HDAC inhibition upregulates the expression of angiostatic ADAMTS1

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Abstract HDAC inhibitors are promising anticancer agents that induce cell cycle arrest and apoptosis. However, the role of HDACs in cancer progression, such as angiogenesis and metastasis, remains largely unexplored. Among various HDAC inhibitors, we demonstrate that TSA and SAHA upregulated the expression of angiostatic ADAMTS1 in A549 cells. HDAC6 inhibitor tubacin, and knockdown of HDAC6, also lead to ADAMTS1 upregulation. By reporter, DAPA, and ChIP assays, the proximal GC boxes were demonstrated to be essential for ADAMTS1 induction. Decreased binding of SP1 and HDAC6 to the ADAMTS1 promoter after TSA treatment was also seen. These data suggest the involvement of HDAC6 and SP1 in the HDACi-induced expression of angiostatic ADAMTS1.

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1. Introduction

Removing the acetyl group added by histone acetyltransferase (HAT) has been identified as the first step in gene silencing [1]. Abnormal histone deacetylation not only results in gene silencing but also reduces DNA repair [2], thereby accelerating cancer initiation and progression. Histone deacetylases (HDACs), which are responsible for removing acetyl groups from histones, were upregulated in tumors derived from several origins [3]. Moreover, global hypoacetylation of histone H4 represents a hallmark of human tumors [4]. Thus, HDACs have become the emerging target for cancer therapy, and small molecule inhibitors of HDACs have shown promising clinical applications as antitumor agents [5]. HDAC inhibitors (HDACi) cause histone hyperacetylation and reactivation of tumour-suppressor genes. For example, HDACi have been shown to induce cyclin-dependent kinase (CDK) inhibitors such as p21 [6], and to activate the death-receptor-mediated extrinsic apoptotic pathways [7,8]. Although these effects were reported to contribute to their antitumor activity, the effects of HDACi on cancer progression, such as angiogenesis and metastasis, are largely unexplored.

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Abbreviations: HDAC, histone deacetylase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif

ADAMTS1 is an extracellular metalloproteinase known to participate in various biological processes that includes inflammation, angiogenesis, and development of the urogenital system [9]. It belongs to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif) family [10,11], and contains the propeptide region, a metalloproteinase domain, two disintegrin loops and three C-terminal thrombospondin type 1(TS) motifs [12]. ADAMTS1 cleavages aggrecan, an extracellular matrix found mostly in cartilage, and versican, a widely distributed extracellular matrix component [13]. Although metalloproteinase was generally thought to promote the invasion of tumor cells, ADAMTS1 is reported to be a potent inhibitor of angiogenesis by sequestration of VEGF165 and release of angioinhibitory thrombospondin 1/2 [9,14–16]. Lower expression of ADAMTS1 is observed in non-small-cell lung cancer (NSCLC) [17]. According to its angioinhibitory ability and silencing in NSCLC [17], it is possible that ADAMTS1 may serve as a tumor suppressor in NSCLC. Therefore, uncovering the mechanism of its silencing may facilitate the development of NSCLC therapy.

In this study, we found broad spectrum HDACi, TSA and SAHA [3] were capable of inducing ADAMTS1 expression. A549 cells with reduced expression of HDAC6, a member of class Iib HDAC, showed higher expression of ADAMTS1. A specific inhibitor of HDAC6, tubacin [18], also induced ADAMTS1 expression. By reporter, DAPA and ChIP assays, the proximal GC boxes are demonstrated to be essential for ADAMTS1 induction, and decreased binding of SP1 and HDAC6 to ADAMTS1 promoter after TSA treatment was also seen. These data suggest that HDAC6 and SP1 are involved in the HDACi-induced expression of angiostatic ADAMTS1.

2. Materials and methods

2.1. Cell culture and reagents

A549 human lung carcinoma cells from ATCC were cultured in DMEM supplemented with 10% fetal bovine serum. A549 variants with intact HDAC6 expression (pSuper) or reduced HDAC6 expression by RNA interference (HD6KD) were kindly provided by Dr. Yao (Department of Pharmacology and Cancer Biology, Duke University) [19]. Tubacin and niltubacin were provided by Dr. Ralph Mazitschek, who are supported by ICG (Initiative for Chemical Genetics – National Cancer Institute) [18]. The ADAMTS1 promoter constructs (1186 bp and /GC box mutants were generous gifts from Dr. JoAnne S. Richards [20].

2.2. Western blot analysis

Following treatment with HDAC inhibitors, total cell lysates or nuclear extracts were prepared and subjected to SDS-PAGE using adequate percentage polyacrylamide gels.


