CEBPD Reverses RB/E2F1-Mediated Gene Repression and Participates in HMDB-Induced Apoptosis of Cancer Cells

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Abstract

**Purpose:** Recent evidence indicates that a tumor suppressor gene CEBPD (CCAAT/enhancer-binding protein delta) is downregulated in many cancers including cervical cancer, which provides a therapeutic potential associated with its reactivation. However, little is known for CEBPD activators and the effect of reactivation of CEBPD transcription upon anticancer drug treatment. In this study, we identified a novel CEBPD activator, 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione (HMDB). The purpose of this study is to characterize the mechanism of HMDB-induced CEBPD activation and its potential effect in cancer therapy.

**Experimental Design:** Methylation-specific PCR assay, reporter assay, and chromatin immunoprecipitation (ChiP) assay were performed to dissect the signaling pathway of HMDB-induced CEBPD transcription. Furthermore, a consequence of HMDB-induced CEBPD expression was linked with E2F1 and retinoblastoma (RB), which discloses the scenario of CEBPD, E2F1, and RB bindings and transcriptional regulation on the promoters of proapoptotic genes, PPARG2 and GADD153. Finally, the anticancer effect of HMDB was examined in xenograft mice.

**Results:** We demonstrate that CEBPD plays an essential role in HMDB-mediated apoptosis of cancer cells. HMDB up-regulates CEBPD transcription through the p38/CREB pathway, thus leading to transcriptional activation of PPARG2 and GADD153. Furthermore, increased level of CEBPD attenuates E2F1-induced cancer cell proliferation and partially rescues RB/E2F1-mediated repression of PPARG2 and GADD153 transcription. Moreover, HMDB treatment attenuates the growth of A431 xenografts in severe combined immunodeficient mice mice.

**Conclusions:** These results clearly demonstrate that HMDB kills cancer cells through activation of CEBPD pathways and suggest that HMDB can serve as a superior chemotherapeutic agent with limited potential for adverse side effects. Clin Cancer Res; 16(23); 5770–80. ©2010 AACR.
Anticancer Drug, HMDB, Activates Tumor Suppressor CEBPD

Translation Relevance

Reactivation of tumor suppressors is one of the most important strategies in the development of cancer chemotherapy drugs. CEBPD, a tumor suppressor, is inactivated in many cancers including cervical cancer, hepatocellular carcinoma, breast cancer, and leukemia, indicating it can serve as a drugable target. For the first time, the authors provide new insight for the potential of HMDB serving as an anticancer drug, and they also highlight the CEBPD induction as an important therapeutic anticancer drug effect.

translational relevance

In conclusion, these results demonstrate that HMDB kills cancer cells through activation of CEBPD pathways and suggest that HMDB can serve as a superior chemotherapeutic agent with limited potential for adverse side effects.

Materials and Methods

Materials
Antibodies against CEBPD, CREB, Sp1, COX-1, and p38 were purchased from Santa Cruz Biotechnology. Phosphorylated CREB and p38 antibodies were purchased from Cell Signaling Technology. RB protein antibody was purchased from BD Biosciences. Assay kits for the detection of plasma levels of creatine kinase and blood urea nitrogen (BUIN) were purchased from BioSystems S.A. Aspartate transaminase (AST) and alanine transaminase (ALT) assay kits were purchased from Human GmbH. HMDB was purchased from Sigma. Dominant-negative p38 and CREB expression vectors were constructed by Wang et al. (18, 19).

Cell culture and treatments
A431-, HeLa-, and E1A-immortalized wild-type and Cebpd+/-- MEFS (mouse embryonic fibroblasts) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. When cells were 80% confluent, HMDB (60 μmol/L) was added to the cells for the indicated times.

Plasmid transfection and reporter assays
Cells were transfected with plasmids using Lipofectamine 2000 according to the manufacturer’s instructions. Transfectants were cultured in complete medium with or without treatment for 12 hours. Luciferase activity was measured in the lysates of transfectants.

