BHLH transcription factor DEC2 regulates pro-apoptotic factor Bim in human oral cancer HSC-3 cells

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ABSTRACT

DEC1 (BHLHE40/Stra13/Sharp2) and DEC2 (BHLHE41/Sharp1) are basic helix-loop-helix (bHLH) transcription factors that are involved in the regulation of apoptosis, cell proliferation, circadian rhythms and the response to hypoxia. We previously showed the functional effects of DEC1 and DEC2 on apoptosis in human breast cancer MCF-7 cells. However, the roles of DEC1 and DEC2 in oral cancer are poorly understood. We examined whether DEC1 and DEC2 are involved in the regulation of apoptosis in human oral cancer HSC-3 and CA9-22 cells. The expression of DEC2 was upregulated by cis-diamminedichloroplatinum (II) (cisplatin: CDDP) treatment in HSC-3 cells, whereas CDDP treatment had little effects on the expression of DEC2 in CA9-22 cells. We showed that DEC2 overexpression inhibits pro-apoptotic factor Bim and inhibits apoptosis induced by CDDP in HSC-3 cells, whereas it had little effects on apoptosis in CA9-22 cells. DEC1 overexpression had little effects on apoptosis induced by CDDP in these cells. We also found that CDDP upregulated the amounts of DEC2 in the nucleus in HSC-3 cells. These results suggest that DEC2 has anti-apoptotic effects on apoptosis induced by CDDP in HSC-3 cells.

Oral cancer accounts for 2% of all new cancer cases and 1.7% of all cancer related deaths worldwide (9). Ninety percent of all oral cancer arises from epithelial squamous cells (27). Despite advances in diagnosis and treatment, there has been little improvement in survival rate for patients with oral squamous cell carcinoma (11).

Cis-diamminedichloroplatinum (II) (CDDP) or cisplatin is an efficient DNA-damaging agent and is one of the first-line chemotherapeutic agents employed for treatment of a wide variety of cancers (2, 28). CDDP treatment affects mitogen-activated protein kinases (MAPKs), p53, Bax, Bcl-2, Bcl-XL, Bim, PERIOD 1 (PER1), PER3 and activates caspases and the cleavage of poly (ADP-ribose) polymerase (PARP) (4, 7, 15, 25, 26, 30). Bim (Bcl-2-interacting mediator of cell death) is a member of the Bcl-2 homology 3 (BH3)-only proteins (BOPs) of the Bcl-2 family and exists as three splice variants, BimS (short form), BimL (long form) and BimEL (extra long form) (6, 13, 21). All isoforms promote apoptosis, and it seems that BimEL is the most detectable isoform in apoptosis reactions (8, 19, 20, 23). Bim binds all of the pro-survival Bcl-2 like proteins, thereby relieving their inhibition of Bax or Bak and inducing apoptosis of various tumor cells, such as lung cancer, breast cancer, osteosarcoma, melanoma and gingival cancer (1, 8, 25).
Differentiated embryonic chondrocyte (DEC) 1 and DEC2 are basic helix-loop-helix (bHLH) transcription factors. We have reported that DEC1 and DEC2 are involved in the regulation of apoptosis, cell cycle, response to hypoxia and circadian rhythms (14, 18, 24, 29). DEC1 is overexpressed in tumor regions of breast, colon and oral cancers (3, 5, 17). However, the role of DEC2 in tumor is poorly understood. Our previous data suggested that DEC1 and DEC2 have opposite properties in regulating apoptosis, that is, DEC2 has anti-apoptotic, whereas DEC1 has pro-apoptotic effects in human breast cancer MCF-7 cells (18, 29).

In the present study, we investigated the role of DEC1 and DEC2 on apoptosis induced by CDDP in human oral cancer CA9-22 and HSC-3 cells.

The expression of DEC2 was upregulated by CDDP in HSC-3 cells, and inhibited apoptosis induced by CDDP in HSC-3 cells, involving Bim downregulation.

