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Advances in Brief

Histone Deacetylase Inhibitor Up-Regulates RECK to Inhibit MMP-2 Activation and Cancer Cell Invasion[1]

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Abstract

Histone deacetylase (HDAC) inhibitors are known to exert antimetastatic and antiangiogenic activity *in vitro* and *in vivo*. RECK is a membrane-anchored glycoprotein that negatively regulates matrix metalloproteinases (MMPs) and inhibits tumor metastasis and angiogenesis. In this study, we test the possibility that HDAC inhibitor may increase RECK expression to inhibit MMP activation and cancer cell invasion. Our results showed that trichostatin A (TSA) up-regulated RECK via transcriptional activation in CL-1 human lung cancer cells. Flow cytometric analysis demonstrated that RECK protein on cell surface was increased after treatment of TSA. Moreover, up-regulation of RECK expression by TSA attenuated MMP-2 activity. To explore whether HDAC inhibitor-induced inhibition of MMP-2 activation is indeed mediated via RECK, we used small interference RNA (siRNA) to block RECK expression and found that inhibition of RECK by siRNA abolished the inhibitory effect of TSA on MMP-2 activation. In addition, TSA suppressed the invasive ability of CL-1 cells. Taken together, this study reveals a novel mechanism by which HDAC inhibitors suppress tumor invasion and provides a new strategy for cancer therapy.

Introduction

The MMPs[3] are a family of zinc-dependent endopeptidases that are involved in diverse cellular processes. The *MMP* gene family consists of at least 20 enzymes and may be subgrouped into different types based on substrate specificity and sequence characteristic (1). MMPs are synthesized as inactive precursors and are activated by proteolytic cleavage. Therefore, MMP activity can be regulated by modulation of gene expression, control of proenzyme processing, and direct inhibition of enzymatic activity.

The *RECK* gene was isolated as a transformation suppressor gene by using an expression cloning strategy designed to identify human cDNA inducing flat reversion in a v-Ki-*ras*-transformed NIH3T3 cell line (2).

This gene encodes a membrane glycoprotein that may inhibit tumor metastasis and angiogenesis by negatively regulating MMP activity (3, 4). Whereas RECK mRNA is expressed in most of normal human tissues and untransformed cells, it is undetectable in many tumor cell lines or in cells artificially expressed active oncogenes (3). In addition, clinical study also indicated that patients with high RECK expression in tumor tissues showed better survival, and such tumors were less invasive (5).

These data suggest that *RECK* is a novel and critical suppressor gene for metastasis and angiogenesis.

HDAC inhibitors are a novel class of anticancer drugs that may inhibit growth and induce apoptosis of cancer cells (6, 7). These inhibitors were shown to stimulate the expression of growth-inhibitory genes like $p21^{Waf1}$ and $p27^{Kip1}$ to inhibit proliferation of cancer cells (8). Additionally, these inhibitors also reduced the expression of antiapoptotic genes like *bcl-2* and *bcl-xL* to trigger cell apoptosis (9, 10). Recent studies demonstrated that HDAC inhibitors exerted antimetastatic and antiangiogenic effect *in vitro* and *in vivo* (11, 12).

Interestingly, two recent investigations demonstrated that HDAC inhibitors apicidin and depudecin induced morphological reversion of *ras*-transformed fibroblasts and suppressed *ras*-induced invasive phenotype, a characteristic very similar to the effect of RECK on *ras*-transformed NIH3T3 cells (13, 14). Therefore, we tested the possibility that *RECK* may be a target gene for HDAC inhibitors and may be involved in the inhibition of tumor metastasis by HDAC inhibitors.

Materials and Methods

Cell Line and Plasmids. CL-1 human lung cells were provided by Dr. Kuo Min-Liang (National Taiwan University, Taipei, Taiwan) and was routinely cultured in DMEM/F12 medium supplemented with 10% heat-inactivated FCS and antibiotics. Mouse RECK promoter-luciferase plasmid was a generous gift of Dr. Noda, Kyoto University, Kyoto, Japan (15). TSA was obtained from Biomol (Polymouth Meeting, PA). Anti-RECK antibody was purchased from MBL (Nagoya, Japan). LipofectAMINE reagent was purchased from Invitrogen (Carlsbad, CA) and luciferase assay system was from Promega (Madison, WI).