Foci formation assays
Cells (500), including E1A-immortalized wild-type and Cebpd+/-- MEFS and transfected A431 and HeLa cells, were cultured for 10 to 14 days. Colonies were detected by methyl blue staining, and the number of colonies was counted for statistical analysis (n = 3, triplicate).

Reverse transcription (RT)-PCR
Total RNA was isolated after A431 cells were stimulated with HMDB. CEBPD-, PPAR2-, and GADD153-specific primers were used for analysis, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as a control. The specific primers used were: for human CEBPD, 5’-AGCGCAACACATCGGCGTG-3’ and 5’-GGCGGCTGCAGGTATGGGTC-3’; for PPAR2, 5’-GGATTTCCCTCT-3’ and 5’-CATGATAC-3’; and for GAPDH, 5’-GACTTCAAGTTGTCATCAC-3’ and 5’-TCTTATTTCACCTCACTCACTCACT-3’. Amounts of PCR product were determined using the IS-1000 digital imaging system.

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Evaluation of CEBPD expression in human cervical tissue samples

Immunohistochemistry was performed on 4-μm thick, formalin-fixed, paraffin-embedded tissue sections from representative tissue blocks of normal cervical tissue (n = 10), cervicitis (n = 10), and cervical carcinoma (n = 10). Sections were deparaffinized, hydrated, and immersed in a citrate buffer at pH of 6.0 in a microwave for epitope retrieval. Endogenous peroxidase activity was quenched in 3% hydrogen peroxidase for 15 minutes and sections were then incubated with 10% normal horse serum to block nonspecific immunoreactivity. Primary CEBPD antibody (1:50) was subsequently applied and detected by using the DAKO EnVision kit. Only tumor cells that demonstrated moderate-to-strong nuclear staining were regarded as having positive staining, and the expression in each sample was graded, with 0 for no staining, 1 for reactivity in less than 25% of cells, 2 for reactivity in 25% to 49% of cells, 3 for reactivity in 50% to 75% of cells and 4 for reactivity in more than 75% of cells. The difference in CEBPD expression levels in these samples was calculated using a chi-square test.

Immunoprecipitation assay

A431 cells that were transfected with the pcDNA3-HA or pcDNA3-HA/CEBPD expression vectors were lysed by a freeze–thaw method (buffer containing 20 mmol/L of HEPES [pH = 7.9], 0.2 mmol/L of EDTA, 2 mmol/L of MgCl2, 1 mmol/L of DTT, 0.1 mmol/L of KCl and 10% glycerol). α-HA antibody (1 μg) was incubated with 200 μg of lysate at 4°C overnight. The immune complex was washed 3 times with PBS and then separated by 10% SDS-polyacrylamide gel electrophoresis for Western blot with the indicated antibodies.

Short hairpin RNA (shRNA) assays

The lentiviral expression vectors pLKO.1-shLuciferase containing 5’-CTTCGAATGTTCCGTTGGT-3’ and pLKO.1-shCEBPD containing 5’-GGTGTCGCTCAGAAAC-3’ were obtained from the National RNAi Core Facility located at the Genomic Research Center of the Institute of Molecular Biology, Academia Sinica. The virus was produced from Phoenix cells co-transfected with the pMD2.G and psPAX2 vectors along with the pLKO.1-shLuc or pLKO.1-shCEBPD vector.

