Histone Deacetylase 1 Contributes to Cell Cycle and Apoptosis

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Histone deacetylases (HDACs) are generally thought to play important roles in human disease. However, little information is available concerning the specific functions of individual HDACs. We previously reported on transgenic mice that expressed human HDAC1 and experienced steatosis and nuclear pleomorphism in their hepatic tissues. To find out if the over-expression of HDAC1 contributes to the expression of genes related to the cell cycle, apoptosis, and lipid metabolism that eventually contribute to the pathological changes in the livers of the transgenic mice, the expression profiles of the related genes in liver tissues were determined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. The activated human HDAC1 significantly induced the expression levels of mRNA for p53, PPAR-gamma and Bak and reduced the p21 expression level compared with the levels in control littermates. However, the protein levels of p53 and PPAR-gamma were significantly decreased. In conclusion, our results indicate that HDAC1 can regulate gene expression at the mRNA and protein levels independently and that this may be a potential cytopathic factor for hepatic tissue in transgenic mice that over-express HDAC1.

Key words histone deacetylase 1; steatosis; apoptosis; cell cycle

Chromatin structure has a central role in the regulation of gene expression,[1] and several mechanisms play important roles in chromatin remodeling events by the modification of histone amino-termini that extend from the core particles of chromatin. Among these functions, histone acetylation is particularly important because gene activity was first correlated with this event.[2] The status of histone acetylation is controlled by the competition between two classes of enzymes, histone deacetyltransferases (HDACs) and histone acetyltransferases (HATs).[3] These enzymes participate in a variety of cellular processes including transcription, DNA replication, and cell cycle progression. Owing to their important roles in the regulation of these events, it has been suggested that the enzymes that affect the status of histone acetylation are associated with proliferative diseases such as tumors.[4] In fact, HDAC has recently attracted interest as a novel anti-tumor therapeutic target. HDAC inhibitors (HDACi) have been shown to induce growth arrest, differentiation, and apoptosis of cancer cells *in vitro* and *in vivo*.[5]

The class I enzyme HDAC1 was the first mammalian deacetylase identified.[6] Numerous transcription factors, including regulators of the cell cycle, differentiation, and development, have been shown to associate with HDAC1, thereby mediating the repression of specific target genes.[7—9] The disruption of both HDAC1 alleles results in embryonic lethality as a result of severe proliferation defects and retardation in development.[10] Moreover, the loss of HDAC1 leads to significantly reduced overall deacetylase activity, and no alternate pathway replaces it.[10] On the other hand, several types of human cancer are known to have higher HDAC1 activities than normal cells.[11] MS-27-275, a new HDACi that preferentially inhibits HDAC1, was recently identified.[12] Along with TSA, MS-27-275 increased histone H4 acetylation and induced apoptosis in the human colon cancer cell line SW620. Collectively, these findings indicate that HDAC1 has important roles in development and proliferative diseases such as cancer and that it is not replaced by an alternate enzyme.

We have reported on transgenic mice that over-expressed human HDAC1 in many tissues. However, the pathological changes, including steatosis and nuclear pleomorphism, were specifically found in liver tissues.[13] To find out if the over-activation of HDAC1 was a potential factor contributing to the cellular pathological changes through the regulation of the related genes, the expression profiles of the genes related to the cell cycle, apoptosis, and lipid metabolism were determined in this study.

METHODS AND MATERIALS

Experimental Animals We previously reported on transgenic mice that expressed the human HDAC1 gene in many tissues.[13] However, significant pathological changes were only detected in the hepatic tissue of the transgenic mice compared with that of the control littermates. To determine if the over-expression of HDAC1 contributes to gene expression, which may be related to the liver steatosis and nuclear pleomorphism, the hepatic tissues were sampled from HDAC1 transgenic mice at 6 months of age. In addition, to evaluate the data from the liver, splenic tissues [the HDAC1 transgene was expressed at a lower level in splenic tissue than in other tissues according to a reverse transcription-polymerase chain reaction (RT-PCR) assay[13]] were also sampled and evaluated at the same time.

