EUROPEAN JOURNAL OF CANCER 47 (2011) 1747–1758

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PERIOD1 (PER1) has anti-apoptotic effects, and PER3 has pro-apoptotic effects during cisplatin (CDDP) treatment in human gingival cancer CA9-22 cells

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ARTICLE INFO

Article history:
Received 1 January 2011
Received in revised form 21 February 2011
Accepted 28 February 2011
Available online 1 April 2011

Keywords:
CA9-22
CDDP
PER1
PER3
Bim
Apoptosis

ABSTRACT

PERIOD (PER) proteins are transcriptional regulators that are involved in circadian rhythms, sleep homeostasis, cell proliferation and tumour progression. We previously showed that the expression of PER1 was related to the regulation of apoptosis in human pancreatic cancer and hepatocellular carcinoma cells. However, the significance of PER in oral cancer has not been reported, and the detailed molecular mechanisms by which anti-tumour drug induces apoptosis in gingival cancer cells are not well understood. We examined whether PER1 and PER3 are involved in the regulation of apoptosis in human gingival cancer CA9-22 cells. The expression of PER1 and PER3 was upregulated and downregulated, respectively, by *cis*-diamminedichloroplatinum (II) (cisplatin: CDDP) treatment in CA9-22 cells, whereas CDDP treatment had little effects on the expression of PER1 and PER3 in human gingival fibroblasts (HGF-1). We found that short interference RNA (siRNA)-mediated knockdown of PER1 enhanced apoptosis of CA9-22 cells, and that PER1 regulated the amount of Bim, an apoptosis-related molecule. On the other hand, PER3 knockdown had an inhibitory effect on the apoptosis of CA9-22 cells induced by CDDP treatment. These results suggest that the alternation of expression of PER1 and PER3 was related to the apoptosis of CA9-22 cells. Furthermore, PER1 was intensely stained in the gingival cancer tissues, whereas PER3 was significantly stained in the non-tumour tissues by immunohistochemistry.

These findings suggest that PER1 and PER3 have anti-apoptotic and pro-apoptotic effects in human gingival cancer CA9-22 cells, respectively. The balance of PER1 and PER3 may modulate apoptotic reactions in gingival cancer cells.

1. Introduction

The frequency of oral cancer is about 3% of all cancers, and tongue and gingival carcinoma make up a high proportion of oral cancer cases. In addition, the incidence of oral cancer is increasing worldwide and so its diagnosis and treatment are critical issues.[1] Despite improvements in surgery, chemotherapy and radiation therapy, the overall 5-year survival rate for oral cancer remains at 50% and has not significantly improved in the past 30 years.[2,3]

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2011.02.025

cis-Diamminedichloroplatinum (II) (cisplatin: CDDP) is one of the most important anti-tumour drugs, and CDDP treatment induces DNA-damage and leads to apoptosis in various solid tumours.[4] CDDP treatment also affects transcription, translation, DNA repair and the cell cycle,[4–6] although some tumours are resistant to CDDP treatment. Several mechanisms are thought to be involved in the acquisition of resistance to CDDP. Tumours that are resistant to CDDP often show decreased drug accumulation and increased DNA-repair activity and transcription factor activity.[4,7] However, the details of the molecular mechanisms responsible for CDDP resistance are still unknown.

The induction of apoptosis is mainly regulated by the balance of pro- and anti-apoptotic factors. Bax, Bim, Bid and Bok are pro-apoptotic, while Bcl-2, Bcl-X_L and Mcl-1, which belong to the Bcl-2 family, are anti-apoptotic proteins.[8] The overexpression of Bcl-2 and Bcl-X_L is observed in the majority of oral cancers and correlates with chemotherapy resistance.[9,10] CDDP treatment has been shown to affect the expression of Bcl-2, Akt, X-linked inhibitor of apoptosis protein (XIAP), and Bim, and to activate the cleavage of caspases and poly (ADP-ribose) polymerase (PARP). CDDP treatment-induced apoptosis is also associated with p53 dependent and independent responses.[11] Bim is a Bcl-2 homology 3 (BH3)-only protein that has three isoforms (BimS, BimL and BimEL) and plays a critical role in initiating apoptosis in various cells.[12] The amount of Bim also correlates with apoptosis induced by various anti-tumour drugs, such as paclitaxel or gefitinib.[12,13]

PERIOD was first identified as a clock gene[14] and is known to have three isoforms (*PER1*, *PER2* and *PER3*). The three *PER* isoforms have similar structures[15,16] and work as transcriptional regulators of circadian rhythm; *PER1* and *PER2* are dominant negative regulators, while *PER3* is thought to be an output gene. Recently, it has been reported that PER1 was correlated with tumour progression.[17–21] However, a role of PER3 in tumours is poorly understood. PER1 has pro-apoptotic effects in prostate and colon cancers[22,23], whereas PER1 has anti-apoptotic effects in pancreatic cancer and hepatic carcinoma cells.[17] PER1 regulates apoptosis via p53-dependent or -independent responses. PER1 also regulates the expression of anti- and pro-apoptotic proteins in various tumours.[17,22] However, actions of PER in oral cancer cells have not been reported.

In the present study, we examined the effects of PER1 and PER3 on the apoptosis of human gingival cancer CA9-22 cells. PER1 knockdown enhanced the apoptosis induced by CDDP treatment and upregulated the expression of Bim. On the other hand, PER3 knockdown decreased the apoptosis induced by CDDP treatment. Our results suggest that PER1 and PER3 are anti-apoptotic and pro-apoptotic, respectively, in human gingival cancer cells.

2. Materials and methods

2.1. Cell culture and treatment

The human gingival cancer cell line CA9-22 was obtained from the Japanese Cancer Research Resources Bank. Normal human gingival fibroblasts (HGF-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in Dulbecco's Modified Eagle's Medium-high glucose (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% foetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. In some experiments, the cells were incubated with CDDP (Sigma) at various concentrations for 24 h.

2.2. Human tissues

Well, moderately, or poorly differentiated human gingival cancer tissues ($n = 13$) were obtained at around 12:00 or 13:00 (Table 1). Histological specimens were retrieved from the archives of Hirosaki University Hospital under the guidelines produced by the Japanese Society of Pathology. We examined 11 surgical resection specimens and 2 biopsy specimens from 13 patients and analysed PER1 and PER3 protein expressions in the tumours and the adjacent non-tumour tissue.

Table 1 – Immunohistochemical detection of PER1 and PER3 proteins in human gingival cancer tissues.

Cases	Age (year)/sex	Differentiation of squamous cell carcinoma	PERI protein expression		PER3 protein expression	
			Tumour cells	Adjacent non-tumour cells	Tumour cells	Adjacent non-tumour cells
1	66/M	Well	Strong	Weak	Weak	Strong
2	80/F	Well	Strong	Weak	Strong	Strong
3	80/F	Poorly	Strong	Weak	Strong	Strong
4	75/F	Poorly	Strong	Weak	Weak	Strong
5	85/F	Well	Strong	Weak	Weak	Strong
6	85/F	Well	Strong	Weak	Weak	Strong
7	53/M	Well	Strong	Weak	Weak	Strong
8	59/F	Moderately	Strong	Weak	Weak	Strong
9	76/F	Well	Strong	Weak	Weak	Strong
10	62/M	Well	Strong	Weak	Weak	Strong
11	52/M	Well	Strong	Weak	Weak	Strong
12	70/M	Well	Strong	Weak	Strong	Strong
13	68/M	Moderately	Strong	Weak	Weak	Strong

Table 2 – The sequences and product sizes of the primer sets used in RT-PCR.