MATERIALS AND METHODS

Cell culture and treatment. Human oral cancer HSC-3 and CA9-22 cells were obtained from the Japanese Cancer Research Resources Bank. These cells were cultured in Dulbecco’s Modified Eagle’s Medium-high glucose (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂. In some experiments, the cells were incubated with CDDP (Sigma) at various concentrations for 24 h. Since many of the HSC-3 cells died when treated with 100 μM of CDDP for 24 h, we treated them with CDDP up to 50 μM for 24 h.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated, and first-strand cDNA was synthesized as described previously (18). RT-PCR was performed using an aliquot of first-strand cDNA as a template under standard conditions with Taq DNA polymerase (QIAGEN, Hilden, Germany). The primers were designed to perform optimal RT-PCR by DNASIS software, and the primers used were as follows: DEC1-F: 5'-GTCTGTGAGTCACTCTTCAG-3', DEC1-R: 5'-GAGTCTAGTTCTGTTTGAAGG-3', DEC2-F: 5'-CACCTTGGACGTTTCTTTGAG-3', DEC2-R: 5'-GAGAGTGGGAATTGATGCAC-3', GAPDH-F: 5'-CCACCCATGGCAAATTCCATGGCA-3', GAPDH-R: 5'-AGACACCTGGTGCTCAGTGTAGC-3'. The amplified products of DEC1, DEC2 and GAPDH were 534 bp, 502 bp and 696 bp in length, respectively. The cDNAs for DEC1, DEC2 and GAPDH were amplified for up to 25 cycles. The PCR products were separated on 1.5% (w/v) agarose gels.

**DEC1 and DEC2 overexpression.** DEC1 and DEC2 overexpression was induced using pcDNA vector as described previously (18). After transfection, the cells were incubated for 24 h and subjected to Western blotting.

Western blotting. Cells were lysed using M-PER lysis buffer (Thermo Scientific, Rockford, IL, USA), and their protein concentration (10 μg) was determined using the bicinchoninic acid (BCA) assay. The lysates were subjected to SDS-PAGE, and the proteins were transferred to PVDF membranes (Immobilon P; Millipore, Tokyo, Japan), which were then incubated with antibodies. The ECL, ECL-plus, or ECL-advance Western Blotting Detection System (Amersham, Uppsala, Sweden) was used for detection.

Antibodies. The membranes for Western blotting were incubated with antibodies specific to DEC1 (1 : 10,000; Novus Biologicals, Inc), DEC2 (1 : 20,000; H-72; Santa Cruz Biotechnology, Inc), poly (ADP-ribose) polymerase (PARP) (1 : 1,000; Cell Signaling Technology, Inc), cleaved caspase-3 (1 : 1,000; Cell Signaling Technology, Inc), cleaved caspase-8 (1 : 20,000; Cell Signaling Technology, Inc), Bid (1 : 10,000; Cell Signaling Technology, Inc), Bim (1 : 10,000; Cell Signaling Technology, Inc), Bax (1 : 5,000; Santa Cruz Biotechnology), and actin (1 : 30,000; Sigma), followed by horseradish peroxidase-conjugated secondary antibody (IBL, Fujioka, Gunma, Japan). Can Get Signal immunoreaction enhancer solution (TOYOBO, Osaka, Japan) or Immunoshot immunoreaction enhancer solution (Cosmobio, Co, LTD, Tokyo, Japan) was used to dilute the primary antibody.

Immunofluorescent staining. Immunofluorescent staining was performed as described previously (29). The permeabilized cells were then washed in phosphate-buffered saline (PBS) twice and treated with 5% normal horse serum in PBS for 30 min (to minimize the non-specific adsorption of antibodies), before being incubated with anti-DEC1 (1 : 300), or DEC2 (1 : 300) antibodies at 4°C overnight. The cells were then incubated for 1 h with goat anti-rabbit IgG antibody conjugated to Alexa 488 dye (Molecular Probes, Inc, Tokyo, Japan), while nucle-
ar staining was performed using 4', 6-diamidino-2-phenylindole (DAPI). These cells were visualized using confocal laser scanning microscopy (LSM 710; Zeiss, Wetzlar, Germany).

