Histone deacetylases 1, 6 and 8 are critical for invasion in breast cancer

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Abstract. Histone deacetylases (HDACs) are associated with the development and progression of cancer, but it is not known which of the HDAC isoforms play important roles in breast cancer metastasis. This study identified the specific HDAC isoforms that are necessary for invasion and/or migration in human breast cancer cell lines. MDA-MB-231 cells were significantly more invasive and expressed higher levels of matrix metalloproteinase-9 (MMP-9) compared to MCF-7 cells. We compared the expression of HDAC isoforms between MCF-7 and MDA-MB-231 cells and found greater expression of HDAC4, 6 and 8 in MDA-MB-231 cells by RT-PCR and Western blot analyses. In addition, apicidin, a histone deacetylase inhibitor, was shown to attenuate the invasion, migration and MMP-9 expression in MDA-MB-231 cells. Using specific siRNAs directed against HDAC1, 4, 6 and 8, we show that inhibition of HDAC1, 6 and 8, but not HDAC4, are responsible for invasion and MMP-9 expression in MDA-MB-231 cells. We analyzed the invasiveness of MCF-7 cells overexpressing HDAC1, 4, 6 or 8 and found that overexpression of HDAC1, 6 or 8 increased invasion and MMP-9 expression. By developing HDAC isoforms as potential biomarkers for breast cancer metastasis, the present study can be extended to developing therapies for breast cancer invasion.

Introduction

Histone deacetylases (HDACs) are emerging as critical regulators of cell growth, differentiation and apoptosis. HDAC activity in human tumors leads to conformational changes within the nucleosome, which results in the transcriptional repression of genes during migration and metastasis (1). Changes in HDAC expression levels have been associated with clinical prognosis in patients with invasive cancer, including breast cancer (2,3). HDAC1 (4-6), HDAC2 (7,8) and HDAC3 (5) have been found to be expressed at higher levels in cancer tissues. Proliferation of lung, colon and cervical cancer cell lines is reduced after HDAC8 knockdown (9), and HDAC6 mediates estrogen-induced increase in cell motility (10). However, the molecular mechanisms underlying the HDAC isoform functions during invasion remain unclear.

Cancer invasion and metastasis are the leading causes of mortality in patients with breast cancer. Tumor metastasis consists of cell adhesion, invasion and angiogenesis (11). Matrix metalloproteinases (MMPs) have been recognized as important mediators of extracellular matrix (ECM) degradation (12). MMPs and MMP inhibitors have been extensively investigated in human breast cancer clinical studies (13,14). It has been reported that MMP-2 has a role in the H-ras-induced invasive phenotype of MCF10A human breast epithelial cells (15), analogous to the role of MMP-9 expression in the metastatic phenotype of transformed rat embryo cells (16).

This study was aimed at determining the role of HDAC-dependent invasion and migration in breast cancer and examining the HDAC-dependent regulation of MMP-9 expression in breast cancer cell migration and invasion.

Materials and methods

Cell culture. Human breast cancer MDA-MB-231 and MCF-7 cells were obtained from ATCC (Manassas, VA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and incubated in a humidified atmosphere containing 5% CO2 at 37°C.

Reagents. Apicidin was obtained from Sigma (St. Louis, MO). Antibodies against HDAC1 and 8 were purchased from Millipore (Billerica, MA). Antibodies against HDAC2, 3 and 4 were obtained from Abcam (Cambridge, MA). Antibodies against HDAC6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Reverse transcription-PCR. Total cellular RNA (1 μg) isolated from cultured cell lines was used for reverse transcription. The
cDNA was then amplified with primer sets for HDAC1, 2, 3, 4, 6, 8, MMP-9, 2 and GAPDH. Primer sequences are as follows: HDAC1 forward, 5'-gAAAtctAtcgccctcAcA-3' and reverse, 5'-ttgcc AcAgAAccAccAgtA-3'; HDAC2 forward, 5'-A tAAA gcc Actgccg AA gAA-3' and reverse, 5'- tcctcc Agccc AAttAAcAg-3'; HDAC3 forward, 5'-A cgtg g gc A Act tcc Act Ac-3' and reverse, 5'- gActcttggtg AAgccttgc -3'; HDAC4 forward, 5'-A g cgtg Ag cA AgAtcctc At-3' and reverse, 5'- gcc AAgtActc Agcgtctcc -3'; HDAC6 forward, 5'- tAtctg cccc Agt Acct tcg -3' and reverse, 5'- ggAcAtccc AAtcc AcAAtc-3'; HDAC8 forward, 5'- ggtg Acgtgtctg Atgt tgg -3' and reverse, 5'- gAcActtgcc AAttccc Act-3'; GAPDH forward, 5'- cAt c t t c cAg gAg c gAgA-3' and reverse, 5'- ctgcttc AccAccttcttg At-3'; MMP-9 forward, 5'- cAtcgtc AtccAgtttgg -3' and reverse, 5'-gAtggAttggccttgg AA-3' and MMP-2 forward, 5'-ggcctcctcctg AcAttgAcctt-3' and reverse, 5'-ggcctcgtAtAccgcAtcAAtc-3'.

Western blotting. Cells were plated in 6-well plates and treated with apicidin for 24 h. After treatment, cells were harvested and washed twice with PBS at 4°C. Total proteins were prepared by cell lysis in 200 µl of ice-cold RIPA buffer, and lysates were resolved by 10% or 12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with blocking buffer containing 5% non-fat dry milk for 1 h at room temperature and incubated overnight at 4°C with the antibodies indicated. After washing for 1 h with TBS containing 0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat antibodies for 1 h. After washing three times with TBS containing 0.1% Tween-20, the immunoreactive bands were visualized using ECL.