RNA Extraction and RT-PCR. Cells were treated with vehicle or TSA (100 nM) for 48 h. Total RNA was isolated from cells, and RECK expression was examined by using OneStep RT-PCR kit according to the manufacturer's protocol (Qiagen). β-actin was used as an internal control to check the efficiency of cDNA synthesis and PCR amplification. cDNA synthesis was performed at 50°C for 30 min, and the condition for PCR was 30 cycles of denaturation (94°C/1 min), annealing (60°C/1 min), extension (72°C/1 min), and 1 cycle of final extension (72°C/10 min). The predicted sizes for PCR products for RECK and β-actin were 477 and 315 bp, respectively. The primers used were: RECK-forward, 5′-CCTCAGTG AGCACAGTTCAGA-3′; RECK-reverse, 5′-GCAGCACACACACTG CTGTA-3′; β-actin-forward, 5′-TCCTGTGGCATCCACGAAACT-3′; and β-actin-reverse, 5′-GAAG-CATTTGCGGTGGACGAT-3′. After reaction, PCR products were separated on a 2% 0.5× TBE agarose gel, stained with ethidium bromide and visualized under UV light.

Western Blot Analysis. Cells were treated with vehicle or TSA for 48 h and were harvested in a lysis buffer, and equal amount of cellular proteins was subjected to SDS-PAGE, as described previously (16). Proteins were transferred to nitrocellulose membranes, and blots were probed with anti-RECK antibody. Enhanced chemiluminescence reagents were used to depict the protein bands on the membranes.

Flow Cytometric Analysis. Cells were treated with vehicle or TSA for 48 h. After treatment, cells were fixed with 4% formaldehyde, stained with anti-RECK antibody, and subjected to flow cytometric analysis, as described previously (16).

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[3] The abbreviations used are: MMP, matrix metalloproteinase; HDAC, histone deacetylase; TSA, trichostatin A; RT-PCR, reverse transcription-PCR; siRNA, small interference RNA; VEGF, vascular endothelial growth factor.

Promoter Activity Assays. Promoter activity of *RECK* gene was analyzed as described previously (17). In brief, cells were plated onto 6-well plates at a density of 100,000 cells/well and grown overnight.

Cells were cotransfected with 2 μg of full-length mouse RECK promoter-luciferase vector and 1 μg of cytomegalovirus-β-galactosidase plasmid.

After transfection, cells were treated with vehicle or TSA for 48 h, and luciferase activity was determined by using an assay system according to the procedure of the manufacturer (Promega) and was normalized for β-galactosidase activity.

Gelatin Zymography. Cells were treated with vehicle or TSA in serum-free medium for 48 h. Conditioned medium was collected and concentrated by using Centricon YM-50 columns (Amicon, Bedford, MA). Cell number was determined by using the hemocytometer. Conditioned medium from an equal number of cells was separated by 10% acrylamide gels containing 0.1% gelatin (Invitrogen). The gels were incubated in 2.5% Triton X-100 solution at room temperature with gentle agitation to remove SDS and were soaked in reaction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, and 0.5 mM $ZnCl_2$] at 37°C overnight. After reaction, the gels were stained for 1 h with staining solution (0.1% Coommassie Brilliant Blue, 30% methanol, and 10% acetic acid) and were destained in the same solution, but without Coommassie Brilliant Blue. Gelatinolytic activity of MMPs was visualized as a clear band against a dark background of stained gelatin.

***In Vitro* Invasion Assays.** *In vitro* invasion assay was performed by using 24-well transwell units with polycarbonate filters (pore size, 8 μm) coated on the upper side with Matrigel (Becton Dickinson Labware, Bedford, MA). Cells were treated with vehicle or TSA for 24 h. Cells were harvested and 5×10^3 cells in 100 μl of medium containing vehicle or TSA were placed in the upper part of the transwell unit and were allowed to be invasive for 24 h. The lower part of the transwell unit was filled with 10% FCS medium. After incubation, nonmigrated cells on the upper part of the membrane were removed with a cotton swab. Migrated cells on the bottom surface of the membrane were fixed in formaldehyde, stained with Giemsa solution, and counted under a microscope.

siRNA Experiments. siRNA designed to target RECK 5′-AAGACC CAGCCCUUGCCUCAA-3′ (sense strand) and a nonspecific RNA 5′-AACGUUGCGAUAGCGUAGUAC-3′ was synthesized (Dharmacon Research Inc.). Cells were transfected with double strand RNA by using the LipofectAMINE reagent. After transfection, cells were incubated in 10% FCS medium for 48 h. RECK expression was investigated by RT-PCR. For analysis of MMP-2 activity, cells were transfected with double-strand RNA and were cultured in serum-free medium containing vehicle or TSA for 48 h. Conditioned medium was collected and subjected to gelatin zymography as described above.

