) or DEC1 pcDNA under the normoxic (20% O2) or hypoxic (2% O2) condition as mentioned above. The cells were then fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) in phosphate buffered saline (PBS) for 15 min, permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 20 min and finally stained in hematoxylin and eosin.

Statistical analysis. Each experiment was repeated at least three times and data are presented as means ± standard deviation. The ordinary one way ANOVA analysis was performed using GraphPad Prism7 (GraphPad Software, Inc., La Jolla, CA, USA) for comparisons more than two groups. Student’s t test was used to assess the significance between two groups. *P < 0.05 or **P < 0.01 were considered statistical significance.
Results

Exposure to hypoxia enhanced the cell viability of HepG2 cells

We confirmed the effect of hypoxia on cell viability of HepG2 cells by MTS assay. Cell viability of HepG2 cells cultured under hypoxia was increased when compared with those cultured under normoxic condition. Moreover, the increase rate gradually slowed down with the passage of time (Fig. 1).

Exposure to hypoxia affected DEC1 and E-cadherin expression

To examine the time dependent changes of DEC1 and E-cadherin expression, RNAs or proteins of HepG2 cells incubated under hypoxia of various periods were lysed for analyses. As shown in Fig. 2A, DEC1 mRNA exhibited an expression pattern with two peaks at 2 h and 24 h separately. Whereas the mRNA level of E-cadherin decreased to the lowest level after exposing to hypoxia for 12 h. In addition, by western blotting analysis, we found the expression of HIF-1α was induced with one peak at 6 h under hypoxic condition. Different from the expression of mRNA, protein level of DEC1 increased after exposed to hypoxia for 12 h, and reached its peak at 18 h. While the E-cadherin protein level decreased to the lowest at 6 h, after that, its expression was gradually rebounded. Followed by a relative higher peak at 24 h, continuous hypoxic condition culture significantly decreased E-cadherin protein expression (Fig. 2B).

DEC1 overexpression under hypoxia altered the cell morphology of HepG2 cells

Morphological changes of the cells transfected with pcDNA (the empty vector used for control) or DEC1 pcDNA under the normoxic or hypoxic condition were observed using H-E staining. As shown in Fig. 3, pcDNA-transfected cells under normoxia tightly connected with each other and formed cell clusters (Fig. 3A), while those under hypoxia exhibited loose cell-cell connection and showed a single cell pattern (Fig. 3B). In addition, DEC1 overexpression under hypoxia not only decreased the cell adhesion, but also altered the cell morphology to fibroblast-like shape (Fig. 3C).

To find the reason of this phenomenon, we examined effects of DEC1 on E-cadherin, one of the most important epithelial markers. DEC1 pcDNA was transiently transfected into HepG2 cells. DEC1 overexpression inhibited expression of HIF-1α and E-cadherin in mRNA/protein levels (Fig. 4, A and B) under hypoxia. To analyze the role of endogenous DEC1, we introduced DEC1 siRNA into HepG2

Table 1 Sequences of the primer sets and the products sizes of real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product size (bp)</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>DEC1</td>
<td>76</td>
<td>F: 5'-GAAAGGATCGCGCGCAATTA-3'</td>
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<tr>
<td></td>
<td></td>
<td>R: 5'-CATCCTCGAAGACTGATAT-3'</td>
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<td>E-cadherin</td>
<td>75</td>
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<td>HIF-1α</td>
<td>81</td>
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<tr>
<td></td>
<td></td>
<td>R: 5'-AGTGTCACGCGGAGGACATA-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>150</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CATCCTCGAAGACTGATAT-3'</td>
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F: forward primer; R: reverse primer

Fig. 1 Hypoxia increased the cell viability of HepG2 cells. HepG2 cells cultured in 96-well plates were exposed to normoxia (20% O₂) or hypoxia (2% O₂) for 24, 48 and 72 h, and cell viability was analyzed by MTS assay. The values are shown as a percentage of the normoxia. Each value represents the mean ± standard deviation of 3 independent experiences (*P < 0.05 or **P < 0.01, compared with normoxia).
cells. Since endogenous DEC1 was sharply depressed under hypoxia at 48 h (Fig. 2B), we examined the transfection effect of DEC1 siRNA at 2 h (Fig. 5A). DEC1 knockdown increased HIF-1α under hypoxic condition, while it increased E-cadherin under both the normoxic and hypoxic condition (Fig. 5B). These data indicate the possibility that DEC1 is a transcriptional suppressor of HIF-1α and E-cadherin.

