Inhibition of Histone Deacetylase Activity Promotes Invasion of Human Cancer Cells through Activation of Urokinase Plasminogen Activator*

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Histone acetylation plays an important role in chromatin remodeling and gene expression. The molecular mechanisms involved in differential regulation of urokinase plasminogen activator (uPA) gene expression are not fully understood. In this study, we investigated whether histone deacetylation was involved in repression of uPA expression in human cancer cells. Induction of uPA expression by histone deacetylase (HDAC) inhibitors trichostatin A (TSA), sodium butyrate, and scriptaid was observed in all three different types of human cancer cells examined. Chromatin immunoprecipitation assays showed that the induction of uPA expression by TSA was accompanied by a remarkable increase of acetylation of histones H3 and H4, which are associated with the uPA promoter region in human cancer cells. These results were further substantiated by the findings of a restriction enzyme accessibility assay and TSA-stimulated uPA promoter activity through the inhibition of HDAC activity. In vitro Matrigel invasion assays showed that induction of uPA expression by HDAC inhibitors in human cancer cells resulted in a significant increase of cancer cell invasion. Furthermore, HDAC1 knockdown by small interference RNA stimulated uPA expression and cancer cell invasion. In conclusion, this study demonstrates the important role of histone modifications in regulating uPA gene expression and raises a possibility that the use of HDAC inhibitors in patients as cancer therapy may paradoxically establish metastasis through up-regulation or reactivation of uPA.

Tumor invasion and metastasis are the major characteristics of aggressive phenotypes of various human cancers, and therefore, the major causes of cancer deaths (1). Cancer cells must acquire several properties to disseminate from the primary tumor, including the ability to degrade and migrate through the extracellular matrix, a process called invasion (2, 3). Invasion is one of the first steps in the metastatic cascade and is a strong indicator of tumor progression. Tumor invasion and metastasis are often associated with increased expression of extracellular matrix-degrading proteases, among which urokinase plasminogen activator (uPA) is of central importance (4, 5). Mounting evidence from laboratories suggests a role for uPA in the invasion of cancer cells as well as the risk for a relapse in cancer patients (6–10).

Tumor invasion is mediated by uPA through the conversion of plasminogen to plasmin, which degrades basement membranes (11, 12). Additionally, binding of uPA with its receptor uPA receptor activates the Ras/extracellular signal-regulated kinase pathway, which in turn, leads to cell proliferation, migration, and invasion (13). Several studies using uPA inhibitors (9) or uPA gene-silencing approaches (14, 15) have confirmed the important role of uPA in the processes of tumor invasion and metastasis. Because uPA is crucial for invasion and metastasis, we are interested in understanding how its transcriptional activity is regulated by epigenetic mechanisms in human cancer cells.

Epigenetic mechanisms play crucial roles in the regulation of gene expression by affecting chromatin accessibility. DNA methylation and histone modifications are two important epigenetic mediators of transcriptional repression (16, 17). A previous study showed that repression of uPA gene expression in breast cancer cells is associated with methylation of its promoter (18). This study further showed that the repression of uPA in prostate cancer cells is due in part to the presence of methylated cytosines throughout its promoter (19). We recently showed that uPA expression is triggered by promoter demethylation in prostate carcinomas and in metastatic prostate cells (20). However, the functional relevance of histone modifications in the regulation of the uPA gene expression is unknown.

An increasing body of evidence indicates that changes in chromatin structure by histone modification appear to play an important role in the regulation of gene transcription. Acetylation of core histone unpacks the condensed chromatin and renders the target DNA accessible to transcriptional machinery, hence contributing to gene expression (21). In contrast,

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‡ The abbreviations used are: uPA, urokinase plasminogen activator; HDAC, histone deacetylase; HDAI, histone deacetylase inhibitor; TSA, trichostatin A; NaB, sodium butyrate; SCR, scriptaid; 5-aza, 5-aza-2'-deoxycytidine; siRNA, small interfering RNA; shRNA, small hairpin RNA; RNAi, RNA interference; ChIP, chromatin immunoprecipitation; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CMV, cytomegalovirus.
deacetylation of core histones increases chromatin condensation and prevents the binding between DNA and transcriptional factors, which lead to transcriptional silence (22, 23). Histone acetyl transferases and histone deacetylases (HDACs) regulate the acetylation of histones and interact with components of the transcription machinery (24, 25). Several studies have shown that the inhibition of HDACs can induce gene expression in non-expressing cells (26–29).