Cell viability assay. The cell viability assay was performed using the MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay as described previously (25).

RESULTS

Effects of CDDP on the expression of DEC1 and DEC2 in CA9-22 and HSC-3 cells

First, we analyzed effects of CDDP on the expression of DEC1 and DEC2 in CA9-22 and HSC-3 cells. Fifty and 100 μM of CDDP treatment for 24 h increased the amounts of cleaved PARP and Bim, whereas it decreased the endogenous DEC1 and DEC2 mRNA and protein expression in CA9-22 cells (Fig. 1A). In HSC-3 cells, 10 μM of CDDP

![Fig. 1](image.jpg)

CDDP affects the expression of DEC1 and DEC2. CA9-22 and HSC-3 cells were treated with various concentrations of CDDP. After 24 h, total RNA was prepared and subjected to RT-PCR analyses of DEC1, DEC2 and GAPDH (left panel of A and B). CA9-22 and HSC-3 cells were treated with CDDP for 24 h. Cells were lysed, and the lysates were subjected to Western blot analyses of DEC1, DEC2, cleaved PARP, Bim and actin (right panel of A and B).
slightly increased the amounts of cleaved PARP and BimEL (Fig. 1B). In addition, 20 and 50 μM of CDDP significantly increased the amounts of cleaved PARP and BimEL. The expression of DEC1 was decreased in 20 and 50 μM of CDDP, whereas the expression of DEC2 was significantly increased in 20 and 50 μM of CDDP.

DEC2 inhibits Bim and apoptosis induced by CDDP in HSC-3 cells
Using transient transfection, we next examined whether the overexpression of DEC1 or DEC2 affects apoptosis. DEC2 overexpression in the presence or absence of CDDP (50 or 100 μM) had little effect on the amounts of cleaved PARP, caspase-3, caspase-8 and BimEL in CA9-22 cells, whereas DEC2 overexpression in the presence of CDDP (20 or 50 μM) significantly decreased the amounts of cleaved PARP, caspase-3, caspase-8 and BimEL in HSC-3 cells (Fig. 2A and B). On the other hand, DEC2 overexpression had little effect on the expression of Bid and Bax in HSC-3 cells. DEC1 overexpression in the presence of CDDP (50 μM) had little effect on the amounts of cleaved PARP, caspase-3 and caspase-8 both in CA9-22 and HSC-3 cells (Fig. 2C and D).

Effect of CDDP on the amounts of nuclear/cytoplasmic DEC
We investigated whether CDDP affects the amounts of nuclear/cytoplasmic DEC1 and DEC2 by immunofluorescent staining. CDDP had little effect on the amounts of nuclear/cytoplasmic DEC1 in CA9-22 and HSC-3 cells, whereas it significantly increased the amounts of DEC2 in the nucleus of HSC-3 cells (Fig. 3A and B). CDDP had little effect on the amounts of nuclear/cytoplasmic DEC2 in CA9-22 cells.

DEC2 overexpression inhibits cell death in HSC-3 cells
We also examined cell viability using the MTS-assay (Fig. 4A and B). DEC2 overexpression and CDDP had little effect on the cell viability in CA9-22 cells, whereas it significantly increased the cell viability compared with that in CDDP-treated control HSC-3 cells. DEC2 overexpression in the absence of CDDP had little effect on the cell viability in these cells.