Results

TSA Stimulates RECK Expression in Lung Cancer Cells. CL-1 cells were incubated with vehicle or TSA and were harvested for different investigations. As indicated in Fig. 1*A*, RT-PCR analysis showed that TSA up-regulated RECK mRNA level in CL-1 cells. In accordance with the result of RT-PCR analysis, we found that RECK protein level was increased after treatment of TSA (Fig. 1*B*). We also addressed whether TSA stimulated RECK via transcription activation. Because human RECK promoter has not been cloned yet, we used mouse RECK promoter-luciferase construct to address this question. Our data showed that TSA potently stimulated RECK promoter activity (Fig. 1*C*). In addition, flow cytometric analysis indeed demonstrated that TSA increased RECK protein level on cell surface (Fig. 1*D*). These results indicate that TSA up-regulates RECK expression in CL-1 cells.

TSA Inhibits MMP-2 Activity via RECK. Because our data showed that TSA increased RECK expression on the cell surface of CL-1 cells, we investigated whether TSA-induced RECK might lead to the inhibition of MMP activity. Gelatin zymography indicated that significant MMP-2 activity was detected in the conditioned medium of CL-1 cells (Fig. 2*A*). Similar results have been observed in our previous study (18). Treatment of TSA obviously reduced MMP-2 activity. However, it should be noted that TSA did not show any inhibitory effect on the expression of MMP-2 in CL-1 cells (Fig. 2*B*). Therefore, attenuation of MMP-2 activity in the conditioned medium by TSA is not caused by down-regulation of MMP-2 expression. We hypothesize that TSA may induce RECK to suppress MMP-2 activity. To test this hypothesis, we used specific siRNA to suppress RECK expression and investigated whether the siRNA might antagonize the inhibitory action of TSA on MMP-2 activity. Our data demonstrated that siRNA specifically suppressed RECK, but not β-actin, expression in a dose-dependent manner in CL-1 cells (Fig. 3*A*). In addition, a nonspecific double-strand RNA did not affect RECK expression under similar experimental condition (data not shown). In accordance with our hypothesis, RECK-specific siRNA inhibited TSA- stimulated RECK expression (Fig. 3*B*) and counteracted TSA-induced down-regulation of MMP-2 activity (Fig. 3*C*). These results strongly support the notion that TSA acts via RECK to inhibit MMP-2 activity.

TSA Suppresses the Invasive Ability of CL-1 Cells. Because TSA might activate RECK expression and attenuate MMP-2 activity, we tested whether TSA might suppress the invasive ability of CL-1 cells.