HDAC Activity Assay The nuclear extracts from the tissues were used to detect HDAC activity with a fluorometric detection HDAC assay kit (Upstate U.S.A., Inc., New York) according to the manufacturer's instructions. Briefly, incubation was performed at 37 °C for 30 min with HeLa nuclear cell extracts and the HDAC assay substrate. After 30 min,

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Table 1. Primer Sequences for RT-PCR

Genes	Forward (5′→3′)	Reverse (5′→3′)
HDAC1	ATGACCAACCAGAACACTAACGAG	TCAGGCCAACTTGACCTCTTCTTT
HDAC2	ATGACAAACCAGAACACTCCAGAAT	TCAAGGGTTGCTGAGTTGTTCTGA
HDAC3	ATGGCCAAGACCGTGGCGTATTTC	TCAACTTTCCTTGTCGTTGTCATGG
HDAC4	ATGTCCCTGCACCGCTATGACGA	CTACAGTGGTGGTTCCTCCTCCA
HDAC5	ATGCCCATTGCCCAGGAGTTCTC	TCACAGGGCAGGCTCCTGCTCC
HDAC6	ATGAACAGCTTGATGCTGACACAAG	TTAGTGTGAGTGGGGCATGTCCTC
HDAC8	ATGTCTGATGTTGGCCTGGGGAAA	CTAGACCACATGCTTCAGATTCCC
PPAR-alpha	ATGAACAAGGTCAAGGCCCGGGT	TCAGTACATGTCTCTGTAGATCTCT
PPAR-delta	ATGTCGCACAACGCTATCCGCTTT	GTCATCGAGCTCCAGCGCATTGA
PPAR-gamma	ATGAATTCCTTAATGATGGGAGAAG	GCCTGGGCGGTCTCCACTGAGA
FADD	ATGGAGCTCAAGTTCTTGTGCCGC	TCAGGGTGTTTCTGAGGAAGACAC
Bad	ATGGGAACCCCAAAGCAGCCCTC	GGAGCCTCCTTTGCCCAAGTTTC
Bak	ATGGCATCTGGACAAGGACCAGG	TCATGATCTGAAGAATCTGTGTACC
Bax	ATGAAGACAGGGGCCTTTTTGCTA	TCAGCCCATCTTCTTCCAGATGGT
Bcl-xL	ATGTCTCAGAGCAACCGGGAGCT	TCACTTCCGACTGAAGAGTGAGCC
BcL-2	ATGGCGCAAGCCGGGAGAACAGG	TCACTTGTGGCCCAGGTATGCAC
p53	ATGACTGCCATGGAGGAGTCACAG	CTAGCAGTTTGGGCTTTCCTCCTTG
p21	GTCCAATCCTGGTGATGTCC	GTTTTCGGCCCTGAGATGT
GAPDH	TCCACCACCCTGTTGCTGTA	ACCACAGTCCATGCCATGCCATCAC

Activator Solution was added, and the mixture was incubated for an additional 10 min at room temperature. Fluorescence was measured using a SpectraMax Gemini XS fluorescence plate reader (Molecular Devices) with excitation at 360 nm and emission at 460 nm.

RT-PCR Analysis RT-PCR was performed to detect whether the expression of HDAC1 influences the expression of genes regulated in the cell cycle, apoptosis, and lipid metabolism and the expression of mouse HDACs in hepatic and splenic tissues. Total RNA was isolated from mouse tissues using the TRIzol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA, U.S.A.). RT-PCR was performed using a reverse transcription system (Promega Corp., Madison, WI, U.S.A.) according to the manufacturer's instructions. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a loading control. The primers for mouse HDAC family members (HDAC1, -2, -3, -4, -5, -6, and -8), PPARs (PPAR-alpha, -delta, and -gamma), cell-cycle regulated genes (p53 and $p21^{Waf1/Cip1}$), and apoptosis regulated genes (FADD, Bcl-2, Bcl-xL, Bax, Bad, and Bak) were designed according to the gene sequences obtained from PubMed databases (Table 1). A single band and correctly sized RT-PCR product indicated the target genes.