**HIF-1α knockdown failed to alter the expression of DEC1**

Previous study has reported that HIF-1α binds to hypoxia responsive element (HRE) in the promoter region of DEC1 to activate the transcription of DEC1 mRNA (13). To investigate whether HIF-1α upregulates DEC1 in HepG2 cells, we introduced HIF-1α siRNA into HepG2 cells. Our result showed that HIF-1α knockdown had no effect on the DEC1 protein level (Fig. 6). Concluding the present results with those of other group (13), we thought DEC1 and HIF-1α might regulate each other mutually.

**DISCUSSION**

Hypoxic microenvironment existed in various kinds of solid tumors including hepatocellular carcinoma and contributed to cancer development and metastasis (21). As a hypoxia-inducible gene, the role of DEC1 under hypoxia was still unclarified. In the present study, we focused on the functional analysis of DEC1 in hepatocellular carcinoma HepG2 cells under hypoxic condition. DEC1 overexpression facilitated the morphological changes caused by hypoxia. Loss of E-cadherin may contribute to these changes since expression of HIF-1α and E-cadherin was markedly suppressed in mRNA/protein levels...
DEC1 promotes hypoxia-induced EMT via HIF-1α and E-cadherin

Fig. 3 DEC1 overexpression attributed to the cell morphological changes of HepG2 cells. Morphological changes were evaluated with hematoxylin and eosin (H-E) staining in HepG2 cells transfected with either pcDNA or DEC1 pcDNA, following exposure to hypoxia (2% O₂) for 48 h. The pcDNA-transfected HepG2 cells cultured under normoxia were used as control. A representative image of at least two independent experiments with similar results is shown. DEC, differentiated embryonic chondrocyte expressed gene; pcDNA, empty vector control; DEC1 pcDNA, DEC1 overexpression vector.

Fig. 4 Differentiated embryonic chondrocyte expressed gene (DEC) 1 overexpression decreased the transcription and translation of HIF-1α and E-cadherin. HepG2 cells were treated as described above, and mRNA or protein was prepared from the cells and subjected to quantitative real-time PCR or western blotting analyses for the expression of HIF-1α and E-cadherin. One representative of at least 3 independent experiments with similar results is shown.
When exposed HepG2 cells to hypoxia, we found the peak levels of HIF-1α and DEC1 proteins appeared at different time points. HIF-1α protein reached its peak at 6 h, and that of DEC1 was at 18 h. After that, the expression of HIF-1α was gradually decreased and kept above the base level until 24 h. These data suggested that HIF-1α may function at the early stage of hypoxia since the expression of HIF-1α returned to the basal level at 48 h. On the other hand, DEC1 protein was ascended little by little until 18 h. From then on, the expression of DEC1 was sharply decreased. As the induction of HIF-1α and DEC1 under hypoxia was different from each other, we presumed they functioned distinctly in EMT caused by hypoxia. We previously reported that DEC1 negatively correlated with E-cadherin in pancreatic adenocarcinoma PANC-1 cells (17). The current study showed DEC1 overexpression prevented E-cadherin while DEC1 knockdown promoted its expression in HepG2 cells. As is known that loss of E-cadherin is one of the key criteria of EMT, to elucidate the mechanism through which DEC1 regulating E-cadherin would help us understand the roles of DEC1 in EMT processes.

DEC1 is a well-known controller of mammalian molecular clock (5). Recently, the chronochemotherapy based on the clock genes attracted more and more attention (14). Rhythm analysis of DEC1 under hypoxic condition could be taken into consideration in our future study.

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Acknowledgements

This work was supported by JSPS KAKENHI, Grants-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant no. 26870028).

REFERENCES