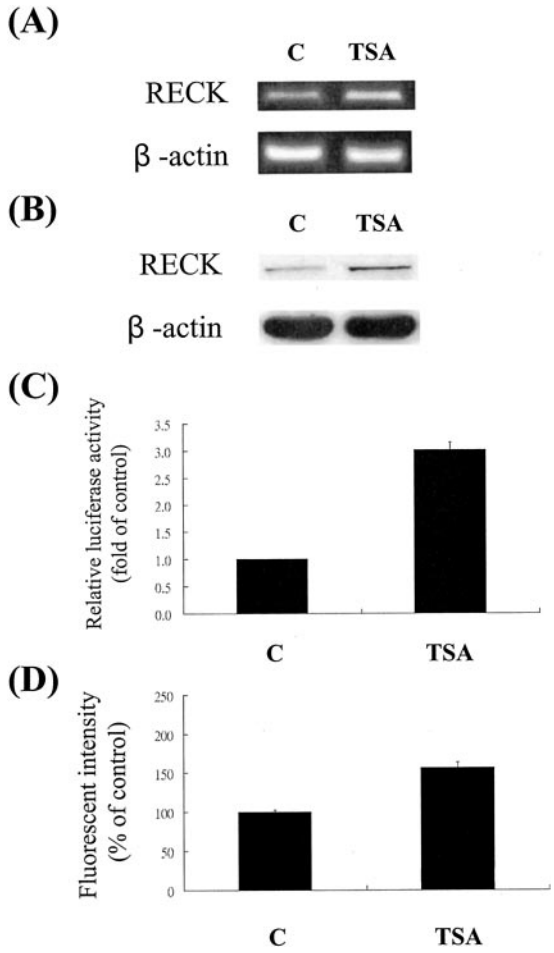

Fig. 1. TSA up-regulates RECK expression in human lung cancer cells. Cells were treated with vehicle or with TSA (100 nM) for 48 h and were harvested for analysis. *A*, RT-PCR was performed to investigate RECK mRNA level in vehicle- [*C* (control)] or in TSA-treated cells. *B*, RECK protein level was examined by Western blot analysis. *C*, cells were cotransfected with 2 μg of full-length mouse RECK promoter-luciferase vector and 1 μg of cytomegalovirus-β-galactosidase plasmid. After transfection, cells were treated with vehicle [*C* (control)] or with TSA for 48 h, and luciferase activity was determined and was normalized for β-galactosidase activity. *D*, cells were fixed with 4% formaldehyde and blocked by 5% nonfat milk for 30 min at room temperature. Cells were incubated with anti-RECK antibody for 1 h followed by incubation with FITC-conjugated secondary antibody for another 1 h. Cells were then subjected to flow cytometric analysis as described in "Materials and Methods."

As shown in Fig. 4, our results demonstrated that TSA significantly reduced the number of penetrated cells in cell invasion assays. Taken together, these results indicate that HDAC inhibitors may up-regulate RECK expression to inhibit MMP-2 activity and cell invasion.

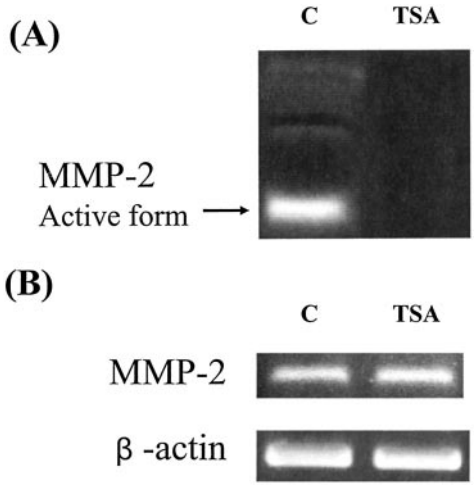

Fig. 2. TSA inhibition of MMP-2 activity is not caused by suppression of MMP-2 expression. Cells were treated with vehicle or with TSA (100 nM) in serum-free medium for 48 h. Conditioned medium was then collected for zymographic assay, and cells were harvested for RT-PCR analysis. *A*, MMP-2 degrading activity in the conditioned medium was assessed by gelatin zymography as described in "Materials and Methods." *B*, MMP-2 mRNA level of vehicle [*C* (control)] or TSA-treated cells was investigated by RT-PCR.

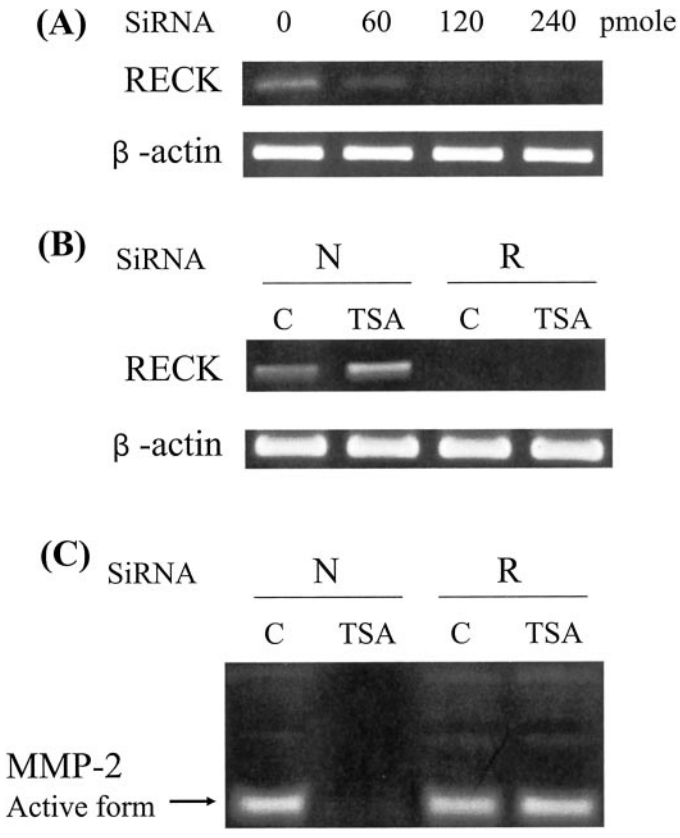

Fig. 3. RECK-specific siRNA antagonizes the inhibitory action of TSA on MMP-2 activation. *A*, cells were transfected with various amounts of siRNA, and RECK expression was investigated by RT-PCR. *B*, cells were transfected with nonspecific (*N*) or RECK (*R*) siRNA. After transfection, cells were cultured in serum-free medium containing vehicle [*C* (control)] or TSA (100 nM) for 48 h. Cells were harvested for analysis of RECK expression by RT-PCR. *C*, conditioned medium was collected for gelatin zymography. Experiments were repeated three times with similar results.