Western Blotting The whole-tissue lysates were prepared by homogenizing tissues that were freshly retrieved or flash-frozen in extraction lysis buffer. The protein concentration of the cell extracts was measured using the Bradford protein assay (Bio-Rad, Hercules, CA, U.S.A.). Equal amounts (30 μg) of protein were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (IPVH00010, Millipore Corp., Bedford, MA, U.S.A.). The membranes were probed with primary and secondary antibodies, and positive bands were detected by means of an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL, U.S.A.). The antibodies used in Western immunoblotting were anti-p53 (#2524, Cell Signaling, Frankfurt, Germany), anti-PPAR-gamma (#2492, Cell Signaling), anti-GAPDH (Trevigen, Gaithersburg, MD, U.S.A.); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Amersham Biosciences, Buckinghamshire, U.K.), and HRP-conjugated goat anti-rabbit IgG (A9169, Sigma, St. Louis, MO, U.S.A.).

Statistical Analysis The differences between the experimental groups were tested for statistical significance using the Chi-square test and Student's *t*-test. p-values<0.05 were considered to be significant.

RESULTS

Enzymatic Activity of Human HDAC1 Protein in Transgenic Mice Our previous studies have shown that the HDAC1 protein is exclusively located in the hepatocyte nucleus, and the activity of HDAC is significantly higher in the hepatic tissues of the transgenic mice than in the tissues of the control littermates.[13] To confirm if the HDAC activity is significantly increased in other tissues, splenic tissues were selected for HDAC activity assays because the lowest expression of the human HDAC1 transgene occurs in the spleen, compared with expression in the other tissues of transgenic mice.[13] The results showed that the transgenic hepatic and splenic tissues both had significantly higher HDAC activity compared with that in the tissues of normal littermates (Fig. 1). No difference was detected in HDAC activity levels with age or between lineages (data not shown). The expression of human HDAC1 in transgenic mice efficiently and stably increased the HDAC activity. To detect the possible induction of other HDACs by HDAC1 transgene expression, the expression of mouse HDACs was examined by RT-PCR analysis. The results showed that the over-expression of the HDAC1 transgene did not influence the expression of mouse HDACs (HDAC1, -2, -3, -4, -5, -6, and -8) (Fig. 2). This observation confirmed that the significantly higher HDAC activity detected is mainly attributable to the over-expressed HDAC1 transgene.

The Expression of p53 and $p21^{Waf1/Cip1}$ in Transgenic Tissues The deacetylation of the p53 protein by HDAC1 has been shown to play an important role in the regulation of p53 functions.[15] To determine whether this is true for our transgenic mice *in vivo*, the expression of p53 was examined at the mRNA and protein levels. The p53 mRNA level was

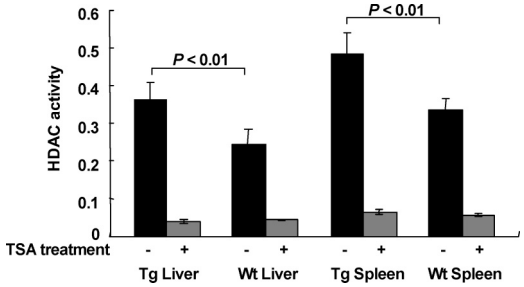

Fig. 1. Comparison of HDAC1 Activity in Hepatic and Splenic Tissues between Transgenic and Normal Littermates at 6 Months of Age

Total nuclear extracts were prepared from non-transgenic and transgenic hepatic and splenic tissues. The soluble (2 mg each) were subjected to HDAC activity assays. The deacetylase activity assay was performed with a fluorescent substrate and measured in the presence or absence of TSA (1 mM). Data are shown as the mean±S.D. of three independent experiments. TSA, trichostatin A; Wt, control littermates; Tg, transgenic mice. ($n \geq 4$)