Fig. 1. CCAAT/enhancer-binding protein delta (CEBPD) functions as a tumor suppressor but is inactivated in cervical cancer specimens. A, CEBPD expression is low in normal cervical tissue and is significantly elevated in inflamed cervical epithelium (P = 0.004). CEBPD expression is diminished in cervical cancer (P = 0.003). B, Cebpd-deficient MEFs have a higher rate of proliferation. Foci assays were performed as described in the “Materials and Methods” section. C, increased levels of CEBPD attenuate E2F1-induced proliferation. Foci assays were performed with cells that were co-transfected with a vector encoding E2F1 and with a control vector or one encoding CEBPD. The quantifications results of colony numbers of which size were larger than 100 μm. A very similar pattern was observed in 2 independent experiments. The total DNA concentration for each transfection was matched to equal with pcDNA3 vector. Figure is representative from 1 experiment in triplicate. Data are shown as the mean ± S.E.M. *, P < 0.05 by Student’s t test. HE, Hematoxylin and Eosin stain.
Severe combined immunodeficient mice (SCID) were used for animal studies.

Chromatin immunoprecipitation (ChIP) assays were performed as described (16, 20). Briefly, A431 cells were treated with 1% formaldehyde for 15 minutes. Cross-linked chromatin was then isolated and sonicated to an average size of 500 bp. DNA fragments were immunoprecipitated with antibodies specific for CEBPD, RB, E2F1, or control rabbit IgG at 4°C overnight. Cross-linking was reversed and immunoprecipitated chromatin was amplified with primers corresponding to specific regions of the genomic loci. The ChIP primers for the COX-1 promoter are as follows: 5'-GGGGTACCTCGACAATCCCAGTGGATGGATACC-3' and 5'-TGGCTTTGGGTCACGAG-GCTTCACG-3'.

Animal studies
Female, 6- to 8-week-old NOD (non-obese diabetic)/SCID mice were obtained from the Laboratory Animal Center of the National Cheng Kung University, Tainan, Taiwan. A431 cells (2 × 10⁶) in 0.2 mL of PBS were inoculated subcutaneously into the right flank of the mice. After 8 days, when macroscopic tumors (50–100 mm³) had formed, animals (n = 5 per group) were placed randomly into 2 groups as follows: (1) control group, which received identically vehicle (corn oil); and (2) experimental group, treated with HMDB. HMDB, dissolved in corn oil, was given intraperitoneally at doses of 50 mg/kg/day 5 days per week for 6 weeks. Animal weight and tumor dimensions were measured every 2 days with calipers, and tumor volumes were estimated using 2-dimensional measurements of length and width and were calculated with the formula: [l × (w)²] × 0.52, where l is length and w is width. At the end of the experiment, all animals were sacrificed and blood samples were collected in heparinized tubes. Plasma was separated from red blood cells by centrifugation (4,700 rpm for 10 minutes). The plasma levels of creatine kinase, BUN, AST, and ALT were measured, following the procedures stated in the assay protocols.

Results

Inflammation-induced CEBPD is inactivated in cervical cancer specimens
CEBPD is responsive to proinflammatory stimuli, such as IL-1β and TNFα (21, 22). In addition, the CEBPD gene was observed to be inactivated in cervical cancer through increased hypermethylation of its promoter (14). However, these phenomena have not yet been linked with clinical specimens. In the current study, we quantified CEBPD...
expression in normal, inflamed, and cancerous cervixes. As shown in Figure 1A, the expression level of CEBPD was low in the normal cervix but significantly elevated in inflamed cervical epithelium (P = 0.004). Moreover, inactivation of CEBPD was observed in a majority of cervical cancers relative to inflamed cervical epithelium (P = 0.003). Our previous study demonstrated that the hypermethylation of CpG islands within the CEBPD promoter plays an important role in CEBPD silencing in cervical cancer (14). To address whether the inactivation of CEBPD can enhance proliferation, a foci assay was performed with wild-type and Cebpd-deficient MEFs (23). Our results suggest that the loss of Cebpd expression leads to enhanced proliferation (Fig. 1B). In addition, E2F1 demonstrates oncogenic activity in the induction of cancer cell proliferation (24, 25). To address whether CEBPD can serve as a negative regulator of E2F1-induced proliferation in A431 and HeLa cells, these cells were co-transfected with vectors encoding CEBPD and with empty vector or vector encoding E2F1, and foci assays were performed. As shown in Figure 1C, the overexpression of CEBPD attenuates E2F1-induced proliferation. Taken together, these results suggest that the incremental insensitivity of CEBPD to external stimuli, such as EGF (16) or TNFα (22), could benefit the E2F1-mediated tumorigenesis.