Gene	Product size (bp)	Primer sequences
PERIOD (PER)1	264	F: 5′-GACATGAGTGGCCCCCTAGA-3′ R: 5′-CAGTGGTCTCCAGCAGGGCTG-3′
PER3	144	F: 5′-CCTATTGATTTCCCACCTTCC-3′ R: 5′-CAACAGCACIGGGTAAGAAGCTC-3′
Bim	166	F: 5′-AGCACCCATGAGTTGTGACAAATC-3′ R: 5′-CGTTAAACTCGTCTCCAATACGC-3′
Bax	487	F: 5′-GTTTCATCCAGGATCGAGCAG-3′ R: 5′-CATCTTCTTCCAGATGGT-3′
BcI-X_L	161	F: 5′-CATGGCAGCAGTAAAGCAAG-3′ R: 5′-GCATTGTTCCCATAGAGTTCC-3′
Bid	365	F: 5′-TGTTCTGACAACAGCTTCCG-3′ R: 5′-CAGCATGGTCTTCTCCTTC-3′
c-Myc	254	F: 5′-GAAGAAATTCGAGCTGCTGC-3′ R: 5′-CACATACAGTCCTGGATGATG-3′
p53	870	F: 5′-ATGGAGGAGCCGCAGTCAGATCCTA-3′ R: 5′-TCAGAGATTCTCTTCCTCTGTGCGC-3′
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	695	F: 5′-CCACCCATGGCAAATTCCATGGCA-3′ R: 5′-AGACCACCTGGTGCTCAGTGTAGC-3′

F: forward primer; R: reverse primer.

2.3. *Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR*

Total RNA was isolated using an RNeasy RNA isolation kit (QIAGEN, Hilden, Germany). First-strand cDNA was synthesised from 1 μg of total RNA using ReverTra Ace (TOYOBO, Osaka, Japan). RT-PCR was performed using an aliquot of first-strand cDNA as a template under standard conditions with *Taq* DNA polymerase (QIAGEN). The PCR products were separated on 1.5% (w/v) agarose gels. The signal intensities were compensated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal controls. The real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems, Tokyo, Japan). The sequences and product sizes of the primer sets are shown in Tables 2 (RT-PCR) and 3 (real-time PCR).

2.4. *Knockdown of PER1 or PER3 by RNA interference*

Short interference RNA (siRNA) against PER1 and PER3 was synthesised by QIAGEN. The sequences of the sense and anti-sense PER1 siRNA were 5′-r (CGCUCGCCCUGGCCAAUAA) d (TT)-3′ and 5′-r (UUAUUGGCCAGGGCGAGCG) d (GG)-3′, respectively. The sequences of the sense and anti-sense PER3 siRNA were 5′-r (GGUGUAUAAUUGGAUUCAA) d (TT)-3′ and 5′-r (UUGAAUCCAAUUAUACACC) d (TT)-3′, respectively. The negative control (scrambled) siRNA sequences were as follows: 5′-r (UUCUCCGAACGUGUCACGU) d (TT)-3′ and 5′-r (ACGUGACACGUUCGGAGAA) d (TT)-3′. For the siRNA transfection experiments, CA9-22 or HGF-1 cells were seeded at 5×10^4 cells per 35-mm well. After 24 h, the siRNA were transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were incubated for 48 h and subjected to various analyses.

2.5. *Overexpression of PER1 or PER3*

The expression plasmids for mouse PER1 pcDNA were described previously.[24] FLAG-PER3 pCS2 was given from Dr. T. Pieler (Georg August Univ). CA9-22 cells were seeded at 1×10^5 cells per 35-mm well. Twenty four hours later, PER1 pcDNA or FLAG-PER3 pCS2 was transfected into the cells using Lipofectamine LTX (Invitrogen). After 4 h of transfection, the medium was changed, and the cells were incubated for an additional 24 h and then subjected to Western blot analysis.

Table 3 – The sequences and product sizes of the primer sets used in real-time PCR.

Gene	Product size (bp)	Primer sequences
PER1	68	F: 5′-CGATGCCAACAGCAATGG-3′ R: 5′-CGCTGAGATGCGCCTCTAG-3′
PER3	75	F: 5′-GCCTTACAAGCTGGTTTGCAA-3′ R: 5′-CTGTGTCTATGGACCGTCCATTT-3′
Differentiated embryo-chondrocyte (DEC1)	76	F: 5′-GAAAGGATCGGCGCAATTAA-3′ R: 5′-CATCATCCGAAAGCTGCATC-3′
DEC2	78	F: 5′-CGCCCATTCAGTCCGACTT-3′ R: 5′-CGGGAGAGGTATTGCAAGACTT-3′
18S rRNA	150	F: 5′-GTAACCCGTTGAACCCCATT-3′ R: 5′-CCATCCAATCGGTAGTAGCG-3′

F: forward primer; R: reverse primer.

2.6. Western blotting

The cells transfected with siRNA or plasmid DNA were lysed using M-PER lysis buffer (Thermo Scientific, Rockford, IL, USA). Their protein concentration was determined using the bicinchoninic acid (BCA) assay. The lysates (5 μg protein for the HGF-1 cells and 15 μg protein for the CA9-22 cells) were subjected to SDS–PAGE and the proteins were transferred to PVDF membranes (Immobilion P, Millipore, Tokyo, Japan). The membranes were incubated with antibodies specific to PER1 (1:1000), which were purchased from Trans Genic Inc., Hyogo, Japan; PARP (1:1000), cleaved caspase-3 (1:500), procaspase-9 (1:2000), Bim (1:10,000), Bok (1:10,000), and Bid (1:10,000), which were purchased from Cell Signaling Technology, MA, USA; Bax (1:1000) and c-Myc (1:10,000), which were purchased from Santa Cruz Biotechnology, CA, USA; p53 (1:1000), which were purchased from Abcam, Cambridge, UK; Bcl-X_L (1:20,000), which were purchased from Trevigen, MD, USA; and actin (1:10,000) (Sigma), followed by horseradish peroxidase-conjugated secondary antibody (IBL, Gunma, Japan). Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO) was used to dilute the primary antibody. The ECL, ECL-plus, or ECL-advance Western Blotting Detection System (Amersham, Uppsala, Sweden) was used for detection.