In this study, we examined human uPA mRNA, uPA promoter activity, and acetylation of histones associated with uPA in human cancer cells treated with inhibitors of HDACs. We found that HDAC inhibitors induce uPA expression and activity in human cancer cells, resulting in enhanced cancer cell invasion. Our results show that histone deacetylation plays a central role in the transcriptional regulation of the uPA gene in cancer cells and that use of HDAC inhibitors results in the epigenetic activation of uPA.

EXPERIMENTAL PROCEDURES

Reagents—TSA, SCR, and 5-aza-2’-deoxycytidine (5-aza) were purchased from Sigma. TSA and SCR were dissolved in Me2SO; 5-aza was dissolved in phosphate-buffered saline. Sodium butyrate (NaB) solution was purchased from the Upstate Group, Inc. (Lake Placid, NY).

Cell Lines and Culture Conditions—Human neuroblastoma cells (SK-N-BE and SK-N-AS) and human prostate cancer cells (LNCaP and PC3) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SF-3061 human meningioma cells were provided by Dr. Anita Lal (University of California, San Francisco, CA). LNCaP cells were cultured in RPMI medium supplemented with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate (Invitrogen). PC3, SF-3061, SK-N-BE, and SK-N-AS cells were cultured in advanced Dulbecco’s modified Eagle’s medium. Both media contained 10% fetal bovine serum (GIBCO Invitrogen) and 5% penicillin/streptomycin. Cells were maintained in a 37 °C incubator with a 5% CO2 humidified atmosphere.

Drug Treatments—Cells were seeded at a density of 1 × 10^6 cells/100-mm dish and allowed to attach over 24 h. To reactivate uPA, we carried out HDAC inhibition treatment by adding 100 nM trichostatin A to the culture medium for 8 h or by treating cells for 12 h in medium supplemented with 1 mM NaB or 2 μM SCR. We carried out demethylating treatments using 5-aza (0–25 μM) for 5 days, replacing the drug and medium 24 h after the beginning of the treatment. For the synergetic study, cells were first incubated with 25 μM 5-aza for 72 h at 37 °C, followed by 100 nM TSA for an additional 24 h. The treated cells were washed once with phosphate-buffered saline. Cells were allowed to recover for 24 h in drug-free medium in a 37 °C incubator with a 5% CO2 humidified atmosphere.

RT-PCR Analysis—Cellular RNA was isolated from SK-N-BE, SK-N-AS, SF-3061, and LNCaP cell lines using the Qiagen RNeasy kit. RNA (1 μg) was treated with DNase (10 units/μg of RNA for 1 h) and used as a template for the RT reaction (20 μl). The RT reaction mix (Invitrogen) contained 1 μl (10 pm) of primers. The resultant cDNA was then used in PCR reactions and analyzed by gel electrophoresis. We used the following primers for PCR: uPA-sense, 5’-TGC GTC GTG ACACA-3’, and uPA-antisense, 5’-CTA CAG CGC GCA GCT GTG TG-3’; GAPDH-sense, 5’-GGT AGT CAA CCG ATT TGG TCG TAT-3’, and GAPDH-antisense, 5’-AGC CTT CTC CAT GGT GGT GAA GAC-3’. PCR conditions were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The final extension was at 72 °C for 5 min. All reactions were performed in triplicate. No reverse transcriptase or no template served as the negative controls.

Fibrin Zymography—The enzymatic activity and molecular weight of electrophoretically separated forms of uPA were determined in conditioned medium of human cancer cell lines SK-N-BE, SK-N-AS, SF-3061, and LNCaP by SDS-PAGE as described previously (30). Briefly, the SDS-PAGE gel contains acrylamide to which purified plasminogen and fibrinogen were substrates before polymerization. After polymerization, equal amounts of proteins in the samples were electrophoresed and the gel was washed and stained as described previously (30).

