Abstract. Histone deacetylase (HDAC) inhibitors are a new class of anticancer agents that act by inhibiting cancer cell proliferation and inducing apoptosis both \textit{in vitro} and \textit{in vivo}. This study examined the anti-tumor effect of apicidin on human endometrial cancer Ishikawa cells in an animal model by inhibiting specific HDAC expression. Nude mice were injected subcutaneously (s.c.) with Ishikawa cells, and the levels of cell proliferation and apoptosis were measured in the tumor tissues after an apicidin treatment. The expression patterns of a specific HDAC class by apicidin were measured in Ishikawa endometrial cancer both \textit{in vitro} and \textit{in vivo}. The tumor volume and weight were measured after the apicidin treatment. Apicidin significantly increased the acetylated histone H3 levels in an Ishikawa cell culture but the levels of HDAC3 and HDAC4 expression were significantly decreased. Apicidin suppressed the tumor growth of transplanted Ishikawa cells, the expression of proliferative cell nuclear antigen (PCNA) and vascular endothelial growth factor (VEGF) in tumor xenograft model, respectively. The inhibitory effect of apicidin on tumor growth was mediated in part through the down-regulation of HDAC3 and HDAC4. We suggest that apicidin is an effective anti-tumor agent on human endometrial cancer cells, and acts by regulating cell proliferation and apoptosis through the down-regulation of HDAC3 and HDAC4.

Introduction

Histone deacetylases (HDACs) play a key role in the epigenetic regulation of genes by catalyzing the removal of acetyl groups, stimulating chromatin condensation and promoting transcriptional repression (1). The loss of expression of tumor suppressors, cell-cycle inhibitors and differentiation factors or apoptosis inducers by histone hypo-acetylation would be advantageous to a cancer cell (2). The overexpression of HDAC enzymes has been observed in many primary human cancer tissues, including stomach, colon, breast, and prostate cancer (3-6). Inhibitors of HDAC enzymes have emerged as new targets for cancer therapy (7-10).

The inhibition of HDAC activity can reverse epigenetic silencing that is commonly observed in cancer, and various HDAC inhibitors have been developed for cancer therapy (11). Several types of HDAC inhibitors with a variety of chemical structures have been identified from natural and synthetic sources. HDAC inhibitors can be divided into five structural classes, such as hydroxamic acid-derived compounds, cyclic peptides, short-chain fatty acids, benzamides and ketones (12,13). Although HDAC inhibitors show a broad spectrum of anti-tumor activities, the concentration of HDAC inhibitor and time course of the experiments were slightly different depending on the cell type both \textit{in vitro} and \textit{in vivo} (14,15).

Apicidin, a cyclic tetrapeptide isolated from \textit{Fusarium} sp., was first reported to be a reversible inhibitor of the \textit{in vitro} development of Apicomplexan parasites. Apicidin acts by inhibiting parasitic (including \textit{Plasmodium} species) HDAC enzyme and was only later shown to have anti-proliferative and cyto-differentiation activity on mammalian cells (16). Apicidin inhibits cell proliferation in several human cancer cell lines, including leukemia, cervical cancer, gastric cancer and breast cancer (17). It was previously reported that apicidin induces the acetylation of histones and has anti-tumor properties on endometrial cancer cells by inducing selectively the genes related to cell cycle arrest and apoptosis \textit{in vitro} (18). However, it is still unclear if apicidin acts selectively against
the various HDAC isoforms in human endometrial cancer cells and the anti-tumor effect on Ishikawa endometrial cancer cells in vivo has not been reported.

This study examined the expression of specific HDAC isoforms and the selective HDAC inhibitory effects of apicidin in Ishikawa cancer cells both in vitro and in vivo. In order to further examine the anti-tumor effect of apicidin on Ishikawa cancer cells, Balb/c nude mice bearing an Ishikawa endometrial cancer xenograft were treated with apicidin, and its effect of tumor growth was monitored. The effect of apicidin on inducing apoptosis and HDAC inhibition was analyzed in vivo.