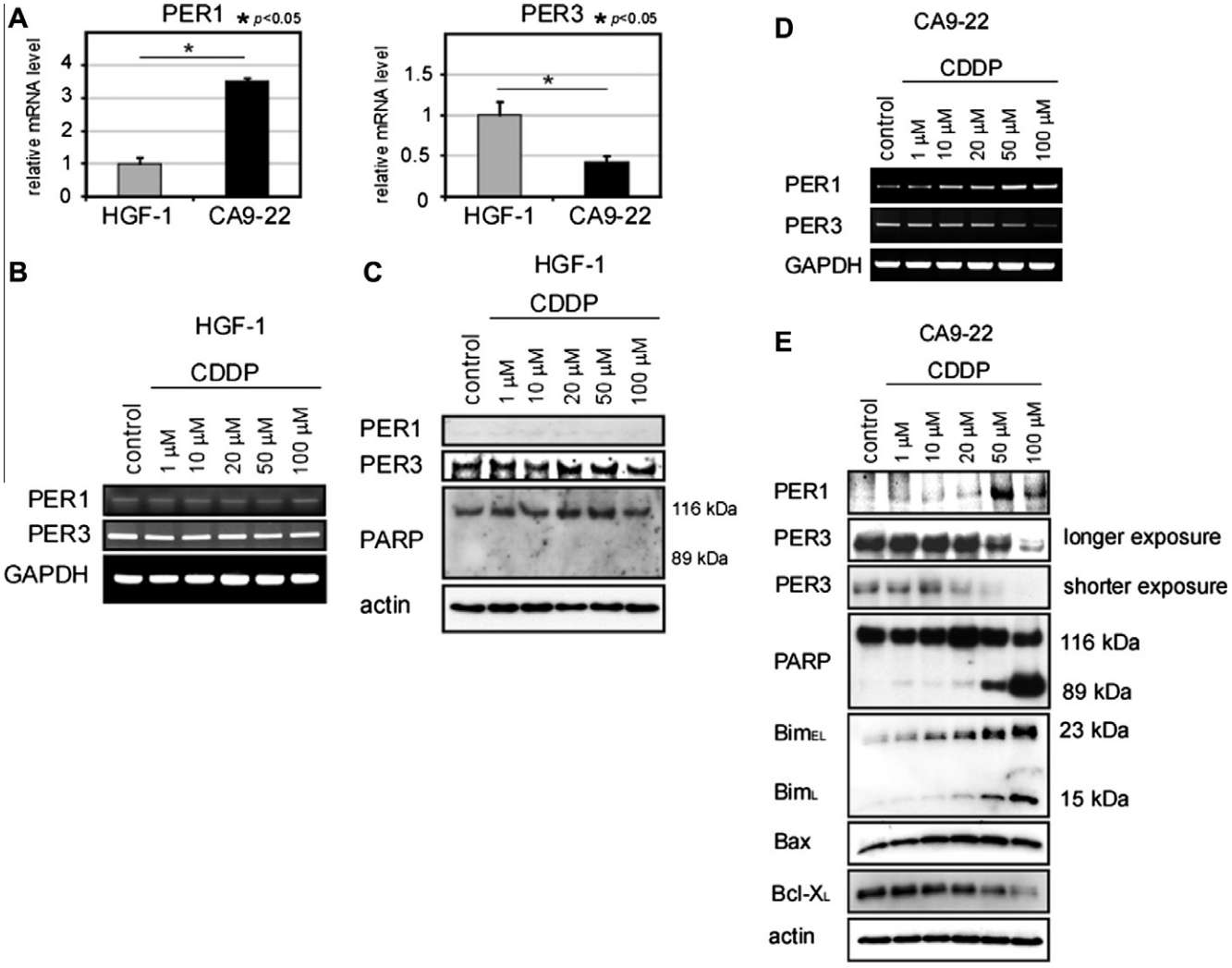

Fig. 1 – PER1 expression was upregulated and PER3 expression was downregulated by *cis*-diamminedichloroplatinum (II) (CDDP) treatment in CA9-22 cells. (A) Human gingival fibroblasts (HGF)-1 and CA9-22 cells were cultured, and total RNA was prepared and subjected to real-time PCR. Each value represents the mean ± SE (*bars*) of three independent experiments $^{*}p < 0.05$, according to the *t*-test. (B) HGF-1 cells were treated with various concentrations of CDDP for 24 h. The control cells were treated with CDDP-diluted buffer phosphate-buffered saline (PBS). Total RNA was prepared and subjected to reverse transcription-polymerase chain reaction (RT-PCR) analyses of PER1, PER3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C) HGF-1 cells were treated with CDDP for 24 h. The cells were lysed, and the lysates were subjected to Western blot analyses of PER1, PER3, poly (ADP-ribose) polymerase (PARP) and actin. (D) CA9-22 cells were treated with CDDP for 24 h. Total RNA was prepared and subjected to RT-PCR analyses of PER1, PER3 and GAPDH. (E) CA9-22 cells were treated with CDDP in a concentration-dependent manner for 24 h. The cells were lysed, and the lysates were subjected to Western blot analyses of PER1, PER3 (longer and shorter exposure), PARP, Bim, Bax, Bcl-X_L and actin. One representative of at least three independent experiments with similar results is shown.

2.7. Immunohistochemistry

The expression of PER1 and PER3 in the gingival cancer cells was examined by immunohistochemistry using serial sections of deparaffinised tissue. Immunoreactivity was detected using the DAKO ENVISION Kit/HRP (DAB) (DakoCytomation, Kyoto, Japan). The sections were pretreated with L.A.B. solution (Polysciences, Eppelheim, Germany) for 6 min for antigen retrieval. This was followed by incubation overnight at 4 °C with anti-PER1 (1:400) or PER3 (1:400) antibodies diluted in Can Get Signal Immunostain Solution (TOYOBO). The sections were then incubated with the HRP-conjugated secondary antibody. Finally, the sections were counterstained with Mayer's haematoxylin.

2.8. Immunofluorescent staining

CA9-22 cells were seeded in a four-chamber slide glass and incubated overnight. The cells were then washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min, before being permeabilised with 0.2% Triton-X-100 in PBS for 10 min. The permeabilised cells were then washed in PBS twice and treated with 5% normal horse serum in PBS for 30 min (to minimise the non-specific adsorption of antibodies), before being incubated with anti-PER1 (1:50) or anti-PER3 (1:50) 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 nuclear staining was performed using 4′,6-diamidino-2-phenylindole (DAPI), Hoechst 33258 or TUNEL. The TUNEL assay was carried out using the click-iT TUNEL Alexa Fluor Imaging Assay kit (Molecular Probes), in accordance with the manufacturer's instructions. The cells were visualised using confocal laser scanning microscopy (Leica, Solms, Germany) and the numbers of cells that were intensely stained with Hoechst 33258 or TUNEL were counted.