Chromatin Immunoprecipitation Assay—ChIP assays were performed as per the manufacturer’s instructions (catalog no. 17–295, Upstate Biotechnology, Lake Placid, NY). In brief, cells (~1 × 10^6 cells/100 mm dish) were fixed with formaldehyde at a final concentration of 1% and incubating for 10 min at 37 °C. The cells were washed twice with ice-cold phosphate-buffered saline containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A), harvested, and treated with SDS lysis buffer for 10 min on ice. The resulting lysates were sonicated to shear the DNA to fragment lengths below 1000 bp (amplitude 60%, 4 × 10 s, Fisher Sonic Dismembrator 60, Pittsburgh, PA). After pre-clearing the lysates, 4 μg of specific antibodies (anti-acetylated histone H3, anti-acetylated histone H4, anti-HDAC1, anti-HDAC3, and anti-HDAC7, Cell Signaling Technology Inc., Beverly, MA) were used to immunoprecipitate the protein-DNA complexes. Antibody controls were also included for each ChIP assay; no precipitation was observed. The antibody-protein complexes were collected using salmon sperm DNA-protein A-agarose slurry and washed several times as per the manufacturer’s instructions. The immunocomplexes were eluted with 1% SDS and 0.1 mM NaHCO3, and the cross-links were reversed by incubation at 65 °C for 4 h in the presence of 200 mM NaCl. The samples were treated with proteinase K for 1 h, and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The recovered DNA was resuspended in 30 μl of H2O and used as templates for PCR of uPA or β-actin gene promoters. The following primers were used for PCR: uPA promoter-sense, 5’-CAG GTG CAT GGG AGG AAG C-3’, and uPA promoter-antisense, 5’-AGG GGC GCC GGC CCC GCG GCG G-3’; β-actin promoter-sense, 5’-CCA ACG CCA AAA CTC TCC C-3’, and β-actin promoter-antisense, 5’-AGC CAT AAA AGG CAA CTT TCG-3’. Initially, PCR was performed with different numbers of cycles or dilutions of input DNA to determine the linear range of the amplification; all results shown fall within this range. Following 30 cycles of amplification, PCR products were run on 2% agarose gels and analyzed by ethidium bromide staining.

Restriction Enzyme Accessibility Assay—The nuclei of SK-N-BE, SK-N-AS, SF-3061, LNCaP, and PC3 cells were extracted...
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according to published methods (31) and digested with restriction enzymes PvuII or PstI (New England Biolabs, Ipswich, MA). DNA was then extracted from the digested nuclei with the proteinase K/phenol procedure (32). DNA from PvuII-digested nuclei was amplified by PCR with primers uPA-F1 (5′-CAC CGG GAC TGC CCC AG-3′) and uPA-R (5′-GGC CAC CGG GAC TGC CCC AG-3′) and electrophoresed on a 2% agarose gel (Fig. 3A). The absence or presence of a 925-bp fragment indicates that PvuII is or is not accessible to the chromatin in the region of uPA promoter, respectively. To access the input of chromatin, another upstream primer, uPA-F2 (5′-CAC CGG GAC TGC CCC AG-3′) was also mixed in the PCR (Fig. 3A). The amount of PvuII-digested chromatin was represented by the ratio of 925:241 bp. Similarly, DNA from PstI-digested nuclei was also amplified by PCR with primers uPA-F1, uPA-F2, and uPA-R. The absence or presence of a 925-bp product (compared with a control of 241 bp) indicates that PstI is or is not accessible to the chromatin, respectively.

Promoter Activity Assay—A PCR product spanning nucleotide positions −562 to +83 of uPA promoter sequence (GenBank™ accession number X02419) was amplified using LNCaP genomic DNA and subsequently cloned into the pGL3 basic plasmid (Promega, Madison, WI). The uPA-luc construct and control null vector without the uPA promoter insert (pGL3) were transiently transfected into LNCaP cells using the FuGENE HD transfection method (Roche Applied Science) with a β-galactosidase plasmid for normalization. The cells were treated with 100 nM TSA for 8 h following transfection. The cells were harvested 24 h after TSA treatment, and promoter activities were determined using the luciferase assay system as recommended by Promega Corp.

Matrigel Invasion Assay—We used 6.5 mm-diameter Transwell inserts (Costar, Cambridge, MA) with the 8-μm pore membranes coated with Matrigel (BD Biosciences, Bedford, MA) to assess the invasive potentials of human cancer cells before and after treatment with HDAC inhibitors. Cells were detached, washed twice in phosphate-buffered saline, and resuspended in serum-free advanced Dulbecco’s modified Eagle’s medium. A total of 5 × 105 cells in 0.2 ml was placed in the upper chamber of a Transwell, and the lower chamber was filled with 400 µl of advanced Dulbecco’s modified Eagle’s medium/10% fetal bovine serum. After a 24-h incubation period, the cells in the upper chamber that did not migrate were gently scraped away, and adherent cells present on the lower surface of the insert were stained with Hema-3 and photographed. To determine the importance of uPA in HDAI-induced invasion, LNCaP cells stably overexpressing uPA or vector-based shRNA against uPA were used in the Matrigel invasion assay along with each control cell. Cells were detached and subjected to HDAI-induced in vitro Matrigel invasion assay as described above. Construction and characteristics of the uPA shRNA vector have been previously described (20). For stable expression of uPA, LNCaP cells were transfected with the neomycin-selectable pCMV-uPA plasmid or with a control, neomycin-resistant expression vector pCMV. Stable transfection was performed using 5 µg/ml DNA and 10 µl/ml Lipofectin reagent (Invitrogen) following the manufacturer’s protocol. The selection medium containing 1 mg/ml Genetecin (G418, Invitrogen) was added to the cells 72 h after transfection to select for neomycin-resistant transfectants.