Materials and methods

Chemicals. Unless stated otherwise, apicidin [cyclo(N-O-methyl-L-tryptophanyl-L-isoleucinyl-D-pippecolinyl-L-2-amino-8-oxodecanoyl)] and all other chemicals were purchased from Sigma (St. Louis, MO, USA). Apicidin was dissolved in sterile dimethyl sulfoxide (DMSO) to generate a 5 mM stock solution, and stored at -80°C. Subsequent dilutions were made in RPMI-1640 (Gibco, Rockville, MD, USA).

Cell culture. The Ishikawa cancer cells were kindly provided by Dr Jacques Simard (CHUL Research Center, Quebec, Canada). The cells were maintained as monolayers at 37°C in DMEM (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) in an atmosphere containing 5% CO2/air. For the apicidin treatment, the cells were plated for 48 h in DMEM containing 10% FBS. The medium was then changed to DMEM containing 5% charcoal-dextran treated FBS (CD-FBS, Gibco) with various concentrations of apicidin.

Cell viability. Cell growth was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) dissolved in phosphate-buffered saline (PBS). The cultures were initiated in 96-well plates at a density of 1x10^4 cells per well. The cells were allowed to attach for 48 h before being exposed to apicidin. At the end of the treatment period, 15 μl of the MTT reagent was added to each well. After 4 h incubation at 37°C, the supernatant was aspirated, and the formazan crystals were dissolved in 100 μl DMSO at 37°C for 10 min with gentle agitation. The absorbance per well was measured at 540 nm using a VERSAmax Microplate Reader (Molecular Devices Corp., USA).

Western blot analysis and antibodies. The cells were then washed twice with PBS, suspended in a lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, phenylmethylsulfonyl fluoride 100 μg/ml, aprotinin 2 μg/ml, pepstatin μg/ml, and leupeptin 10 μg/ml, and placed on ice for 30 min. The suspension was collected after centrifugation at 15,000 x g for 15 min at 4°C. The animal tissues were homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 100 mM phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After incubation for 30 min at 4°C, the homogenate was centrifuged at 16,000 x g for 20 min at 4°C, and the supernatant was collected to determine the protein concentration. The protein concentrations were quantified using a Bio-Rad protein assay reagent (Bio-Rad Laboratories) according to the manufacturer’s protocol. The whole cell lysates and tumor homogenates (50 μg) were resolved on SDS-polyacrylamide gel in an 8-12% gel, transferred to a polyvinylidene difluoride (PVDF) membrane (NEN life Science, Boston, USA), and probed sequentially with the antibodies against acetylated H3 (Upstate, Lake Placid, NY, USA), p21 (BD Biosciences, San Jose, CA, USA), HDAC1 (Cell Signaling, MA, USA), HDAC2, HDAC3, HDAC4, HDAC5, HDAC7 (all from Cell Signaling) and β-actin antibody (Sigma). The blots were developed using an enhanced chemiluminescence (ECL) kit (Amersham, Cardiff, UK).

In vivo tumor xenograft model. Six-week-old female nude mice (BALB-c nu/nu, Charles River Lab. Inc., Wilmington, MA, USA) were housed under controlled temperature (22±2°C) and lighting (12 h light/dark cycle) contains in filtered-air laminar-flow cabinets and manipulated using aseptic procedures. All animal procedures were approved by the Korea Medical Experimental Animal Care Commission (Daejon, Korea), prior to experimentation. Ishikawa cells (2x10^5) in 0.1 ml serum-free medium containing 50% Matrigel were injected subcutaneously (s.c.) into the upper flank of each nude mouse. When the tumor size reached 200 mm^3, mice were randomized to two groups (n=5). Apicidin 5 mg/kg was administered intraperitoneally to the mice daily for 21 days and the control mice were administered 0.1% DMSO in the same manner. The tumor sizes were measured with calipers and their volumes calculated using the standard formula: width^2 x length x 0.52. The body weights were recorded prior to dosing. The mice were sacrificed at the end of the treatment period. The tumor tissue was excised, weighed, frozen in liquid nitrogen, and stored at -80°C until needed. In addition, the tumor tissue was fixed in 4% paraformaldehyde, and