2.9. 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. HGF-1 or CA9-22 cells were seeded in 96-well plates. The cells were transfected with control siRNA or siRNA against PER1 or PER3. After 48 h transfection, the cells were treated with 50 or 100 μM of CDDP and incubated for 24 h. Then, the cells were added along with the

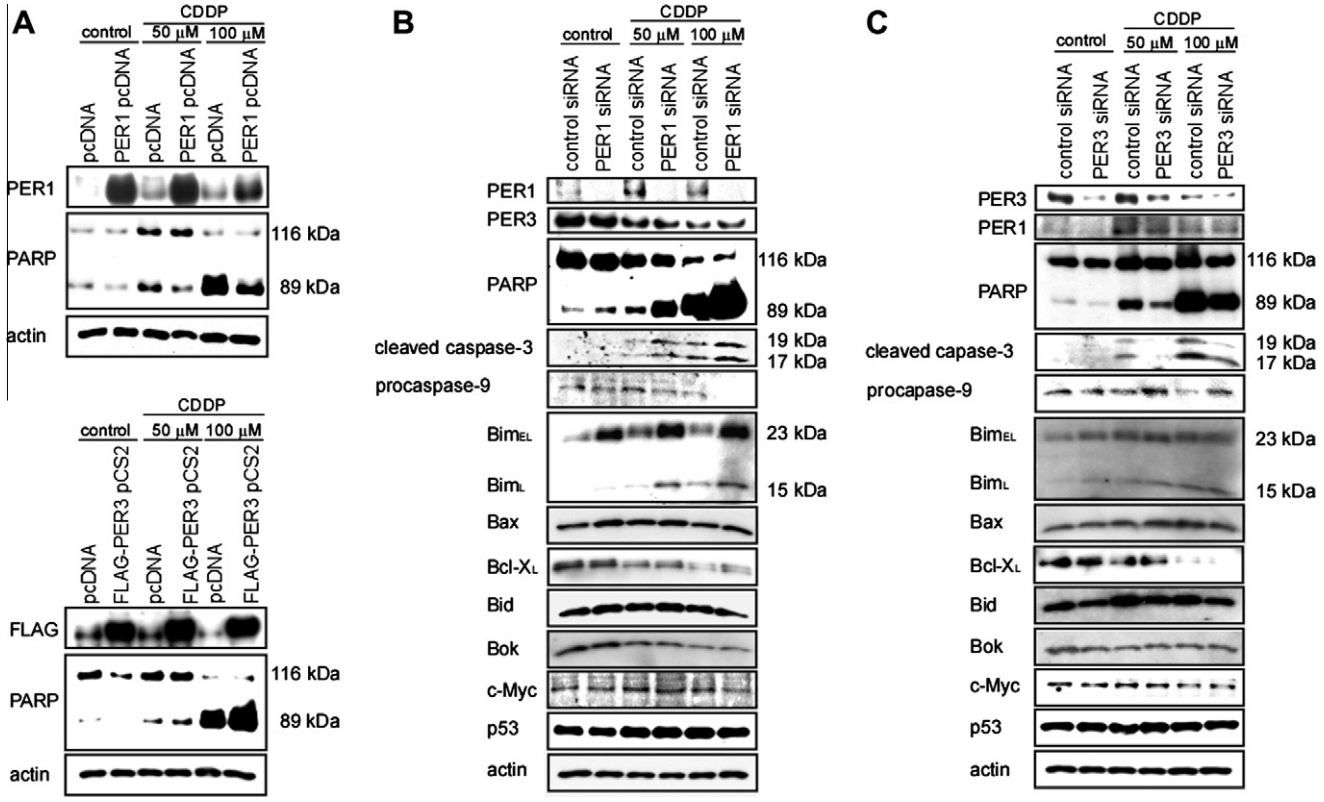

Fig. 2 – Opposing effects of PER1 and PER3 on apoptosis. (A) CA9-22 cells were transfected with an empty vector (pcDNA) or the PER1 or PER3 expression plasmid (PER1 pcDNA or FLAG-PER3 pCS2) and incubated for 4 h. Then, the cells were treated with CDDP (50 or 100 μM) and incubated for an additional 24 h, before being lysed. The lysates were subjected to Western blot analyses of PER1, FLAG, PARP and actin. (B and C) CA9-22 cells were transfected with control short interference RNA (siRNA) or siRNA against PER1 or PER3 and incubated for 48 h. Then, the cells were treated with CDDP (50 or 100 μM) and incubated for 24 h. Cell lysates were prepared from the cells and subjected to Western blot analyses of PER1, PER3, PARP, cleaved caspase-3, procaspase-9, Bim, Bax, Bcl-X_L, Bid, Bok, c-Myc, p53 and actin. One representative of three to five independent experiments with similar results is shown.

Cell Titer 96 AQ_{ueous} One Solution Reagent (Promega Corporation, Medison, WI, USA) to each well and were incubated at 37 °C for an additional 1 h. Absorbance (OD_{490} nm) was measured using a 96-well plate reader.

3. Results

3.1. *CDDP treatment upregulates the expression of PER1, whereas CDDP treatment downregulates the expression of PER3, in CA9-22 cells*

In this study, we used two types of cells to functionally analyse PER1 and PER3, human gingival fibroblasts (HGF-1) and the other is human gingival cancer CA9-22 cells. We investigated the endogenous mRNA expression in these cells by real-time PCR. The expression of PER1 was significantly higher in the CA9-22 cells than in the HGF-1 cells (Fig. 1A). On the other hand, the expression of PER3 was lower in the CA9-22 cells than in the HGF-1 cells.

CDDP is one of the major anti-tumour drugs used for the treatment of oral cancer. We, therefore, investigated whether CDDP treatment affects the expression of PER1 and PER3 in HGF-1 and CA9-22 cells. CDDP treatment had little effect on the expression of PER1 and PER3 in HGF-1 cells (Fig. 1B and C). The amount of cleaved PARP was also not affected by CDDP treatment. However, CDDP treatment upregulated the