Nuclear Extract Preparation and Immunoblot Analysis—Nuclear extracts were prepared from control and TSA-treated SK-N-BE, SK-N-AS, SF-3061, and LNCaP cells using a nuclear extraction kit from Panomics, Inc. (Redwood City, CA) as per the manufacturer’s instructions. Equal amounts of nuclear extracts were resolved by SDS-PAGE and then blotted with rabbit anti-human HDAC1, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, and histone H3 (Cell Signaling Technology, Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) were used for detection of immunoreactive proteins by chemiluminescence (Amerham Biosciences).

Transfection of siRNAs—To silence HDAC1 expression by RNA interference, 150,000 cells per well were seeded in a 6-well plate at least 20 h before transfection. Either small interfering RNAs against HDAC1 or nonspecific control (siControl) were transfected using siLentFect transfection reagent (Bio-Rad) as per the manufacturer’s instructions. Two days post-transfection, the nearly confluent cells were trypsinized, and the cells were used for fibrin zymography, RT-PCR analysis, ChIP assay, and Matrigel invasion assays. The sequences of siRNAs for HDAC1 gene knockdown were as follows: siHDAC1, 5′-TAA GGT TCT CAT ACA GTC G-3′; siHDAC2, 5′-TTT GAA GGT GGA AGA GTT C-3′; and siHDAC3, 5′-TTT AAC AAT AGG GGC ACC TTT C-3′. “Smart pool” siRNAs that combined the above HDAC 1–3 siRNAs targeted against different regions of the HDAC1 mRNA sequence (NM_004964) were used for transfection to increase the knockdown effect.

Densitometry—ImageJ software (National Institutes of Health) was used to quantify the band intensities. Data are represented as relative to the intensity of the indicated loading control.

Statistical Analysis—Statistical comparisons were performed using analysis of variance for analysis of significance between different values using GraphPad Prism software (San Diego, CA). Values are expressed as mean ± S.D. from at least three separate experiments, and differences were considered significant at a p value of <0.05.

RESULTS

Inhibition of Histone Deacetylation Activates uPA Expression—Besides DNA methylation, another epigenetic mechanism by which gene expression can be repressed involves deacetylation of chromosomal histones. Hypoacetylated chromatin is transcriptionally silent (23). Inhibition of histone deacetylation can be accomplished by treatment with HDAIs such as TSA, NaB, and SCR (33). To determine whether HDAI induces acetylation of histones and the concomitant induction of uPA expression, we treated uPA-silenced human cancer cell lines that originated from neuroblastoma (SK-N-BE and SK-N-AS), menigioma (SF-3061), and prostate (LNCaP) with 100 nM TSA for 8 h and performed immunoblot analysis on the nuclear extracts using antibodies to acetylated histones H3 and H4. Accumulation of acetylated histones was observed in TSA-treated human cancer cells (Fig. 1A). If histone deacetylation is associated with transcriptional repression, then histone acetylation following
TSA treatment should lead to uPA expression. According to the RT-PCR results, treatment with TSA induced uPA mRNA expression in all four human cancer cell lines (Fig. 1B, top). Fibrin zymography for uPA expression supported the RT-PCR analyses (Fig. 1B, bottom). A similar trend was observed at both the activity and mRNA levels when these cells were treated with NaB or with SCR (Fig. 1, C and D). These results indicate that the effect of TSA on uPA expression can be extended to other HDAs (i.e. NaB and SCR). They also suggest that the induction of uPA expression and activity by HDAs was not confined to a single type of human cancer cell line model.