The preceding experiments demonstrated that CEBPD expression inhibits tumor formation. Therefore, we wanted to investigate whether CEBPD levels are changed in A431 cells following treatment with clinical anticancer drugs. Interestingly, CEBPD transcription is induced in response to stimulation from multiple anticancer drugs, including dexamethasone (Dex), RA, and 5′-azacytidine (5′-Aza).
Anticancer Drug, HMDB, Activates Tumor Suppressor CEBPD

Figure 3. CCAAT/enhancer-binding protein delta (CEBPD) mediates the induction of PPARG2 and GADD153 transcription following HMDB (1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione) treatment. A, PPARG2 and GADD153 gene expression is coincident with the increase in CEBPD levels after HMDB treatment. Total RNA and lysates from HMDB-treated A431 cells were harvested at the indicated times for RT-PCR with gene-specific primers (left) and Western blot analysis with the indicated antibodies (right), respectively. B, CEBPD is an upstream activator of HMDB-induced PPARG2 and GADD153 transcription. A431 cells were co-transfected with PPARG2 or GADD153 reporters and with a control vector or 1 amount of CEBPD, and cells were treated with HMDB. Figure is representative from 3 experiments in duplicate. Data are shown as the mean ± S.E.M. **, P < 0.01 by Student’s t test. For this assay, 0.5 μg of reporter and 0.5 μg of expression vectors as indicated were co-transfected into A431 cells. The total DNA concentration for transfection was matched to equal with pcDNA3 or related backbone vectors.
GADD153 and PPARG2 is upregulated by HMDB and coincident with CEBPD expression. Experiments were performed to clarify whether CEBPD participates in HMDB-induced activation of GADD153 and PPARG2 transcription. An RT-PCR analysis demonstrated that shRNA-mediated knockdown of CEBPD blocked HMDB-induced PPARG2 and GADD153 transcription, suggesting that CEBPD is an upstream regulator of the expression of these genes (Fig. 3B). In a complementary approach, reporter assays were performed in which exogenous CEBPD was introduced into cells. Wild-type CEBPD trans-activated the PPARG2 and GADD153 reporters, whereas the dominant-negative CEBPD attenuated the HMDB-induced reporter activity of both constructs (Fig. 3C and Supplementary Fig. 4).

**RB negatively regulates CEBPD-mediated activation of PPARG2 and GADD153 transcription**

RB can act as a transcriptional repressor and controls cell-cycle progression, differentiation/survival, and genomic integrity through its interaction with E2F1 (27, 28). CEBPD was demonstrated to bind to RB in an in vitro protein–protein interaction assay (17). However, the details of how RB and CEBPD interact during transcriptional regulation were unknown. An immunoprecipitation (IP) assay was performed with the initial aim of verifying that CEBPD can interact with RB in A431 cells. RB was, in fact, detected in the CEBPD–IP complex (Fig. 4A). Subsequently, we wanted to characterize the function of RB in CEBPD-mediated promoter regulation. Overexpression of RB-attenuated CEBPD-induced GADD153 and PPARG2 promoter activity (Fig. 4B). As shown in Figure 2A, HMDB treatment resulted in decreased RB in A431 cells, while attenuating the levels of the hyperphosphorylated form of RB. These interesting observations led us to subsequently investigate the interplay between RB, E2F1, and CEBPD in the regulation of GADD153 and PPARG2 reporter activity. In these experiments, CEBPD functioned in opposition to E2F1 and RB to activate PPARG2 and GADD153 reporter activity.
Anticancer Drug, HMDB, Activates Tumor Suppressor CEBPD