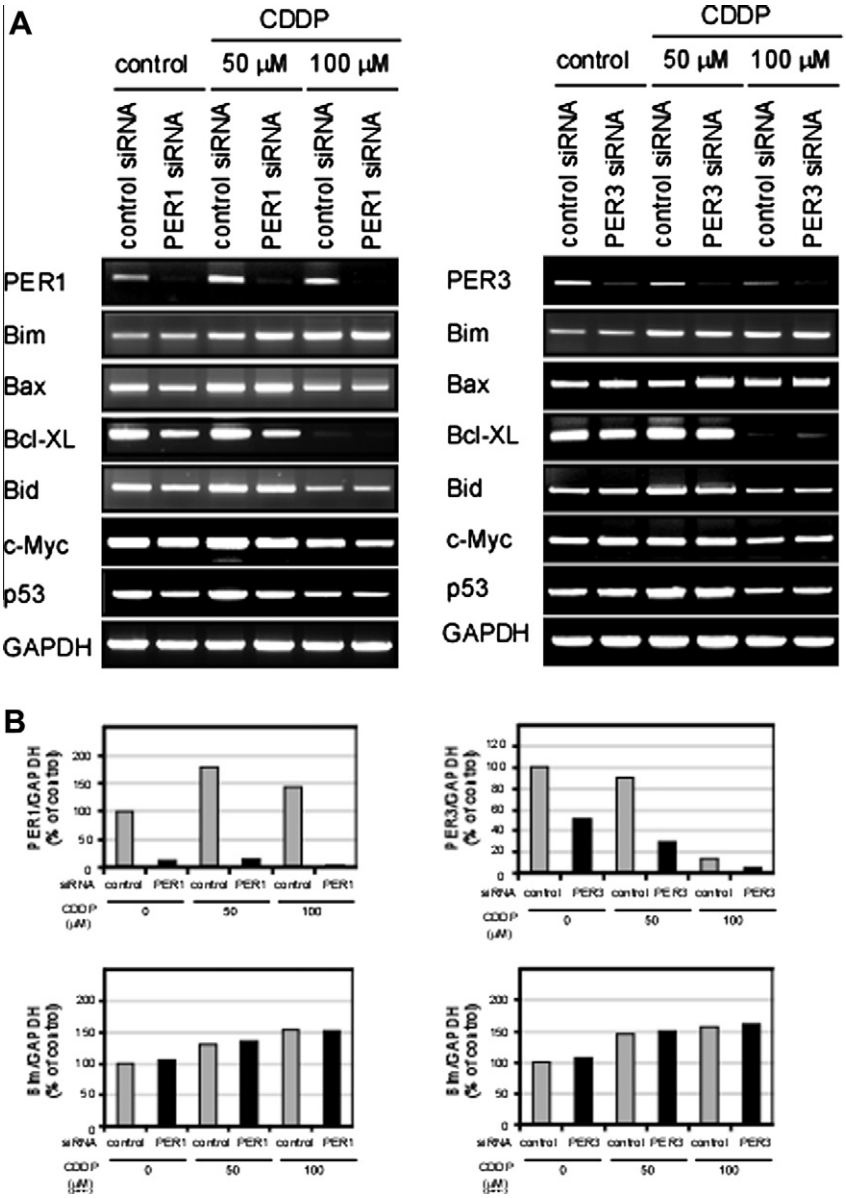

Fig. 3 – Analysis of the mRNA expression of apoptosis-associated genes in PER knockdown cells. (A) CA9-22 cells were transfected with control siRNA or siRNA against PER1 or PER3. After 48 h of transfection, the cells were treated with CDDP (50 or 100 μM) and incubated for 24 h. Total RNA was prepared and subjected to RT-PCR analyses of PER1, PER3, Bim, Bax, Bcl-X_L, Bid, c-Myc, p53 and GAPDH. (B) The intensity of the bands for PER1, PER3 and Bim in control siRNA and PER1 or PER3 siRNA-treated cells with or without CDDP (50 or 100 μM) for 24 h were quantified. One representative of at least three independent experiments with similar results is shown.

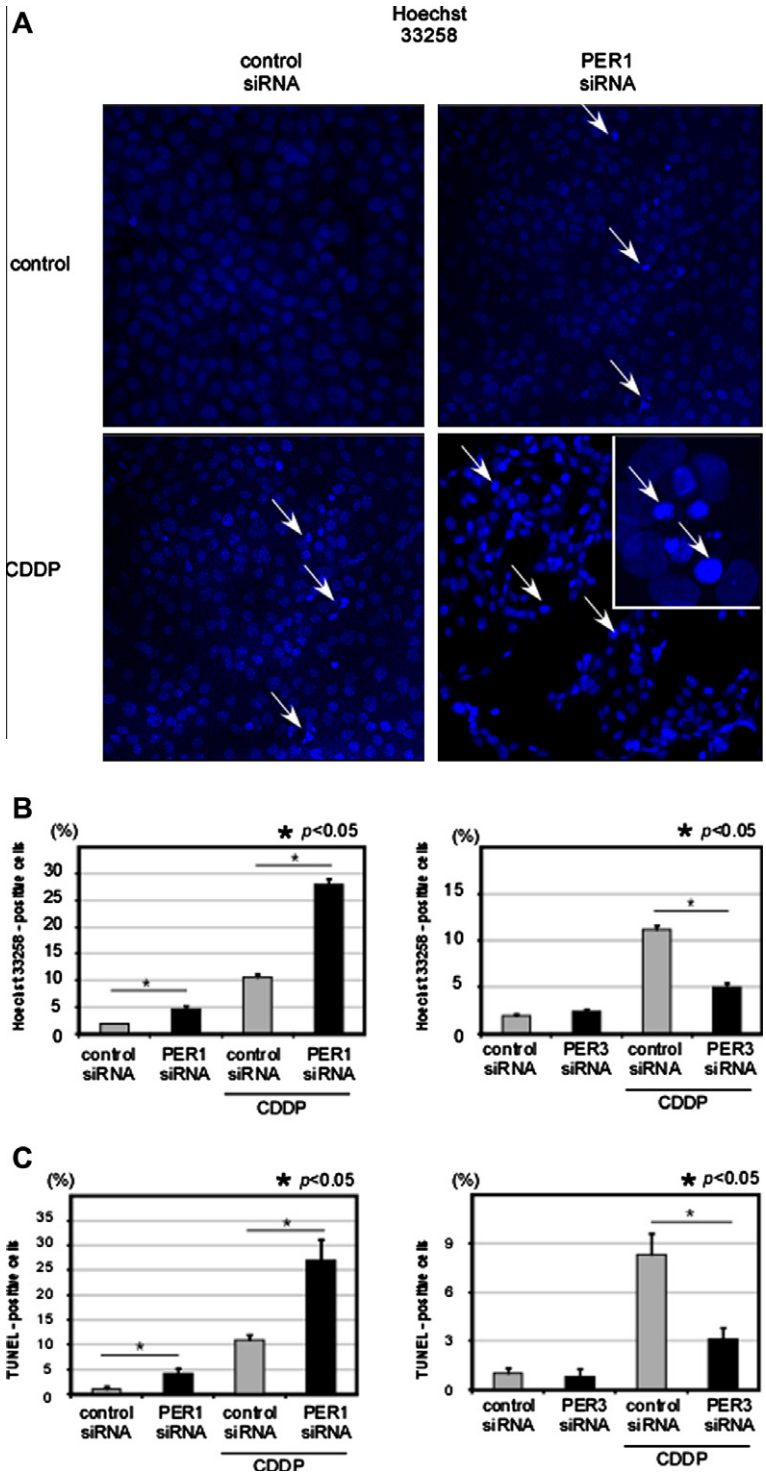

Fig. 4 – PER1 knockdown dramatically increased the apoptosis induced by CDDP treatment. (A) CA9-22 cells were transfected with control siRNA or siRNA against PER1. After 48 h incubation, the cells were treated with or without 50 μM of CDDP for an additional 24 h. The cells were then fixed and stained with Hoechst 33258 (the arrows show nuclear condensation). The inset in the right upper panel shows a higher magnification of the highlighted region. (B) The cells treated with control siRNA or PER1 siRNA or PER3 siRNA in the absence or presence of CDDP and stained with Hoechst 33258. Percentage of cells intensely fragmented and condensed in nuclei with Hoechst 33258 were counted as positive cells. About 100 total cells were counted in individual six random microscopic fields at 40× magnification. Each value represents the mean ± SE (*bars*) of three independent experiments $^{*}p < 0.05$, according to the *t*-test. (C) The cells were treated as described above and stained with TUNEL. The number of cells that were positively stained was counted in six random microscopic fields at 40× magnification. The percentage of positive cells is shown, and each value represents the mean ± SE (*bars*) of three independent experiments $^{*}p < 0.05$, according to the *t*-test.