Because it is known that uPA can be silenced by promoter DNA methylation (18), we examined the effects of the DNA methylation inhibitor 5-aza on the re-activation of uPA in human cancer cell lines by RT-PCR. However, treatment with higher doses (10–25 μM) of 5-aza for 5 days individually or in combination with TSA did not restore or enhance the expression of uPA in all cell lines analyzed (data not shown).

**TSA Induces Accumulation of Acetylated Histones in Chromatin Associated with the uPA Gene**—Previous studies have shown that TSA, as well as other HDAs, induce the accumulation of acetylated histones in human cells (34–37). ChIP analysis was used to examine the effect of HDAC inhibition on the acetylation of histones H3 and H4, which are associated with the uPA gene promoter. Chromatin fragments from human cancer cells cultured with or without TSA for 8 h were immunoprecipitated with antibodies to acetylated histones H3 or H4. DNA from the immunoprecipitates was isolated, and PCR was performed using uPA promoter primers (Fig. 2A). Acetylation of histones H3 and H4 associated with the uPA promoter region in all four human cancer cell lines was undetectable before TSA treatment. However, we observed remarkable increases in the acetylation of histones H3 and H4 in the promoter region of all four human cancer cell lines after treatment with TSA (100 nM, 8 h) (Fig. 2B). The accumulation of acetylated histones H3 and H4 confirmed that histone deacetylation was involved in the transcriptional repression of uPA. We also carried out PCR on the same set of immunoprecipitated DNA fractions for β-actin promoter as a control. The relative levels of acetylated histones H3 and H4 at β-actin promoter was similar in all TSA-treated and untreated cells.

**TSA Treatment Changes Chromatin Conformation around uPA Promoter**—Chromatin conformation near the uPA promoter was studied by restriction enzyme accessibility assay. The nuclei from uPA non-expressing cell lines SK-N-BE, SK-N-AS, SF-3061, and LNCaP and the uPA-expressing cell line PC3 were digested with the restriction enzyme PvuII. The PvuII site is located (+287) in the vicinity of the transcription initiation site (Fig. 3A). DNA extracted from the nuclei was amplified by PCR and analyzed by electrophoresis. The ratio 925:241 bp represents the amount of undigested chromatin at PvuII site (+287) in the uPA promoter (Fig. 3A). The ratio was significantly decreased in PC3 cells compared with the DNA from undigested nuclei, indicating that the DNA sequence is accessible to the enzyme in the chromatin, and the chromatin is open.
in this cell line. The ratio 925:241 bp was not changed after PvuII digestion in SK-N-BE, SK-N-AS, SF-3061, and LNCaP cells, thereby suggesting that the chromatin is closed (Fig. 3B).

To further confirm the alteration in chromatin structure at the uPA promoter region, the effects of the HDAC inhibitor TSA were examined. SK-N-BE, SK-N-AS, SF-3061, and LNCaP cells were treated with 100 nM TSA for 8 h, after which, the chromatin structure of the uPA promoter was examined by PvuII restriction enzyme accessibility assay. PvuII was accessible to the chromatin after TSA treatment (Fig. 3C). Restriction enzyme PstI was also used to analyze its accessibility at position +326 in TSA-treated SK-N-BE, SK-N-AS, SF-3061, and LNCaP cells. A similar result was observed (data not shown).

**TSA Treatment Activates uPA Promoter Activity**—We found that HDAC inhibitors induced uPA expression in human cancer cells (Fig. 1, B–D). To determine whether the induced uPA expression levels following treatment with inhibitors of HDAC were due to increased uPA transcription, we analyzed uPA promoter activities using an uPA promoter-luciferase reporter construct in control and TSA-treated LNCaP cells. A plasmid construct in control and TSA-treated LNCaP cells. A plasmid containing the uPA promoter linked to a luciferase gene was transfected into LNCaP cells. The cells were treated with 100 nM TSA for 8 h and then lysed, and the cell lysates were used to measure luciferase activity. The uPA promoter exhibited induced activity in the presence of TSA (Fig. 4), suggesting that the stimulation of uPA promoter activity was in fact contributing to induce uPA expression.