Small interfering RNA (siRNA) and plasmid transfection. Specific siRNAs for HDAC1, 6, 8 (sc-29343, sc-35544, sc-35548, Santa Cruz Biotechnology) and HDAC4 (J-003497-07-0005, Dharmacon, Lafayette, CO) or a scrambled siRNA (Invitrogen, Carlsbad, CA) were obtained. HDAC1 and HDAC4 constructs were kindly provided by Dr S.L. Schreiber (Howard Hughes Medical Institute, Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA) and Dr E. Seto (Moffitt Cancer Center, University of South Florida, Tampa, FL), respectively. HDAC6 and HDAC8 constructs were purchased from Addgene (Cambridge, MA). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Invasion assay. Invasion assays were performed in triplicate, using a 48-well microchemotaxis chamber with 8-µm-pore membranes (Neuroprobe) pre-coated with 10 µg/ml Matrigel (BD Bioscience). The bottom chamber was filled with 1% FBS/RPMI (MDA-MB-231) or 20% FBS/RPMI (MCF-7), and then cells that had been treated with apicidin for 24 h were placed in the upper chamber and incubated at 37°C for 24 h. The membranes were fixed and stained with Diff-Quik reagents (Dade Behring, Inc., Deerfield, IL).

Results

Apicidin inhibits invasion and migration in breast cancer cells. We first compared the expression of HDAC isoforms in two cell lines, MDA-MB-231 (a highly metastatic breast cancer cell line) and MCF-7 (a less invasive breast cancer cell line). HDAC1 and HDAC2 expression patterns were similar in both cell lines. However, the expression of HDAC4, 6 and 8 was higher in MDA-MB-231 cells compared to MCF-7 cells (Fig. 1).

We next examined whether apicidin, an inhibitor of histone deacetylases, inhibited invasion and migration of MDA-MB-231 cells. As shown in Fig. 2A and B, MDA-MB-231 cells exhibited greater invasion and migration than MCF-7 cells (Fig. 1).

Apicidin treatment of MDA-MB-231 cells inhibited invasion and migration in a dose-dependent manner.

Apicidin has been reported to inhibit MMP-2 activity more than MMP-9 in human breast epithelial cells (17). To test the effect of apicidin on MMP activity, we treated cells with increasing amounts of apicidin and observed mRNA expression using RT-PCR. Apicidin inhibited MMP-9 expression in MDA-MB-231 cells (Fig. 2C) but did not inhibit MMP-2 activity (data not shown).
Specific HDACs involved in invasion and MMP-9 expression.

The comparison of HDAC expression between MDA-MB-231 and MCF-7 cells indicated that high expression of HDAC4, 6 and 8 was significantly associated with invasion. In addition, HDAC1 has been previously demonstrated to be associated with invasion (18). Based on these criteria, HDAC1, 4, 6 and 8 were selected for further study. We used specific HDAC siRNA to determine which HDACs were involved in invasion. Treatment of MDA-MB-231 cells with HDAC1, 6 and 8 siRNAs inhibited invasion (Fig. 3A). Each specific siRNA caused a marked decrease in specific mRNA levels without affecting the mRNA levels of the other HDACs. The knockdown of HDAC1, 6 and 8 caused decrease of MMP-9 mRNA expression (Fig. 3B). However, HDAC4 knockdown did not affect invasion or MMP-9 expression.

Discussion

In this study, we examined whether HDAC isoform status plays a role in the differential responses of MCF-7 and MDA-MB-231 cells. Many reports have correlated HDAC expression levels with clinical prognosis in patients with invasive cancers, including breast cancer (2,3,6,10,19). This study is the first to investigate the expression of various HDAC isoforms. It has been proposed that HDAC expression leads to increased MMP expression and cell invasion. Apicidin prevents an H-ras-induced invasive phenotype (17). Our data suggest that treating
MDA-MB-231 cells with apicidin inhibits invasion, migration and MMP-9 expression in a dose-dependent manner.

HER-2/neu inhibits the metastasis suppressor RECK via an Sp1- and HDAC1-dependent mechanism, to promote cell invasion (20). In addition, HDAC1 has been found to be involved in the migration and invasion of cancer cells (18). In this study, HDAC1 expression was similar in both MDA-MB-231 and MCF-7 cell lines but was also found to be important for invasion. Cell lines representative of ER-positive (MCF-7) and ER-negative (MDA-MB-231) human breast cancer cells can both differentiate to invasion. An ER-negative and invasive human breast cancer cell line, MDA-MB-231, has constitutively higher mitogen-activated protein kinase (ERK1&2/MAPK) when compared to the ER-positive and non-invasive cell lines representative of ER-positive (MCF-7) and ER-negative human breast cancer cell lines. Our findings implicate HDAC1, 6, and 8 in the progression of breast cancer cells.

Our data suggest that inhibitor and siRNA of HDACs abolished breast cancer invasion by regulating the expression of factors involved in the degradation of the ECM. MMP-2, 9, 11, 13 and 14 are known to enhance the invasiveness of MDA-MB-231 cells (25,26). HDACs are known to regulate the expression of MMPs (27,28). Therefore, the identification of cancer-related HDAC isoforms has therapeutic interest in oncology for more specific HDAC inhibitors with fewer side effects for patients.

In conclusion, we have examined the functional role of HDACs in invasion according to their expression in MCF-7 and MDA-MB-231 cells. Our findings implicate HDAC1, 6 and 8 in the progression of breast cancer cells.

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