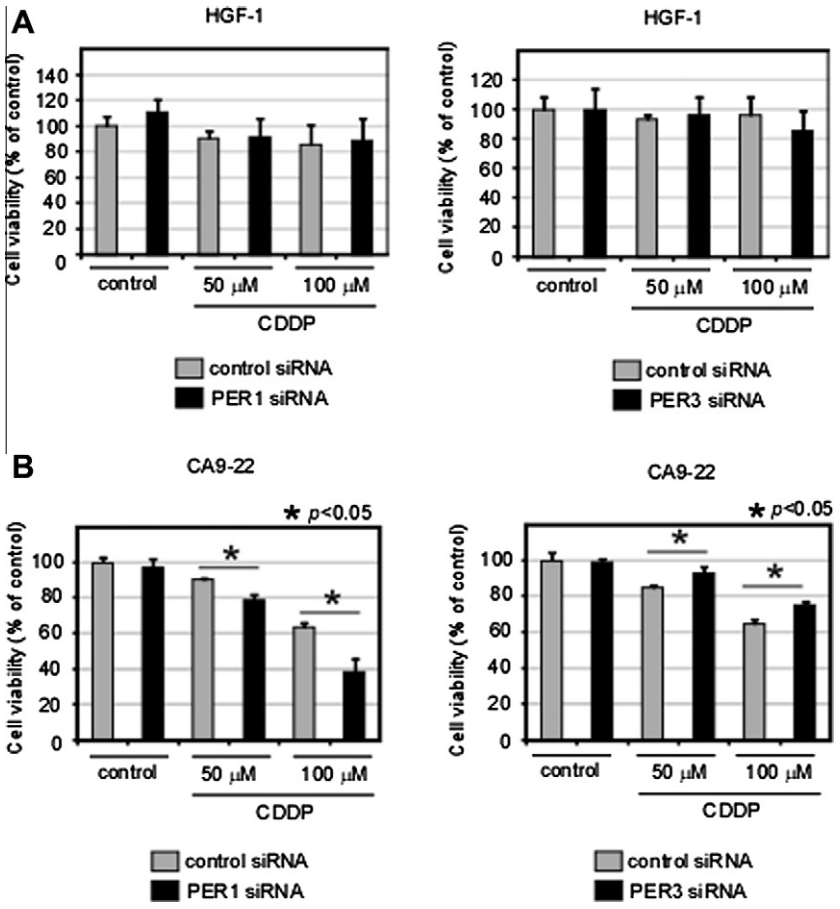

Fig. 5 – Effect of CDDP treatment and knockdown of PER1 or PER3 on cell viability. The cells were treated as described above, and cell viability was measured using an MTS-assay (A: HGF-1 cells and B: CA9-22 cells). The values are shown as a percentage of the control. Each value represents the mean ± SE (*bars*) of three independent experiments (*$p < 0.05$, compared with the control siRNA).

PER1 mRNA and protein expression in CA9-22 cells in a concentration-dependent manner (Fig. 1D and E). On the other hand, the expression of PER3 was reduced by CDDP treatment. The amount of cleaved PARP was increased by treatment with 50 or 100 μM of CDDP for 24 h. The amounts of BimEL and BimL were also increased by CDDP treatment in a concentration dependent-manner, while the expression of Bcl-X_L was decreased. CDDP treatment did not affect the expression of Bax. These findings suggest that CDDP treatment induces apoptosis in CA9-22 but not in HGF-1 cells and that it affects the expression of PER1, PER3, Bim and Bcl-X_L.

3.2. *Overexpression of PER1 and PER3 has opposite effects on apoptosis in CA9-22 cells*

To clarify the biological functions of PER1 and PER3 in gingival cancer cells, we transiently transfected expression vectors for PER1 or PER3 into CA9-22 cells and performed Western blot analyses. PER1 overexpression reduced the cleavage of PARP induced by CDDP treatment, while PER3 overexpression enhanced it (Fig. 2A). These findings suggest that PER1 and PER3 are involved in the regulation of the apoptosis induced by CDDP and play opposing roles in apoptosis.

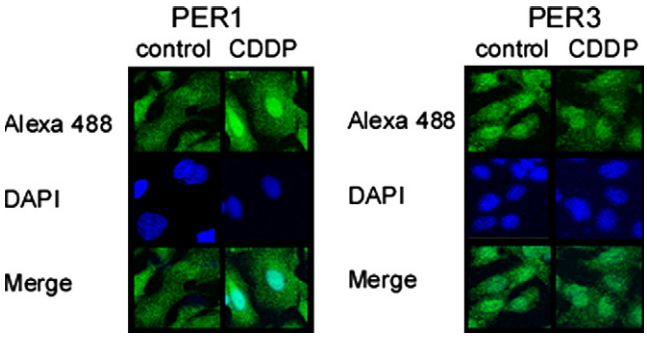

Fig. 6 – Subcellular localisation of PER1 and PER3 in CDDP-treated CA9-22 cells. CA9-22 cells were treated with 100 μM of CDDP for 24 h and fixed. The cells were then incubated with anti-PER1 or PER3 antibody and visualised using Alexa488-conjugated secondary antibody (green). The cells were also counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) to localise the nucleus. One representative result of at least three independent experiments with similar results is shown.