**HDAC Inhibitor Up-regulates uPA to Induce Cancer Invasion**

*uPA Is Essential for HDAC Inhibitors to Stimulate Cancer Cell Invasion*—Previous studies by our group (5, 14, 30) and others (9, 15) established that uPA expression is closely associated with the invasive properties of tumor cells. To determine whether HDACI-induced uPA functionally contributes to the metastatic activity, we examined invasive activity in vitro. We found that SK-N-BE, SK-N-AS, SF-3061, and LNCaP cells treated with HDACIs displayed significant invasion into the Matrigel substrate when compared with untreated counterparts (Fig. 5A). If uPA is important to HDACI-induced invasion, knocking down uPA expression should inhibit HDACI-induced invasion. As shown in Fig. 5B (bottom panels), uPA shRNA, but not scramble shRNA, significantly inhibited the HDACI-mediated induction of uPA, indicating the validity of the gene-silencing effect of the shRNA used in this study. Consistent with the magnitude of the inhibitory effect on uPA expression, uPA shRNA, but not scramble shRNA, almost completely abrogated HDACI-induced invasion, as determined by Matrigel invasion assay (Fig. 5B, top panels). In a parallel experiment, we also examined whether the presence of uPA is sufficient to confer invasion of cells that are normally either low or non-invasive. The uPA-silenced prostate cancer cell line, LNCaP, which has a very low invasive potential (14), was transfected with either vector alone (pCMV) or pCMV-uPA. LNCaP cells stably expressing uPA exhibited a significant increase in invasion through Matrigel as compared with their empty vector-transfected counterparts (Fig. 5, C and D). A similar result was observed in the uPA-silenced, non-invasive prostate cell line RWPE1 (data not shown).

**HDAC1 Is Present in the uPA Promoter Region in the Absence, but Not the Presence, of TSA**—We first examined whether treatment with TSA suppressed the expression of the HDAC protein levels in uPA-silenced cells. Treatment with TSA did not change the expression of the *HDAC1* protein in SK-N-BE, SK-N-AS, SF-3061, and LNCaP nuclear extracts (Fig. 6A). We found that TSA caused little decrease in HDAC3 protein in the nuclear extracts of SK-N-BE, SK-N-AS, and LNCaP cells but not in SF-3061 cells. There were no detectable levels of HDAC4, -5, -6, and -7 proteins in the nuclear extracts of cells cultured without or with TSA (Fig. 6A). To determine whether the activation of uPA by TSA is related to *in vivo* recruitment of HDAC complexes to the promoter, we investigated the association of HDACs with the uPA promoter by ChIP assay. To this end, uPA-silenced cells were treated in the presence or absence of TSA. Cross-linked and sonicated chromatin lysates were immunoprecipitated with specific antibodies against HDAC1, -3, and -7. The precipitated DNA was analyzed by PCR with primers spanning the uPA promoter region (Fig. 6B, top panel). For the positive control, a 3-fold dilution of total input DNA in the absence or presence of TSA was used for the PCR reactions. No PCR products were detected in extracts subjected to immunoprecipitation with control normal rabbit serum. Anti-HDAC1 antibody was able to immunoprecipitate the uPA promoter region in the absence, but not the presence, of TSA (Fig. 6, B (bottom panel) and C). In contrast, no amplification was observed when DNA immunoprecipitated by anti-HDAC3 or anti-HDAC7 antibody was used as a template in either the absence or presence of TSA (Fig. 6, B (bottom panel) and C).
Taken together, these results indicate that HDAC1 is involved in repressing uPA promoter activity and in mediating the TSA response.

**HDAC1 Knockdown Stimulates uPA Expression and Cancer Cell Invasion**—To further confirm that the HDAC1 was responsible for silencing of uPA, the endogenous levels of HDAC1 were knocked down in LNCaP cells by treatment with specific siRNAs capable of degrading mRNA transcripts in a target-specific manner. The siRNAs that were specifically targeted to HDAC1 exhibited a significant reduction in HDAC1 mRNA (Fig. 7A, top). In HDAC1 knockdown cells, a stimulation of uPA expression (Fig. 7A, top) and activity (Fig. 7A, bottom) was observed when compared with the control siRNA-transfected LNCaP cells. Next, we analyzed the specific binding levels of HDAC1 proteins to the silenced uPA promoter. The siRNA coupled-ChIP assay in LNCaP cells revealed that knockdown of HDAC1 resulted in failure of recruitment of HDAC1 on the uPA promoter region (Fig. 7B, right). In addition, in HDAC1 knockdown cells, TSA treatments had a lower effect on HDAC binding to the uPA promoter when compared with the control siRNA-transfected LNCaP cells (Fig. 7B). Finally, the effect of HDAC1 knockdown on invasive ability of LNCaP cells was determined using the Matrigel invasion assay. As shown in Fig. 7 (C and D), knockdown of HDAC1 significantly induced the invasive potential of LNCaP cells. A similar trend was observed in HDAC1 knockdown SK-N-AS cells as well (data not shown). Overall, our results suggest that HDAC inhibitors may up-regulate uPA expression to induce cancer cell invasion.