DISCUSSION
We previously showed that DEC1 has pro- and DEC2 has anti-apoptotic effects by TNF-α, which induces apoptosis in human breast cancer MCF-7 cells, and demonstrated that DEC2 knockdown induced apoptosis, and affected the expression of DEC1, Fas, Bax, and c-Myc (18). However, the role of DEC1 and DEC2 on apoptosis of oral cancer cell is poorly understood. This study focused on the functional analysis of DEC1 and DEC2 during CDDP-induced apoptosis in human oral cancer HSC-3 and CA9-22 cells. DEC1 had little effect on the apoptosis induced by CDDP in these cell lines. Since our previous report showed that DEC1 overexpression affects the regulation of cell cycle, involving reverse expression pattern of cyclinD1 in oral cancer HSC-2 cells (3), DEC1 may contribute to the regulation of cell cycle rather than to the regulation of apoptosis in oral cancer cells.

DEC2 was upregulated by CDDP in HSC-3 cells, and DEC2 overexpression inhibited apoptosis. This result corresponds to our previous report (18). We also found that DEC2 regulates Bim in the presence of CDDP in HSC-3 cells. Bim translocated from cytoplasm to mitochondria when cells were exposed to apoptosis stimuli (22). This observation suggests that DEC2 is related to intrinsic pathway of apoptosis induced by CDDP through mitochondria in HSC-3 cells. However, DEC2 had little effect on the apoptosis induced by CDDP in CA9-22 cells. These differences in the effect of apoptosis by DEC2 may depend on the differences in gene expression among these cells. Epidermal growth factor receptor (EGFR) is closely related to the regulation of apoptosis, and is highly expressed in CA9-22 cells (10, 12, 16). CDDP increased the amount of cleaved PARP at concentration of more than 50 μM in CA9-22 cells, whereas it increased the amount of cleaved PARP at concentration of more than 10 μM in HSC-3 cells. MTS-assay also showed that 50 μM of CDDP increased the cell death more in HSC-3 cells than CA9-22 cells. Therefore, we speculate that DEC2 had little effect on apoptosis induced by CDDP in CA9-22 cells because of biochemical characterization of resistance to apoptosis. We previously showed that anti-apoptotic factor PER1 knockdown was significantly sensitive to apoptosis induced by CDDP in CA9-22 cells (25). These results suggest that PER1 or EGFR knockdown in CA9-22 cells may induce apoptosis, and DEC2 may inhibit apoptosis under these conditions. Future studies should clarify the roles of DEC1 and DEC2 in apoptotic reactions and may contribute to the development of a new strategy against oral cancer by chemotherapy.
DEC2 regulates pro-apoptotic factor Bim

Fig. 2  DEC2 inhibits apoptosis induced by CDDP in HSC-3 cells. CA9-22 cells were transfected with an empty vector (pcDNA) or DEC2 expression plasmid (DEC2 pcDNA) and incubated for 24 h. Then, the cells were treated with CDDP (50 or 100 μM) and incubated for an additional 24 h, before being lysed. The lysates were subjected to Western blot analyses of DEC2, cleaved PARP, caspase-3, caspase-8, Bim and actin (A). HSC-3 cells were treated as above, and the CDDP concentration were 20 or 50 μM and were subjected to Western blot analyses of DEC2, cleaved PARP, caspase-3, caspase-8, Bid, Bim, Bax and actin (B). CA9-22 or HSC-3 cells were transfected with pcDNA or DEC1 pcDNA, and incubated for an additional 24 h, before being lysed. The lysates were subjected to Western blot analyses of DEC1, cleaved PARP, caspase-3, caspase-8 and actin (C and D).
Fig. 3  The amounts of DEC2 in the nucleus were increased by CDDP in HSC-3 cells. CA9-22 or HSC-3 cells were treated with 100 or 50 μM of CDDP, respectively, for 24 h and fixed. The cells were then incubated with anti-DEC1 or DEC2 antibody and visualized using Alexa488-conjugated secondary antibody (Green). The cells were also counterstained with DAPI (Blue) to localize the nucleus. One representative result of at least three independent experiments with similar results is shown (A and B).
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