Figure 1. Effect of apicidin on Ishikawa cells viability. The cells were treated with apicidin at various concentrations (0.01-10 μM) for the indicated times. The level of proliferation was measured using an MTT assay. The percentage of viable cells was calculated as the ratio of the treated to control cells. The data are expressed as the mean ± SD of three independent experiments. *P<0.05 as determined by a Student’s t-test.
embedded in paraffin. Paraffin sections (5 μm) were used for histological analysis. The tumor sections were taken for hematoxylin and eosin (H&E) staining and viewed under an optical microscope (magnification, x200, Leica, Wetzler, Germany). The images recorded by a digital camera (Olympus Optical Co. Ltd., Tokyo, Japan).

**Immunohistochemistry.** Paraffin-embedded tissues were used to identify the proliferating cell nuclear antigen (PCNA) and vascular endothelial growth factor (VEGF). Deparaffinization was achieved with xylene followed by a descending series of ethanol concentrations. Antigen retrieval was carried out in a microwave-heated citrate buffer (pH 6.0) for 20 min. The endogenous peroxidases were blocked with 3% H₂O₂/methanol for 15 min at room temperature. The non-specific epitopes were blocked with 1% normal goat serum for 30 min at room temperature. The tumor sections were incubated overnight at 4°C with PCNA (1:200, Lab Vision Corp., CA, USA) and VEGF (1:130, Santa Cruz, CA, USA) antibodies. The immunoreactions were visualized using a streptavidin-biotin complex method followed by a diaminobenzidine reaction (Zymed, USA). The tumor sections were counterstained with hematoxylin in order to visualize the nuclei. The immunoreactions were viewed under an optical microscope (magnification, x400, Leica), and the images recorded on a digital camera (Olympus Optical Co. Ltd.).

**TUNEL assay.** The level of apoptosis was determined using an ApopTag in situ apoptosis detection kit (Chemicon International, Temecula, CA) according to the manufacturer’s protocol. To determine TUNEL expression in tissue sections, the number of apoptotic events was counted in ten random fields by confocal laser scanning microscopy (TCS SP2, Leica) at a magnification, x400 and that number was divided by the total number of cells per field.

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Figure 2. Effect of apicidin on HDAC isoenzymes levels in Ishikawa cells. Ishikawa cells were treated with apicidin at indicated concentrations for 48 h. Total cell lysates were prepared and the protein was subjected to SDS-PAGE followed by Western blot analysis and chemiluminescent detection. The control cells were treated with the vehicle alone. Western blot analysis was performed with the HDACs isoenzymes, acetylated H3 and p21 antibodies. The amount of protein was normalized by a comparison to the β-actin levels. Representative bands from three independent experiments are shown.

Figure 3. Effect of apicidin on the growth of Ishikawa endometrial cancer xenograft model. (A) An assessment of the body weight between the treated and control mice during entire experimental period (21 days). The body weight of the mice in different groups was recorded every alternate day. (B) Relative tumor volumes in mice inoculated with Ishikawa cancer cells (n=5). The relative tumor volumes of the mice treated with apicidin and the vehicle treated controls. The tumor volumes were measured and transformed subsequently to the relative tumor volume, as detailed in Materials and methods. (C) In situ appearance of tumors in control and apicidin-treated mice. i) Grafted tumors in the vehicle treated control nude mice, ii) grafted tumors in the vehicle treated control nude mice, iii) the gross size of the tumors from the vehicle treated control group, iv) the gross size of the tumors from the vehicle treated control group. Final tumor volumes of Ishikawa xenograft. The data are reported as mean ± SD of five animals. *P<0.05, **P<0.01 as determined by a Student’s t-test compared to the control group.
Statistical analysis. All the data are represented as the mean ± SD. The significance was determined using a paired Student’s t-test. A *p<0.05 and **p<0.01 were considered statistically significant.