**DISCUSSION**

Cell invasion plays a pivotal role in tumor progression and metastasis (1, 3, 38–40). Numerous studies with experimental models indicate that one of the most important components in cancer cell invasion is the production of proteases (3, 39, 41). Among the large number of proteases involved in cellular invasion, uPA is of particular importance because it initiates the activation of metalloproteinases and the conversion of plasminogen to plasmin (42, 43). These proteases confer the ability of cells to degrade the extracellular matrix, thus allowing cells to
overcome the constraints of cell-cell and cell-matrix interaction (44, 45). In addition, the interaction of uPA with uPA receptor also promotes cell motility and proliferation (46–49), and these processes also impact tumor invasion and metastasis. Previous studies by our group (20) and others (18, 19) have established that uPA expression is regulated by DNA methylation.

In addition to DNA methylation, another epigenetic mechanism that frequently controls the transcriptional regulation of genes is the acetylation/deacetylation of chromosomal histones associated with target genes (33). In the present study, we have
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![Graph and Images]

FIGURE 7. Knockdown of HDAC1 by RNA interference induces uPA expression and cancer cell invasion. A, LNCaP cells were transfected with siRNA against HDAC1 (siHDAC1). Specific knockdown of HDAC1 mRNA (top) was monitored by RT-PCR analysis. Numbers indicated below each band are the quantitative values from densitometric analyses. Data shown are normalized to GAPDH mRNA levels in each sample. Fibrin zymography of LNCaP cells transfected with siRNA against HDAC1 (bottom). HDAC1 knockdown relieves HDAC1-mediated repression of uPA activity (bottom). B, top, LNCaP cells were transfected with siControl (left panels) or HDAC1 (right panels) for 48 h, then with 100 nM TSA for 8 h, as indicated, and processed for ChIP assays. The antibodies used were HDAC1 and control normal rabbit serum (NRS). Bottom, the result was quantitatively determined by ImageJ program and presented as the percentage of input signal generated with 2% of the immunoprecipitated chromatin. C, comparison of the in vitro invasive potentials of cells transfected with mock, siControl (siCTL), or siHDAC1. A representative number of invading cells through the Matrigel were counted under the microscope in five random fields at a 200× magnification. Each bar represents the mean ± S.D. of five fields counted. Significant difference from controls (i.e. mock or siControl transfected cells) is indicated by an asterisk (p < 0.05). D, representative invasion photographs from cells transfected with mock, siControl, or siHDAC1 as described in C. (Results are representative of three separate experiments.)

provided evidence for the first time that HDAC1 is involved in the repression of uPA expression in human cancer cell lines SK-N-BE, SK-N-AS, SF-3061, and LNCaP. The repression of uPA in these cells could be explained by the inhibition of HDAC activity. Of the three HDAC inhibitors examined, TSA and NaB were most effective in reactivating uPA expression and activity, followed by SCR (Fig. 1). A lower dose of TSA (100 nM) treatment for as little as 8 h was sufficient for increased expression of uPA in all cell lines studied. In contrast, treatment with higher doses (10–25 μM) of the demethylating agent 5-aza for 5 days failed to reactivate expression of uPA to a level detectable by RT-PCR (data not shown). A previous report has shown that DNA methylation is involved in transcriptional regulation of uPA expression (19). In that report, the silenced uPA gene was reactivated in the prostate cancer cell line LNCaP by treatment with 25 μM 5-aza for 10 days. Another report showed that TSA induces uPA gene expression in the breast cancer cell line MDA-MB-231 by a mechanism independent of DNA methylation (18). In both reports, the chromatin structure of the uPA promoter was not examined. Our experiments have shown that the HDAC inhibitor quickly induces the expression of uPA in a panel of four human cancer cell lines that originated from neuroblastoma (SK-N-BE and SK-N-AS), meningioma (SF-3061), and prostate (LNCaP), further suggesting that HDACs play a general role in regulating uPA expression in human cancer cells.

Histone acetylation is a critical component of chromatin remodeling and transcriptional regulation (50). The acetylation level of core histones results from the balance between the activities of HDACs and histone acetyltransferases. Inhibition of HDACs by TSA leads to activation of only specific target genes through increased histone acetylation (51, 52). Our experiments showed that induction of uPA expression by TSA in human cancer cells was accompanied by a remarkable increase in acetylation of histones H3 and H4 associated with the uPA promoter region (~231 to ~33) (Fig. 2). The increase of core histone acetylation at the promoter region of the uPA gene after TSA treatment indicates that the chromatin structure of uPA promoter may become a loose and non-condensed structure, which is usually necessary for the start of transcription (23, 53).