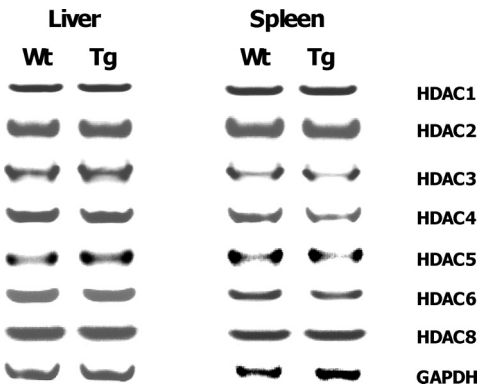

Fig. 2. The Expression of Mouse HDACs in Hepatic and Splenic Tissues of Transgenic Mice

RT-PCR was performed to determine the gene expression at 6 months of age. GAPDH was used as an internal control for RT-PCR. $n=3$ in each group. Wt, control littermates; Tg, transgenic mice.

significantly increased, but the protein level was decreased (Figs. 3A, 4), indicating that over-activated HDAC1 can regulate p53 at the mRNA and protein levels independently. To the best of our knowledge, this is the first time that HDAC1 activity has been shown to regulate p53 transcription. However, the mechanism requires further elucidation. The down-regulation of the protein level of p53 is consistent with the *in vitro* results.[14] Furthermore, $p21^{Waf1/Cip1}$ was significantly decreased in transgenic hepatic tissues according to the RT-PCR assay (Fig. 3B). The decreased $p21^{Waf1/Cip1}$ mRNA level may relate to the decreased p53 protein level. Another possibility is that HDAC1 may regulate $p21^{Waf1/Cip1}$ transcription directly.[5] Associated with the pathological changes of the transgenic liver, nuclear pleomorphism may be influenced by HDAC1 through the regulation of cell proliferation by the $p53/p21^{Waf1/Cip1}$ signaling pathway. In splenic tissue, the expression of p53 was significantly increased at the mRNA level in transgenic mice (Fig. 3A). However, the expression of $p21^{Waf1/Cip1}$ was not affected (data not shown), and no pathological changes were detected in the splenic tissues of our transgenic mice, which indicated that liver cells are more sensitive to the $p53/p21^{Waf1/Cip1}$ signaling pathway and that the function of HDAC1 may vary depending on the cell type.

The Expression of PPARs Peroxisome proliferation-activated receptors (PPARs) have been shown to have important