Results

Apicidin inhibits cell proliferation and induces the acetylated histone levels in Ishikawa cancer cells. Previously, it was suggested apicidin inhibits the proliferation of a variety of human cancer cell lines including SK-OV-3 and Ishikawa cells (18,19). In order to confirm apicidin-induced cytotoxicity on endometrial cancer cells, the cells were treated with varying doses of the drug for 24, 48 or 72 h, and the cell viability was measured using an MTT assay. Apicidin inhibited the proliferation of Ishikawa cancer cells in a concentration-dependent manner, and a significant inhibition was observed even at the low concentration (0.5 μM) after 72 h of treatment (Fig. 1). This dose-response curve suggests that the IC50 value of apicidin is 0.41 μM at 72 h. Moreover, the apicidin treatment reduced significantly the levels of the HDAC3 and HDAC4 proteins in Ishikawa cells, particularly HDAC4 (Fig. 2). In particular, apicidin significantly increased the expression level of p21 in a dose-dependent manner (Fig. 2).

Tumor xenograft model. Athymic nude mouse model using tumor xenografts were established to evaluate the anti-tumor potential of apicidin on Ishikawa cancer cells in vivo. No systemic toxicity including the body weight changes, diarrhea, or other apparent adverse effects were observed in the animals throughout the study period (Fig. 3A). The tumors in the
The tumor effect of apicidin was studied through the induction of anti-cancer activity of apicidin. Tumor cell proliferation and blood vessels correlates with the result that the Ishikawa xenograft as shown in Fig. 4C. These results demonstrate that the in vitro effect of apicidin on the induction of apoptosis is associated with the anti-cancer activity of apicidin in vivo.

Expression of acetylated H3 and HDACs in Ishikawa cell xenografts. To confirm the histone acetylation effect of apicidin in vivo, the levels of acetylated H3 expression in Ishikawa cells xenograft tumor tissue were measured by Western blot analysis. As shown in Fig. 6A, the tumors treated with apicidin showed up-regulation of acetylated H3 and p21 expression compared to those in the vehicle-treated controls. The inhibitory effect of apicidin on the specific HDAC isoforms in tumor xenograft was next investigated. The expression of HDAC3 and HDAC4 was lower in the apicidin-treated Ishikawa cells in vitro compared to those in the vehicle-treated controls. The relative expression levels of HDAC3 and HDAC4 were reduced by 53% and 59% compared to those in the vehicle-treated controls, respectively (Fig. 6B). This shows that the inhibitory effect of tumor growth correlates with the induction of HDAC expression in vivo.

Discussion

Endometrial cancer is one of the most common gynecological malignancies and develops in approximately 142,000 women worldwide with an estimated 42,000 deaths (20). There are two major types (I and II) of endometrial cancer with specific features and different changes in a genetic setting (21). HDAC inhibitors are an emerging class of targeted cancer therapeutics, and may have an important impact on the treatment of both types I and II endometrial carcinoma (22-24). Apicidin is a cyclic peptide group of HDAC inhibitors, and accounts for the in vitro high acetylation level of histones, which correlates with an increase in the transcription of various cancer-related genes (25). It was reported previously that an apicidin treatment increased the levels of acetylated histone and induced cell cycle arrest and apoptosis in Ishikawa endometrial cancer cells (18).

Based on the in vitro anticancer effect of apicidin, the anti-tumor effect of apicidin on Ishikawa endometrial cancer cells was evaluated in the tumor xenograft model using nude mice. Apicidin induced 53% inhibition of tumor growth in the Ishikawa cell xenografts compared to the vehicle alone. These results suggest that apicidin not only inhibits Ishikawa cell growth in vitro but also has a profound effect on the growth inhibition of endometrial tumors in vivo. In order to explore the possible mechanisms underlying tumor growth inhibition evidenced in the apicidin treatment, its effects on tumor cell proliferation was first examined by calculating the proliferative index PCNA on the tumors collected at necropsy from all experiments. The tumor sizes correlated well with...
the expression of PCNA-stained nuclei, as observed by immunohistochemistry. Furthermore, the number of PCNA positive cells was significantly lower in the apicidin-treated group than the vehicle group of mice.