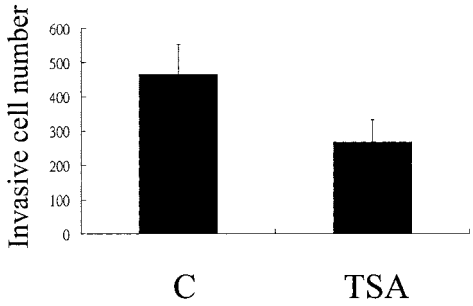

Fig. 4. TSA suppresses invasion of CL-1 lung cancer cells. *In vitro* invasion assay was performed by using 24-well transwell units with polycarbonate filters coated on the upper side with Matrigel. Cells were treated with vehicle [*C* (control)] or TSA (100 nM) for 24 h. Cells (5×10^3) were placed in the upper part of the transwell unit and allowed to be invasive for 24 h as described in "Materials and Methods." Invasive cells were fixed in formaldehyde, stained with Giemsa solution and counted under a microscope. Results shown are mean ± SD from three independent experiments.

Discussion

Lines of evidence have demonstrated that HDAC inhibitors exert potent antimetastatic and antiangiogenic activity *in vitro* and *in vivo* (11, 12). However, the molecular mechanism of this action is largely unknown. A possible candidate that involved in the inhibition of angiogenesis by HDAC inhibitors is VEGF. A recent investigation showed that HDAC inhibitors might inhibit the expression of hypoxia inducible factor-1α (HIF-1α), which in turn suppressed HIF-1α-induced VEGF expression (19). In this study, we reveal a novel mechanism by which HDAC inhibitors inhibit tumor invasion. Our data show that TSA may activate RECK expression to inhibit MMP-2 activity and cancer cell invasion. A nonspecific HDAC inhibitor, butyric acid, also exerts similar action on RECK expression (data not shown). The importance of RECK in the inhibition of MMP-2 activity by HDAC inhibitors is confirmed by using RECK-specific siRNA. This is the first study to show that RECK is a target of HDAC inhibitors and is important for HDAC inhibitor-induced inhibition of cell invasion. Our results also suggest that HDAC inhibitors may act simultaneously via different target proteins (like VEGF and RECK) to suppress angiogenesis and metastasis.

The mechanism by which HDAC inhibitors up-regulate RECK is under investigation in this laboratory. Because our results showed that TSA directly stimulated RECK promoter activity, we think that histone deacetylation might be involved in the control of RECK expression in human cancer cells. Previous work indicated that *ras* oncogene might down-regulate RECK via a Sp1 site located within the −52-bp region of the RECK promoter (15). Recent studies showed that Sp1 might interact with HDACs to repress gene expression. Therefore, it is possible that *ras* activation may stimulate Sp1 to recruit HDACs to RECK promoter and inhibit its expression. This speculation is supported by our recent results.

We used mouse fibroblasts stably transfected with an inducible Ha-*ras* ^{Val12} oncogene under the control of *Escherichia coli lac* operator/repressor system to test whether HDAC inhibitors might counteract *ras*-induced down-regulation of RECK. Our data indicated that *ras* activation increased the binding between Sp1 and HDAC1, and that HDAC inhibitors potently antagonized the inhibitory effect of *ras* on RECK.[4] Thus, histone deacetylation is one of the mechanisms by which cancer cells act to suppress RECK expression.

Another important aspect of this work is that the concentration of TSA used in our study is well tolerated. We found that as little as 100

[4] H-C. Chang, L-T. Liu, and W-C. Hung. Involvement of histone deacetylation in ras-induced down-regulation of the metastasis suppressor RECK, manuscript in preparation.

nM of TSA might effectively stimulate RECK expression in lung cancer cells.

Moreover, a more significant (about 30-fold) increase of RECK promoter activity was seen in CL-1 cells treated with 250 nM TSA. The lack of apparent toxicity of TSA *in vivo* supports the HDAC inhibitors as potential valid therapeutic agents (20). Collectively, our results suggest that HDAC inhibitors may be potentially useful for the treatment of tumor metastasis via the inhibition of MMPs.

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