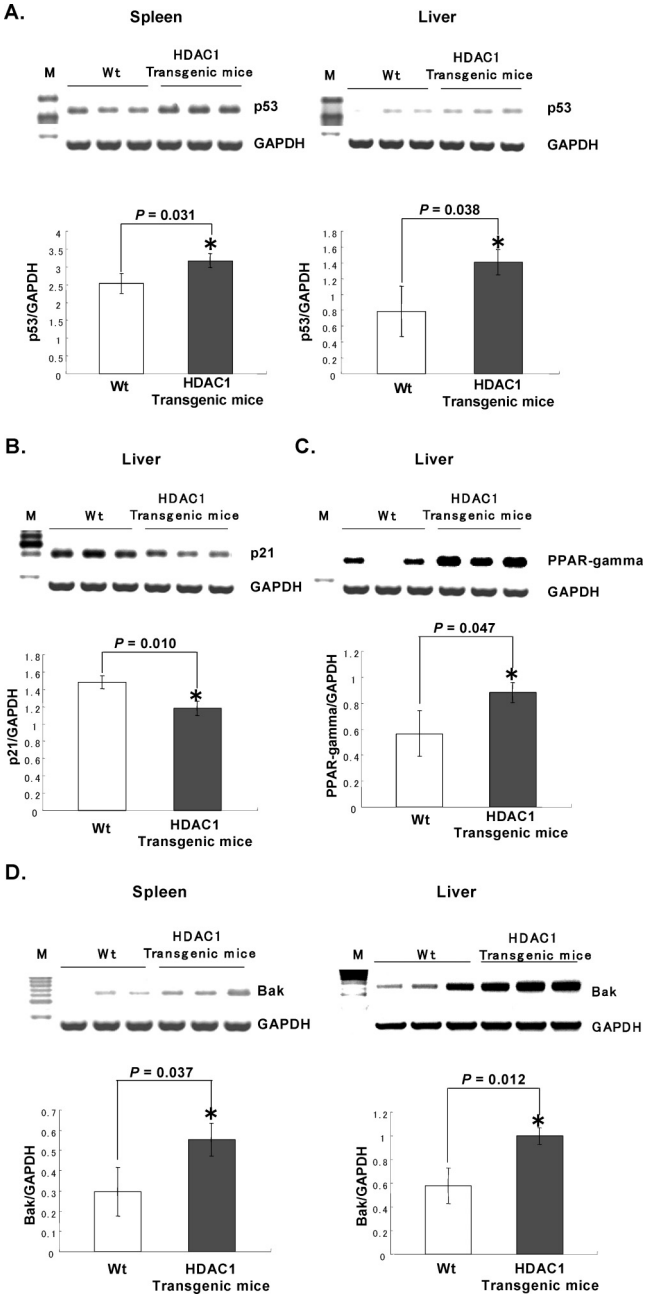

Fig. 3. The Expression of Genes Related to Cell Cycle, Apoptosis, and Lipid Metabolism

To determine the expression levels of p53 (A), p21 (B), PPAR-gamma (C) and Bak (D), RT-PCR was performed using total RNAs extracted from mice tissues of 6 months of age. GAPDH was used as an internal control for RT-PCR (top). And the results were quantified by densitometry and analyzed by Student's *t*-test (bottom). * Indicates significant difference between control littermates and HDAC1 transgenic mice. $n=3$ in each group. Wt, control littermates; Tg, transgenic mice.

functions related to steatosis in the liver.[15,16] Therefore, we determined the expression level of PPAR (alpha, delta, and gamma) genes. There were no changes in the expression of PPAR-alpha and -delta in hepatic tissues, but the expression of PPAR-gamma was significantly increased at the mRNA level and decreased at the protein level (Figs. 3C, 4). There was no difference detected in the expression of PPAR-delta protein in the liver (data not shown). In splenic tissues, the expression of PPAR-alpha and -gamma was barely detectable, and there was no difference in the expression of the PPAR-delta gene at the mRNA level (data not shown). These

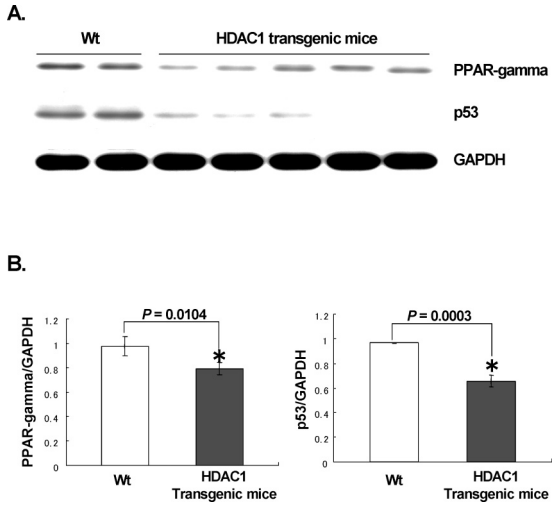

Fig. 4. The Protein Levels of PPAR-gamma and p53 in Liver Tissues as Determined by Western Blot Assay

(A) Western blot analysis for PPAR-gamma and p53 in liver tissues of mice at 6 months of age. GAPDH was used as an internal control. (B) The immunoblots were quantified by densitometry and analyzed by Student's *t*-test. * Indicates significant difference between control littermates and HDAC1 transgenic mice. Wt, control littermates; Tg, transgenic mice.

observations indicate that the activation of HDAC1 can regulate PPAR-gamma at the mRNA and protein levels independently in hepatic tissues. However, the mechanisms remain to be elucidated.