An antitumor effect of HMDB in A431-bearing SCID mice

Previous studies demonstrated that HMDB induced cancer cell apoptosis (4, 5). To assess the potential use of HMDB as an antineoplastic drug, we evaluated its effect on the growth of A431 xenografts in mice. We examined the mice that were administered a single dose of HMDB per day (50 mg/kg, intraperitoneal) (Fig. 6A). Compared with the control, HMDB treatment resulted in a 67% reduction in tumor growth after 6 weeks of treatment. Furthermore, the levels of CEBPD, PPARG2, GADD153, and RB were verified in these experimental mice. As shown in Figure 6C, coincident with reduced tumor size, the higher levels of CEBPD, PPARG2, and GADD153 and decrease of RB were observed in HMDB-administrated A431 xenografts SCID mice. In addition, to determine the toxicity of HMDB in animals, the plasma levels of liver-specific enzymes such as ALT and AST were measured and renal function tests such as BUN and creatinine level detection were performed. The intraperitoneal administration of HMDB at a dose of 50 mg/kg/day for 42 consecutive days was well tolerated, as no toxic deaths or body weight loss were observed during or after treatment (Fig. 6B).

Discussion

It is known that p38 signaling participates in the regulation of apoptotic cell death by upregulating the expression of proapoptotic genes, such as Fas ligand, PPARG2, and GADD153 (29–32). Moreover, p38 is also involved in the negative regulation of cell growth, as it represses cyclin D1 expression and activates p27 (33, 34). The p38 signaling pathway also induces the differentiation or apoptosis of malignant cells and can be activated by chemicals that are used for clinical cancer therapy, such as RA, Dex, and Vitamin D3 (35–37). Previously, our studies suggested that transcriptional activation of CEBPD occurs downstream of the p38/CREB signaling pathway in A431 cells (16), a cervical cell line that is responsive to EGF-induced apoptosis (38). Moreover, upregulation of CEBPD was also observed following treatment with RA, Dex, D3, and HMDB (Supplementary Fig. 1). The overexpression of CEBPD can also downregulate the expression of cyclin D1 and E (8) while upregulating p27 (7). These studies suggest a close relationship between p38 signaling and the activation of CEBPD gene expression.

PPARG2 and GADD153 have been reported to function in tumor suppression (39–42). CDK4 was suggested to derepress RB/E2F1-mediated PPARG2 gene inactivation in adipogenesis (43). In the current study, the promoter activities of the proapoptotic genes, PPARG2 and GADD153, were found to be modulated by RB (Fig. 4). Furthermore, HMDB treatment induced CEBPD expression and decreased RB levels, which were coincident with the induction of PPARG2 and GADD153 transcription (Fig. 2A, Fig. 3A and B). Our molecular evidence links CEBPD and RB in transcriptional regulation following HMDB treatment and suggests that CEBPD enhancement...
Fig. 6. In vivo anti-tumor effect of HMDB (1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione) in A431-bearing severe combined immunodeficient (SCID) mice. Effect of 6 weeks of intraperitoneal administration of HMDB on the size of tumors in A431-bearing SCID mice. A, NOD/SCID mice in 2 groups of 5 were subcutaneously inoculated with A431 cells (2 x 10⁶). Following solid tumor formation, animals were injected intraperitoneally with 0.1 mL of vehicle or HMDB at doses of 50 mg/kg/day. B, evaluation of the toxicity of HMDB in the liver and kidney tissues of the hosts. Following a 6-week drug treatment, all animals were sacrificed and their plasma collected. Plasma levels of the liver-specific enzymes alanine transaminase (ALT), aspartate transaminase (AST) and renal function tests, such as blood urea nitrogen (BUN) and creatinine levels, were measured. Notably, organ function was comparable between the HMDB-treated and vehicle-treated groups. Data from 5 mice in each group are presented as the mean ± SD. Tumor dimensions and animal weight were measured every 2 days. C, increase of CCAAT/enhancer-binding protein delta (CEBPD) and loss of retinoblastoma (RB) are observed in A431-bearing SCID mice. Following a 6-week drug treatment, all animals were sacrificed and the tumors were collected for extractions of protein. The protein samples were performed with Western blot by using specific antibodies. Data in the bottom show the changes of CEBPD and RB levels of with or without HMDB treatment A431 xenofrafts SCID mice.
of GADD153 and PPARG2 transcription can account for the tumor inhibitory effect of HMDB in A431 cells. Furthermore, both gain-of-function and loss-of-function assays demonstrated that RB is important for the E2F1-mediated inactivation of proapoptotic genes (Fig. 4). CEBPD, RB, and E2F1 were demonstrated to interact with each other following treatment with HMDB (Fig. 5), suggesting that the induction of CEBPD by HMDB can reverse RB/E2F1-mediated repression of the PPARG2 and GADD153 genes.