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2.3. Flow cytometry
A549 cells are fixed in 3.7% paraformaldehyde and washed two times with PBS. The cells are blocked and permeabilized with PBS containing 1% BSA and 0.1% Triton X-100, and then incubated with anti-ADAMTS1 antibody for A549 cells for 1 h. After washing, the cells are resuspended in 500 μl PBS for analysis by a FACScalibur flow cytometry system.

2.4. RT-PCR
Total RNA was isolated from A549 cells using Trizol™ Reagent (Life Technology). Reverse transcription reaction was performed using 2 μg of total RNA and reverse transcribed into cDNA using oligo dT primer, then amplified 30 cycles using oligonucleotide primers derived from published ADAMTS1 or β-actin sequence, including 5'-CAGGCTTATGGGTA-3' and 5'-TTCACTTCGATGTGTCGTC-3' (ADAMTS1), or 5'-TGACGGGGTCACCCAGCATCTA-3' and 5'-CTAGAAGCATTTGGGAGG-3' (β-actin). PCR was carried out at 94°C for 30 s, at 55°C for 30 s and 1 min at 70°C for 34 cycles. The PCR products were subjected to 1.5% agarose gel electrophoresis. Quantitative data were obtained using a computing densitometer and ImageQuant Software (Molecular Dynamics).

2.5. Transient transfection and luciferase activity assay
The vectors were transiently transfected into A549 cells with Arrestin transfection reagent. Briefly, 1 μg of plasmid DNA and 5 μL transfection reagents were mixed, and the transfection protocol was carried out according to the manufacturer’s instructions (Promega). Six hour after transfection, the cells were cultured in normal complete medium for another 16 h. The transfected cells with or without HDAC inhibitor treatment were subjected to luciferase assay. Luciferase activities were normalized with the amount of protein in cell lysates.

2.6. DNA affinity protein binding assay
DAPA assay were performed as described [21]. DNA probes containing the sequences of GC boxes of the ADAMTS1 promoter were obtained by PCR amplification using the following pairs of primers labeled with biotin: [5'-TTCTTACCCCCAGAGTGACGA-3'], [5'-TACATAGGGCCCCCTCTCC-3']. The nuclear extract (400 μg) was precleared at 4°C for 1 h with 20 μl of the 4% streptavidin-coated

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Fig. 1. TSA and SAHA induce ADAMTS1 mRNA and protein expression in A549 cells. A549 cells were treated with 1 μM TSA or 5 μM SAHA for 6, 12, 24 or 48 h (A) or treated with 0.5, 1 μM TSA or 3, 5 μM SAHA for 24 h (B), and total RNA (2 μg) was used for RT-PCR as described. In (C), A549 cells were treated with 1 μM TSA or 3 μM SAHA for 6, 12, 24 or 48 h. Whole cell lysate was prepared and subjected to Western blot using Ab specific for ADAMTS1, HDAC6 and Actin. (D) A549 cells were treated with 1 μM TSA or 5 μM SAHA for 24 or 48 h. Cells were fixed and permealized then subjected to flow cytometry as described Section 2.
beads (Sigma) mixed with 50% slurry to reduce nonspecific binding. Precleared nuclear extract was incubated with 4 μg of biotinylated DNA oligonucleotides and 20 μl of 4% streptavidin agarose beads with 50% slurry in 400 μl PBS at 4 °C for overnight with shaking. Beads were then collected by centrifugation at 2000 rpm for 2 min and washed with cold PBS three times. DNA/protein complexes bound to the beads were eluted with 30 μl of Laemmli sample buffer. Nuclear proteins were denatured in a dry bath at 95 °C for 5 min and subjected to SDS–PAGE. Western blot analysis probed with specific anti-SP1 or anti-HDAC6 antibody was performed as described above.

Fig. 2. HDAC6 is implicated in the silencing of ADAMTS1 in A549 cells. (A) The total RNA of A549 cell treated with 1 μM MS275, 1 mM NaB, 5 μM SAHA, 1 μM Oxa, 1 μM TSA or 1 mM VPA for 16 h were harvested then subjected to RT-PCR (B) A549 cells were treated with 5 μM SAHA, 1 μM MS275, 1 mM NaB or 1 mM VPA for 24 h. Cells were fixed and permealized then subjected to flow cytometry. (C) The total RNA of A549 cells treated with 20 μM tubacin or niltubacin for 16 h, and the stable HDAC6 knockdown A549 cells as well as its vector control were harvested then subjected to RT-PCR as described. The expression of HDAC6 in vector control and HDAC6 knockdown cells were examined by western blotting. (D) The A549 cells were treated with 1 μM TSA, 5 μM SAHA or 20 μM tubacin for 24 h. Cells were fixed and permealized then subjected to flow cytometry.
2.7. Chromatin immunoprecipitation assay (ChIP)