The Expression of Genes Related to Apoptosis HDAC1 has been shown to influence apoptosis.[5] To find out if HDAC1 regulates apoptosis by controlling the expression of apoptosis-related genes, the genes were screened by RT-PCR analysis. The results showed that Bak were significantly increased in hepatic and splenic tissues (Fig. 3D). However, no changes in expression were detected for the FADD, Bcl-2, Bcl-xL, Bad, and Bax genes (data not shown). These expression profiles are almost the same between hepatic and splenic tissues. These results indicate that the regulation of apoptosis-related genes by activated HDAC1 may be a common event independent of cell type and that this regulation may play a potential role in the apoptotic process.

DISCUSSION

Our *in vivo* studies indicate that the over-expression of HDAC1 as a transgene can induce significantly higher levels of HDAC1 activity, even in splenic tissues. A large body of literature suggests that the functions of multiple nuclear factors are dependent on the recruitment of HDAC1 activity.[7] The significantly higher HDAC activity detected in the tissues of our transgenic mice indicates that the over-expressed human HDAC1 effectively formed a functional complex with other cofactors to produce its activity. However, this high level of HDAC1 enzymatic activity was not cytotoxic to most cell types *in vivo*, as shown by the absence of developmental defects in our transgenic mice. In addition, most of the tissues examined in our experiments were largely normal histologically through 18 months of age, except for the liver.[13] However, the *in vitro* data suggest that the tightly controlled cell type-specific expression of HDAC1 may be crucial for the regulation of unrestricted proliferation.[10,17] Consequently, based on the results of our *in vivo* data, we propose that HDAC proteins, or HDAC1 in particular, might act primarily as an important modulator of other mechanisms involved in development and in proliferative diseases such as tumorigenesis. As such, the over-expression of HDAC alone would not directly induce disease, but the activity of other molecular regulators would be required. This hypothesis is also supported by the observation that HDAC is over-expressed in most cancer cells, as compared with the expression levels in normal cells.[18] The HDAC inhibitors with anti-cancer activity may disrupt the functions of HDACs that lead to cancer progression in tumor cells.

The chromatin structure has a central role in the regulation of gene expression,[1] and histone deacetylation is particularly important to gene activity.[2] In addition, HDAC1 has been shown to regulate protein levels through the deacetylation of proteins.[14] In this study, we showed that over-activated HDAC1 can regulate gene expression at the mRNA and protein level independently for p53 and PPAR-gamma. Our *in vivo* data indicate that HDAC1 is an important factor in the regulation of gene expression at the levels of transcription and post-transcription. The influence of activated HDAC1 on the expression of genes related to the cell cycle, apoptosis, and lipid metabolism suggests the potential of HDAC1 to participate in pathological cellular changes. However, the observation that only hepatic tissues showed pathological changes indicates that liver cells are more sensitive to over-activated HDAC1.

The development of significantly higher steatosis in the transgenic mice indicates that HDAC1 might be an important factor in the regulation of lipid metabolism. This could be achieved mainly through the regulation of lipid metabolism-related genes. Adipogenesis, glucose homeostasis, and lipid metabolism are the major mechanisms of PPAR-gamma involved in the improvement of insulin resistance.[15,16] However, we detected a significant decrease of PPAR-gamma at the protein level. Although the mechanism remains to be elucidated, the observation suggests that the fatty liver detected in our HDAC1 transgenic mice may occur through other pathways, besides the PPAR-gamma pathway.

Interestingly, we detected the over-expression of p53 and the down-regulation of p21 in transgenic hepatic tissues, indicating that HDAC1 may regulate cell proliferation through the p53 and $p21^{Waf1/Cip1}$ signal pathways. This may be one mechanism related to the pleomorphism detected in the transgenic livers and is the first reported observation that over-activated HDAC1 can regulate p53 and $p21^{Waf1/Cip1}$ at the mRNA level *in vivo*. Further research is needed to understand the mechanism.

In conclusion, our *in vivo* studies showed that HDAC1 can regulate gene expression at the level of transcription and post-transcription. HDAC1 influences the expression of genes related to the cell cycle and apoptosis that have broad impacts, supporting an important role for HDAC1 in proliferative diseases.

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