The inactivation of CEBPD has been observed in several cancers, including breast cancer (10–12), acute leukemia (13), hepatocellular carcinoma, and cervical cancer (14). One therapeutic approach induces cancerous cells to behave like normal cells in what is known as “differentiation therapy” (44). Several studies have demonstrated that CEBPD plays a functional role in differentiation and causes growth arrest (6, 7). In addition, CEBPD is an inducible gene that can be upregulated by several external stimuli, including Dex, RA, and 5′-Aza (7, 14, 45) (Supplementary Fig. 1). However, these clinical anticancer drugs are not ideal because of their induction of hepatotoxicity in cancer patients (46–48). Herein, we demonstrate that the cytotoxicity of HMDB is quite low in cancer cell xenograft mice, as determined by the examination of metabolic indices, body weight, and liver tissue in these mice (Fig. 6B). Moreover, our preliminary results indicate that the cytotoxicity of HMDB is lower than that of 5′-Aza in xenograft mice, suggesting that HMDB could be a nontoxic alternative to anticancer drugs or could be used in combination with current drugs for effective cancer therapy.

Comparison of HMDB with other anticancer drugs in terms of cytotoxicity and efficacy remain to be performed to evaluate whether HMDB is a suitable candidate to replace other marketed drugs. Interestingly, this study has identified the need to determine CEBPD levels when treating patients with current clinical anticancer drugs. Our recent results demonstrate activation of COX-2, which has been suggested to serve as an oncogene, in response to CEBPD induction (49). Additionally, increased levels of MMP1, 3, and 10, which have been suggested to act as inducers of metastasis, are coincident with CEBPD levels (Wang et al., unpublished results). Moreover, overexpression of CEBPD not only induces apoptosis and growth arrest, but also activates inflammation-like responses, such as an increase in genomic instability and cell migration. These intriguing observations reveal another side of CEBPD activity that opposes the side associated with its suspected role as a tumor suppressor. Hence, further investigation is necessary to modulate the inflammation-like responses resulting from CEBPD-inducing anticancer drug treatment to achieve better therapeutic effects in clinical settings. Furthermore, it is well known that anticancer drug resistance may result from a wide variety of mechanisms. One possible mechanism is that some cancer cells can escape the initial cytotoxicity of anticancer drugs and may continuously increase their drug resistance by long-term cellular adaptations. Therefore, treatment with anticancer drugs that potentially cause CEBPD induction, such as p38 activators or DNA methylation inhibitors, may be a double-edged sword, in that they may also lead to the development of drug resistance. This may result from CEBPD not only acting as a proapoptotic activator, but also inducing inflammation-like responses that could be involved in drug resistance. Accordingly, the activation status of CEBPD should be considered before the use of certain anticancer drugs. Another possible strategy could be a combination treatment of CEBPD-inducing anticancer drugs with COX-2, MMP, and/or genomic instability inhibitors to block drug resistance or other adverse effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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