ChIP analysis was performed as described [22]. Immunoprecipitated DNA was purified and resuspended in H2O, and subjected to PCR. To amplify regions of the ADAMTS1 promoter, PCR was performed with the following pairs of primers: [5'AGCGGCCCGACGAGTA-3'] [5'TTCCACGCGAAGAAGGCGG-3']. PCR products were then resolved by 1.5% agarose-ethidium bromide gel electrophoresis and visualized by UV.

3. Results

3.1. TSA and SAHA induce ADAMTS1 expression in A549 cells

We first examined the effect of prototype HDAC inhibitors, TSA and SAHA, on ADAMTS1 expression. The result showed that TSA or SAHA increased the mRNA expression of ADAMTS1 in a time- and dose-dependent manner (Fig. 1A and B). The protein level of ADAMTS1 was also examined by Western blotting and flow cytometry. Upregulation of ADAMTS1 protein in a time-dependent manner was seen after treatment with TSA and SAHA (Fig. 1C and D). These data suggested that epigenetic dysregulation is involved in the silencing of ADAMTS1 in A549 cells and HDAC inhibitors could reactivate its expression.

3.2. HDAC6 is involved in the silencing of ADAMTS1 in A549 cells

We further examined the effects of HDAC inhibitors with different spectrum on ADAMTS1 expression. Cells were exposed to several HDAC inhibitors for 16 h and only the broad spectrum HDACi, TSA and SAHA, were capable to induce ADAMTS1 expression (Fig. 2A and B). TSA and SAHA are capable of inhibiting class I, IIa, IIb and IV HDACs, but MS275, VPA and NaB only inhibit members of class I and IIa HDACs [3]. The class IIb and class IV HDACs may mainly engage in ADAMTS1 regulation. Therefore, we treated A549 cells with tubacin, a specific inhibitor of HDAC6 which belongs to the class IIb HDAC, and found that tubacin but not its inactive analog, niltubacin [18], induced ADAMTS1 expression (Fig. 2C and D). ADAMTS1 expression was also higher in HDAC6 knockdown cells comparing to its vector control (Fig. 2C). The expression of HDAC6 in knockdown cells was confirmed by immunoblot (Fig. 2C). These data implied that HDAC6 is involved in the regulation of ADAMTS1 expression.

3.3. GC boxes are required for TSA- and SAHA-induced ADAMTS1

There are several cis-elements on the ADAMTS1 promoter, such as c-Ets, NF-1, C/EBP and GC boxes. Cells were transfected with either −1186 bp, truncated −375 bp or −151 bp ADAMTS1-Luc. The −151 bp ADAMTS1-Luc reporter was found to be sufficient for the induction of ADAMTS1 promoter activity in response to TSA and SAHA stimulation (Fig. 3A and B), indicating that the inducible region is within the −151 promoter. There are three GC boxes on the ADAMTS1 promoter. The reporter truncated with different GC boxes was examined. Mutation of GC-II and GC-III was found to disrupt the basal promoter activity, their role in TSA-induced promoter activity remained to be identified. However, truncation of GC-I, which possessed unaffected basal promoter activity, significantly attenuated the TSA-induced promoter activity (Fig. 3C), suggesting that GC-I is essential for HDACi-induced ADAMTS1 expression.

3.4. TSA disturbed the binding of HDAC6 and SP1 to ADAMTS1 promoter

Since HDAC6 and GC boxes were implicated in HDACi-induced ADAMTS1 expression, the binding of HDAC6 and SP1/3 to its promoter after TSA treatment was examined by DAPA and ChIP assays. The decreased binding of SP1 to the ADAMTS1 promoter was demonstrated, although partially recovery was seen at 8 h treatment by DAPA assay (Fig. 4A and B). However, ChIP assay showed the invariant binding of SP3 (Fig. 4A and B). The promoter binding was highly correlated with the nuclear amount of SP1, suggesting that HDACi may regulate the localization of SP1 to control its availability for ADAMTS1 promoter. HDAC6 was also removed from ADAMTS1 promoter (Fig. 4A and B) and CBP was recruited upon treatment with HDACi (Fig. 4B). These data imply that the inhibitory role of SP1 and HDAC6 for the regulation of ADAMTS1 is relieved by the inhibition of HDAC, leading to the reactivation of ADAMTS1.