Current knowledge of histone modifications provides an important link between chromatin structures and functions. Generally, acetylation of histones is associated with active chromatin and corresponds to more open conformations (21). According to the histone code hypothesis, in many cases the relationship between the acetylation of core histones and chromatin structure is complex. Specific acetylation of single lysines of histone tails together with other modifications may be crucial for transcriptional regulation (22, 23). The analysis of histone acetylation is extremely useful for the identification of specific features of local chromatin structures. Moreover, increased acetylation of core histones has been demonstrated to correlate with increase in restriction enzyme accessibility of the promoter region of the gene (32, 54). Our restriction enzyme accessibility assays showed a significant difference in chromatin surrounding the uPA promoter region between cells that express high levels of uPA and cells that do not. Indeed, we found that the chromatin configuration was “closed” in the SK-N-BE, SK-N-AS, SF-3061, and LNCaP cell
lines, whereas it was “open” in the uPA-expressing cell lines, including in SK-N-BE, SK-N-AS, SF-3061, and LNCaP, in which expression had been restored with TSA treatment (Fig. 3). This is consistent with our ChIP assay findings that acetylated histones H3 and H4 are much higher in cells expressing uPA. Moreover, TSA activated uPA promoter activity in LNCaP cells (Fig. 4). Future studies will address the mechanism by which TSA modulated the components of the transcriptional complex and HDAC that binds to uPA promoter region. Taken together, all of these findings strongly suggest the involvement of histone deacetylation in uPA gene silencing.

Ample evidence indicated that increased levels of uPA are crucial for tumor cell invasion and metastasis (5, 9, 14, 15). Our in vitro invasion assays showed that HDAI-induced uPA activation might stimulate cancer cell invasion. The importance of HDAI-induced uPA activity to stimulate cancer cell invasion was confirmed by using uPA-specific shRNA (Fig. 5). The effect of HDALs on cancer cell invasion is consistent with those of Mori et al. (55), who observed that TSA treatment enhanced gene expression of both CCR7 and CXCR4 and stimulated the in vitro invasion of melanoma cell lines. A recent study in human endometrial cancer cells also showed that HDALs promote cell migration and invasion (56). In contrast, however, other studies have shown that HDALs suppress cancer cell invasion (57, 58). Variations in cell lines and/or target promoters, which can be regulated by invasive genes through different mechanisms (20, 59), probably account for the variability in the reported effect of HDALs on cancer cell invasion.

Based on these results, it would be logical to ask how uPA is repressed in these cells by HDALs. Our ChIP assays indicate that the induction of uPA in LNCaP cells by TSA is mediated through dissociation of HDAC1 from the uPA promoter (Fig. 6). This loss of HDAC1 associated with the uPA promoter has functional relevance, because treatment of cells with TSA, an inhibitor of HDAC activity, relieves transcriptional repression of uPA. Finally, we sought to confirm the role of HDAC1 in the uPA promoter regulation using RNA interference. Consistent with our ChIP assays demonstrating a crucial role for HDAC1 in TSA-induced increase in acetylation of histones, HDAC1 knockdown significantly increased uPA expression and cancer cell invasion (Fig. 7).

HDALs are currently in clinical trials for cancers, neurodegenerative diseases, and hematologic disorders (60–63). Our study has demonstrated that HDALs are involved in the uPA repression in human cancer cells. The finding, that repressed uPA gene in human cancer cells can be re-activated by the inhibition of HDALs, will not only enhance our understanding in HDAC-mediated uPA gene expression but will also have negative implications for the therapeutic use of HDAC inhibitors in the treatment of cancer. The role of uPA in tumor cell invasion and metastasis is well established (5, 9, 14, 15), and we found that HDALs enhance tumor cell invasion through induction of uPA expression. Therefore, it is noteworthy that the use of HDAL-based cancer therapies in patients may paradoxically establish metastasis through reactivation of uPA. Further investigations of the HDAL effects on uPA promoter activation are essential for understanding the molecular mechanisms underlying the potentially adverse effects of HDALs. Perhaps these investigations can provide the necessary insight to improve the therapeutic efficacy of HDAC inhibitors.

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