Angiogenesis is essential for the development, growth and metastasis of tumors (26). The VEGF stimulates angiogenesis by promoting endothelial cell proliferation, and its expression is regulated by the HDAC activity (27). A previous study reported that the levels of VEGF were elevated significantly in endometrial cancer (28). In the present study, apicidin reduced the levels of VEGF significantly in the apicidin-treated group compared to the vehicle group, which showed increased levels of VEGF. This suggests that apicidin can also induce the anti-angiogenic effects in endometrial cancer development in tumor xenograft model.

Tumor cells deprived of nutrients and survival factors due to the loss of an adequate vasculature may then undergo apoptosis (29). Our previous study revealed that apicidin can induce apoptosis in endometrial cancer cells (18). Therefore, a TUNEL assay was performed to determine if apicidin induce apoptosis in endometrial cancer cells (18). A significantly higher percentage of TUNEL-positive cells were observed in the apicidin treated tumors than in the controls. These results suggest that the induction of apoptosis by apicidin may also be a mechanism for the suppression of Ishikawa xenograft model.

HDAC inhibitors have attracted considerable interest as drug targets in recent years, with several compounds of this class currently in clinical testing (30,31). Despite the broad applications of HDAC inhibitors in cell culture, animal models, and early phase clinical trials, surprisingly little is known about the expression of their targets in cancer tissues (32). The expression of the class I HDACs have been studied in endometrial cancer patients. Each of the class I HDACs was expressed at high levels (HDAC1, 61%; HDAC2, 95%; HDAC3, 83%) in most endometrial carcinomas samples (23). However, the expression of the other class of HDACs in endometrial carcinomas is largely unknown. Our results showed that the levels of HDAC1, 2, 3 and 4 were expressed strongly in Ishikawa cells, whereas HDAC5, 6 and 7 were expressed weakly.

Most HDAC-inhibitors do not selectively inhibit individual HDAC isoenzymes and an individual HDAC inhibitor might have different effects on different biological systems because there are specific differences in HDAC expression according to the tissue- and cell-type localization and targets (33). This showed that the regulation of specific HDAC inhibition by apicidin may also vary according to the cell type and experimental condition. In this study, the inhibition of specific HDAC subtypes to apicidin was evaluated using an Ishikawa cancer cell xenograft model. The administration of apicidin resulted in the repression of HDAC3 and HDAC4, which was observed in apicidin-treated Ishikawa cells in vitro. HDAC3 expression was reported to be associated with a poor prognosis in a large series of gastric, colorectal and prostate cancer samples (34-36). HDAC3 was up-regulated in colon tumors, and the silencing of HDAC3 expression in colon cancer cell lines resulted in growth inhibition, a decrease in cell survival, and increased apoptosis (37). HDAC4 expression was upregulated in breast cancer samples compared to renal, bladder and colorectal cancer (38). HDAC4 (and HDAC6) were reported to bind to and regulate HIF-1α transcriptional activity in renal carcinoma cells. Therefore, targeting of these HDACs may be a means of tumor anti-angiogenesis (39).

Down-regulating HDAC4 expression by small interfering RNA (siRNA) in HCT116 colon cancer cells induced the inhibition of cell growth and induction of apoptosis in vitro, reduced xenograft tumor growth, and increased p21 transcription (40). These studies provide evidence for the potential role of HDAC3 and HDAC4, and suggest that the repression of HDAC3 and HDAC4 by apicidin might be responsible for its anticancer effect both in vitro and in vivo. Further studies on the individual function of HDAC3 and HDAC4 in mediating the components of apicidin response will be needed to determine the inhibitory mechanism of HDAC inhibitors in endometrial cancer.

In conclusion, apicidin showed anti-tumor activity in vivo, leading to the inhibition of cell proliferation and angiogenesis, and the induction of apoptosis in endometrial cancer. The inhibitory effect of apicidin on tumor growth was mediated in part by the up-regulation of acetylated H3 and p21, and the down-regulation of HDAC3 and HDAC4. These results suggest that apicidin is a potential anti-tumor agent against human endometrial cancer.

References