4. Discussion

In this study, we showed that TSA and SAHA induced the expression of angioinhibitory ADAMTS1. The upregulation of ADAMTS1 in HDAC6 knockdown A549 cells was also
seen. Pretreatment with the specific inhibitor of HDAC6, tubacin, increased the ADAMTS1 expression as well. By reporter assay, the GC boxes in the proximal region of ADAMTS1 promoter were demonstrated to be essential for the TSA-induced promoter activity. Moreover, HDAC inhibition disturbed the binding of SP1 and HDAC6 from the ADAMTS1 promoter, thereby leading to the induction of ADAMTS1.

The angioinhibitory properties of HDAC inhibitors have been reported to downregulate proangiogenic genes, leading to their inhibition of neo-vascularization. For example, most HDACi are able to reduce the expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hypoxia-inducible factor-1α (HIF1α), angiopoietin, tunica intima endothelial kinase 2 (TIE2) and endothelial nitric oxide synthase (eNOS) [23–28]. Also, HDACi have been reported to downregulate the expression of chemokine (C-X-C motif) receptor 4 (CXCR4), which is essential for the guidance of circulating endothelial cells to sites of angiogenesis [28,29]. Moreover, HDACi have been shown to suppress endothelial progenitor cell differentiation [28]. However, the effects of
HDACi on transformed cells and angioinhibitory genes are largely unexplored. In this study, we demonstrated that the HDACi, TSA and SAHA, upregulated the angioinhibitory gene, ADAMTS1, in transformed A549 cells. These data suggested that HDACi not only reduce the pro-angiogenesis genes but also induce the angioinhibitory gene. ADAMTS1 is the prototype of the ADAMTS family proteins. It has been reported to bind to VEGF165 and inhibit its binding to VEGF-receptor 2 on endothelial cells [9,16]. The antiangiogenic potency of ADAMTS1 has been described to be greater than that of either thrombospondin 1 or endostatin at the same molar ratio [12]. The binding ability of ADAMTS1 to VEGF165 has been assigned in its thrombospondin motifs [16]. In addition to VEGF165 sequestration, ADAMTS1 also mediates the release of polypeptides from the trimeric structure of both TSP1 and 2 which could generate a pool of antiangiogenic fragments from matrix-bound thrombospondin [15]. Overexpression of the C-terminal region of ADAMTS1, consisting of TSP type I motifs and the spacer region, suppressed Chinese hamster ovary (CHO) tumor growth and metastasis [30].

Although ADAMTS1 has been reported to be silenced in NSCLC [17], approaches to its reactivation in NSCLC has never been reported. In this study, we demonstrated that HDACi, the most promising anticancer agent, was capable of inducing ADAMTS1 in A549 cells. This induction depends on the regulation of HDAC6 and SP1. It has been reported that the C/EBPβ, NF-1-like, SP1/3 binding sites are essential for the ADAMTS1 induction by LH and progesterone receptor in granulosa cell [20]. We showed that the proximal GC-I was required for the HDACi-induced expression of ADAMTS1, suggesting the involvement of SP1. TSA has been demonstrated to decrease the binding of HDAC1 to the MT-1 promoter [31]. In pancreatic adenocarcinoma cell lines, TSA disrupts the association between HDAC1 and SP1, and decreases the binding of HDAC1 to TGFBR2 promoter [32]. In our previous study, we demonstrated that statin and TSA released HDAC1/2 from the p21 promoter [33]. There are also reports demonstrating that SAHA releases HDAC from an Sp1 site [34]. These findings implied that HDACi inhibition may alter the expression of ADAMTS1 promoter-associated proteins. By DAPA and ChIP assays, we demonstrated that Sp1 was dispersed from ADAMTS1 promoter after TSA treatment, suggesting an inhibitory role of Sp1 in ADAMTS1 regulation. The promoter binding of Sp1 was highly correlated with its nuclear amount, suggesting HDACi may regulate the Sp1 localization to control its availability for ADAMTS1 promoter. Meanwhile, HDAC6 was also removed from the ADAMTS1 promoter, while CBP was recruited. This data correspond with the previous finding about the involvement of HDAC6 in ADAMTS1 induction, suggesting that HDAC6 is probably responsible for the epigenetic silence of ADAMTS1 in NSCLC. Overall, our findings offered a novel angioinhibitory effect of HDAC inhibitors and may be helpful for the development of NSCLC therapy.

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References