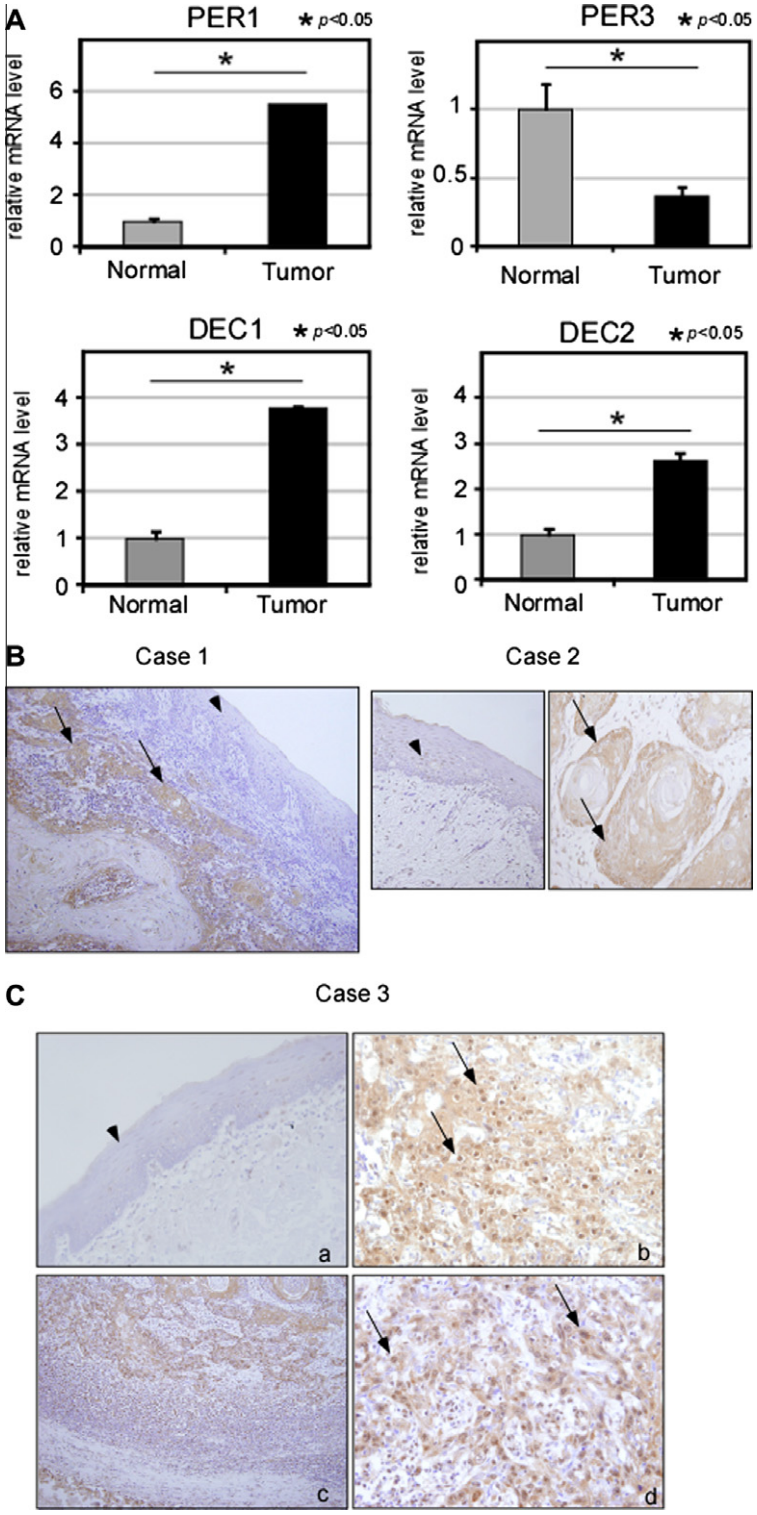

Fig. 7 – PER1 expression is increased in gingival cancer tissue. (A) Total RNA was extracted from gingival cancer and the adjacent non-tumour tissue which was surgically removed from one patient. cDNA was synthesised, and subjected to real-time PCR for PER1, PER3, DEC1 and DEC2. The mRNA expression of PER1, DEC1 and DEC2 was significantly increased in tumour tissues, while PER3 expression was significantly decreased in the tumour tissue. Each value represents the mean ± SE (*bars*) of three independent experiments $^{*}p < 0.05$, according to the *t*-test. (B) The immunohistochemical staining of PER1 was intense in the gingival cancer cells (arrow) from three cases (cases 1 and 2 involved well differentiated gingival cancer, and case 3 involved poorly differentiated gingival cancer; (a) non-tumour tissue, (b) primary region of carcinoma, (c) low magnification of lymph node metastasis, and (d) high magnification of lymph node metastasis), while it was faint in the non-tumour tissue (arrowhead).

3.3. *Effect of the knockdown of PER1 or PER3 on the expression of apoptosis-related proteins in CA9-22 cells*

To further explore the mechanisms by which PERs modulate CDDP-induced apoptosis, we examined whether the transfection of siRNA against PER affected the expression of apoptosis-related factors. CDDP treatment increased the amounts of cleaved caspase-3 and cleaved PARP, whereas it decreased the amount of procaspase-9 (Fig. 2B). PER1 knockdown by siRNA significantly enhanced the CDDP-induced increases of cleaved PARP, BimL and cleaved caspase-3, whereas it decreased the amount of procaspase-9 in the CDDP-treated cells. PER1 knockdown also significantly increased the amount of BimEL without CDDP treatment.

PER1 knockdown had little effect on protein levels of PER3, Bax, Bcl-X_L, Bid, Bok, c-Myc or p53. On the other hand, PER3 knockdown significantly inhibited the CDDP-induced increases in the amounts of cleaved caspase-3 and PARP, whereas it increased the amount of procaspase-9 (Fig. 2C). PER3 knockdown had little effect on protein levels of PER1, Bax, Bcl-X_L, Bid, Bok, c-Myc or p53 or the amounts of BimEL or BimL.

3.4. *PER1 did not affect mRNA expression of Bim in CA9-22 cells*

In order to determine whether PER1 regulates Bim at the transcriptional or post-transcriptional level, we analysed whether PER1 knockdown affects the mRNA expression of Bim using RT-PCR analysis. The results of RT-PCR analyses of PER1, PER3, Bim, Bax, Bcl-X_L, Bid, c-Myc, p53 and GAPDH are shown in Fig. 3A and B. Neither PER1 nor PER3 knockdown affected the expression of Bim. These findings suggest that PER1 regulates the protein level of Bim via a post-transcriptional level.

3.5. *PER1 knockdown induces apoptosis, whereas PER3 knockdown reduces apoptosis in CA9-22 cells*

Nuclear condensation is one of the features of apoptosis. Then, we examined the effect of PER1 or PER3 knockdown and CDDP treatment on nuclear condensation by staining with Hoechst 33258 or TUNEL. Transfection of the cells with PER1 siRNA slightly increased the level of nuclear condensation and significantly increased the nuclear condensation induced by CDDP treatment (Fig. 4A–C). On the other hand, transfection of the cells with PER3 siRNA reduced the amount of nuclear condensation induced by CDDP treatment. There were no differences in nuclear condensation between the control and PER3 siRNA-transfected cells.

We also examined cell viability using the MTS-assay (Fig. 5A and B). In CA9-22 cells, PER1 siRNA and CDDP treatment synergistically reduced the cell viability. However, the viability of HGF-1 cells was not affected by these treatments. On the other hand, PER3 knockdown significantly increased the cell viability of the CDDP-treated cells.

3.6. *Effects of CDDP on the subcellular localisation of PER1 and PER3 in CA9-22 cells*

Using immunofluorescence analysis, we then examined whether CDDP treatment affected the subcellular localisation of PER1 or PER3. As shown in Fig. 6, CDDP treatment increased the amount of PER1 in the nuclei of CA9-22 cells. On the other hand, CDDP treatment did not affect the localisation of PER3.

3.7. *PER1 mRNA and protein expression in tumour tissue and the adjacent non-tumour gingival tissue*

We examined the expression of PER1 and PER3 mRNA in gingival cancer tissues and the adjacent non-tumour tissues by real-time PCR. We also examined the expression of other circadian rhythm-related genes differentiated embryo-chondrocyte (DEC) 1 and DEC2 because DEC1 and DEC2 have been reported to be expressed in various tumours. The expression levels of PER1, DEC1 and DEC2 were significantly increased in the tumour tissue compared with those in non-tumour tissue (Fig. 7A). On the other hand, the PER3 expression in the tumour tissue was decreased compared with that in the non-tumour tissue. We also examined the expression of PER1 and PER3 proteins in gingival cancer tissue by immunohistochemical staining. PER1 was significantly stained in the cancer cells of all 13 cases (arrow) summarised in Table 1, whereas it was faintly stained in stratified squamous epithelium cells (arrowhead), fibroblasts and other non-tumour regions. PER1 was faintly stained only in the nucleus of stratified squamous epithelium cells. On the other hand, it was strongly stained both in nucleus and in cytoplasm of the cancer cells. Photographs of three representative cases are shown in Fig. 7B. On the other hand, PER3 was weakly stained in cytoplasm of the cancer cells of 10 cases, whereas it was strongly stained in cytoplasm of the stratified squamous epithelium cells, fibroblasts and other non-tumour regions.

4. Discussion

It was reported that PER1 is a pro-apoptotic factor in human colon cancer and murine mammary tumour cells.[18,22] On the contrary, it was also reported to be anti-apoptotic in human pancreatic cancer and hepatocellular carcinoma cells.[17] PER1 regulates the expression of molecules involved in apoptosis or the cell cycle,[17,22] and its expression demonstrates a circadian rhythm in some tumour cells.[18,25] However, the effect of PER1 on apoptosis in the presence of anti-tumour drugs is unknown.

Using immunohistochemical analyses, we demonstrated in the present study that PER1 was more strongly expressed in human gingival cancer cells than in adjacent non-tumour tissues. On the contrary, the expression of PER3 was less in tumour cells than in adjacent non-tumour cells. The alternation of the expression of PER1 and PER3 in tumour cells was independent of tumour grade, age or sex. In addition, PER1 was dominantly localised in the nucleus, whereas PER3 was dominantly localised in cytoplasm. These results suggest that PER1 and PER3 are differentially regulated and play opposite roles in development of tumour tissues.

Furthermore, the expression of PER1 protein was upregulated by CDDP treatment in CA9-22, but not in HGF-1 cells and PER1 knockdown enhanced the apoptosis induced by CDDP treatment, and altered the protein expression of apoptosis-related factors, including Bim, the cleaved form of

caspase-3 and pro-caspase-9. These results suggest that PER1 is anti-apoptotic in gingival cancer cells and plays an important role in resistance to CDDP. In addition, PER1 knockdown itself slightly increased apoptosis. Therefore, PER1 may directly regulate apoptosis in gingival cancer cells as well as pancreatic cancer cells, as reported previously.[17] We also found that CDDP treatment increased the expression of PER1 mRNA in CA9-22 cells, whereas it decreased the expression of PER3 mRNA. This suggests that CDDP treatment regulates the transcription of PER1 and PER3 in an opposing manner. Further studies are needed to clarify the details of the mechanism by which CDDP regulates the transcription of PER1 and PER3. In the chemotherapy against oral cancers, 5-fluorouracil or docetaxel is also used. However, there are no reports on the effect of these anti-cancer drugs on PER1 and PER3 expression. This should be clarified by future studies.

Bim is a pro-apoptotic factor related with the pathogenesis of various cancers.[26] However, there are no reports about the role of Bim in gingival cancer. Bim is a p53-independent target of Myc that binds to Bcl-2 or Bcl-X_L.[27,28] The expression of Bim is regulated at both the transcriptional and at post-transcriptional levels.[26,29] Here, we showed for the first time that PER1 regulates the expression of Bim by a post-transcriptional mechanism in tumour cells. Since PER1 knockdown did not affect the expression of Bcl-2, Bcl-X_L or c-Myc, PER1 may selectively regulate the expression of Bim. The mechanisms by which Bim expression is regulated should be clarified in future studies. The inhibition of Bim expression may be involved, at least in part, in the anti-apoptotic properties of PER1.

It was reported that the downregulation of PER3 expression in chronic myeloid leukaemia was correlated with the inactivation of PER3 by methylation.[30] The expression of PER3 was also found to be downregulated in hepatocellular carcinoma cells.[31] However, there has been no information on a relationship between PER3 and apoptosis, and the significance of PER3 in tumour cells is poorly understood. We showed that PER3 is pro-apoptotic in CA9-22 cells. PER3 knockdown decreased the apoptosis induced by CDDP treatment in CA9-22 cells, but we could not elucidate the molecules responsible for the effects of PER3. PER1 knockdown had little effect on the expression of PER3, and PER3 knockdown also had little effect on the PER1 expression. This suggests that PER1 and PER3 expressions are regulated independently. We speculate that PER1 and PER3 work in a compensatory manner. CDDP treatment of CA9-22 cells increased the amount of PER1, but not PER3, in the nucleus. The observed differences between PER1 and PER3 with regard to their roles in the regulation of apoptosis may be related to differences in their subcellular localisation. A similar selective change in nuclear localisation of basic-helix-loop-helix (bHLH) transcription factor DEC1 versus DEC2 was observed in tumour necrosis factor-α treated human breast cancer MCF-7 cells.[32] It was also reported that DEC1 was expressed more in human breast cancer tissue than in the non-tumour tissue.[33] However, there are no reports about the expression of DEC1 in gingival cancer. Therefore, we examined the expression of DEC1 in gingival tumour tissue and found that it was significantly increased in gingival tumour tissue compared with non-tumour tissue. So, DEC1 and DEC2 may also be involved in the pathogenesis of gingival cancer cells.

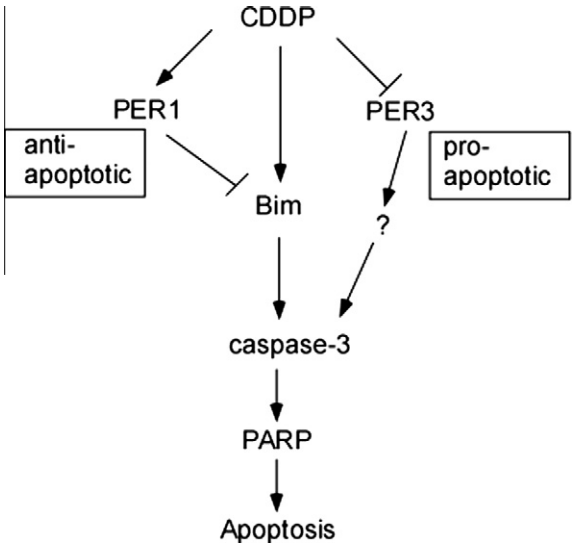

Fig. 8 – Role of PER1 and PER3 in CDDP-induced apoptosis in CA9-22 cells. CDDP treatment upregulates the expression of PER1, whereas it downregulates the expression of PER3. PER1 downregulates the expression of Bim, whereas the downstream factors involved in the pathway mediated by PER3 are unknown.

Clock and brain-muscle-arnt-like-protein 1 (Bmal1) are dominant positive regulators of circadian rhythm. PER negatively regulates the transactivation of Clock/Bmal1.[24] Clock/Bmal1 mutants or knockout mice affected the sensitivity to the anti-tumour drug cyclophosphamide.[34] These findings suggest that clock genes contribute to the regulation of not only circadian rhythm, but also the regulation of apoptosis induced by anti-tumour drugs.

Fig. 8 summarised actions of PER1 and PER3 on apoptosis induced by CDDP treatment in CA9-22 cells. Although the amino acid sequences of PER1 and PER3 share about 40% homology,[16] they have opposite effects on the apoptosis induced by CDDP treatment. Future studies should clarify the roles of PER1 and PER3 in apoptotic reactions and may contribute to the development of a new strategy against gingival cancer resistance to chemotherapy.

Conflict of interest statement

None declared.

Acknowledgements

This study was supported by Grants-in-Aid for Science from the Ministry of Education, Culture Sports, Science, and Technology of Japan; a Grant for Hirosaki University Institutional Research; the Fund for the Promotion of International Scientific Research; the Karoji Memorial Fund for Medical Research; and the Iwadare Scholarship Foundation.

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