Induction of PIG3 and NOXA through Acetylation of p53 at 320 and 373 Lysine Residues as a Mechanism for Apoptotic Cell Death by Histone Deacetylase Inhibitors

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ABSTRACT

Two controversial issues regarding p53 are whether it is involved in apoptosis induction of tumor cells by a histone deacetylase (HDAC) inhibitor and, given that p53 is indeed involved, which genes of acetylated p53 targets are responsible for giving rise to apoptotic death. We, in the present study, first confirmed that some substantial extent of apoptotic cell death was seen when p53-deficient cells (KATO-III) were transfected with wild-type p53 and treated with sodium butyrate (SB) or trichostatin A. By Western blotting, using specific antibodies, we then demonstrated that residues 320, 373, and 382 lysines of p53 were acetylated in KATO-III cells transfected with wild-type p53 (KATO-III/p53) treated with a HDAC inhibitor. However, as revealed by terminal deoxynucleotidyl transferase-mediated nick end labeling staining, only those KATO-III cells transfected with K320R p53 or K373R p53 became insensitive to the HDAC inhibitor, suggesting that these two residues of p53 may be essential for HDAC inhibitor-induced apoptosis, whereas others such as K382R p53 may not. Furthermore, reverse transcription-PCR demonstrated that among various p53-related proapoptotic genes, expression of PIG3 and NOXA was clearly enhanced by SB treatment in KATO-III/p53 cells but not in KATO-III/K320R or KATO-III/K373R cells. Finally, we revealed that apoptosis could be evoked by SB even in cells where p53 mutations occur at residues other than 320 lysine or 373 lysine (TMK-1 and HSC-39 cells) and that this apoptosis was significantly, although not totally, suppressed by the anti-p53 antisense. It was, therefore, concluded that acetylation of the p53 molecule at residues 320 and 373, giving rise to up-regulation of PIG3 and NOXA, is one of the mechanisms for induction of apoptosis by HDAC inhibitors in cancer cells.

INTRODUCTION

Histone deacetylase (HDAC) inhibitors have been shown to exert various antitumor effects such as growth inhibition (1, 2), induction of apoptosis (3–8), induction of differentiation (9, 10) and so forth, and have been recently applied to some clinical studies such as Phase I study of suberoylanilide hydroxamic acids in patients with advanced solid tumors or hematological malignancies (11) and Phase II study of FR901228 in patients with cutaneous T-cell lymphoma or relapsed peripheral T-cell lymphoma. The mechanism underlying these antitumor effects of the HDAC inhibitor is the hyperacetylation of histone (12–14) and/or other factors, thereby bring about conformational change of these proteins to up- or down-regulate the relevant genes. In particular, the release of cytochrome c (9) and activation of caspase-3 (4), which are induced by either the enhancement of Fas (3), Bax (4), or Bcl-2 (5) expressions or suppression of Bcl-3 (6) or Bcl-xL (7, 8) expressions, have been well elucidated. However, involvement of p53, one of the most potent proapoptotic molecules, in tumoricidal effects induced by the HDAC inhibitor has been a controversial issue.

Recently, Luo et al. (15) have demonstrated that apoptosis of a p53-defective human lung cancer cell line, H1299, was induced when the cell was transfected with wild-type p53 and treated with trichostatin A (TSA), a well-known HDAC inhibitor, which inhibits deacetylation of 373 and/or 382 lysine residues of p53, causing an increase of p21/Waf1 expression. Furthermore, substitution of lysine residues 370, 372, 373, 381, and 382 with arginine resulted in an 80% reduction of p21/Waf1 inducibility of wild-type p53 with HDAC inhibitor treatment. Szak et al. (16) showed that acetylation of p53 at 382 lysine and the actual binding of the acetylated p53 to promoters of p21/Waf1, MDM2, and PIG3 genes occurred in a human colon cancer cell line, RKO, which expresses wild-type p53.

Similarly, by applying UV or ionizing radiation to a human osteosarcoma cell line, SAOS-2, which is also p53 defective, and treating it with mutant p53, of which lysine residues 319, 320, 321, 370, 372, 373, 381 or 382 were replaced with arginine, Liu et al. (17), proved that the 320 and 373 lysine residues are indeed targets for acetylation. In concurrence with the above findings, Juan et al. (18) demonstrated a direct binding of HDAC-1, HDAC-2, and HDAC-3 isozymes to p53, deacetylation of 372 and 382 lysine residues by HDAC-1, and subsequent suppression of the Bax gene transcription in HDAC transfectants. Likewise, two independent groups, Vaziri et al. (19) and Luo et al. (20) showed that transfection of another type of HDAC gene, hSir2, brought about deacetylation of 373 and 382 lysine residues to counteract p21/Waf1 expression or apoptosis induced by radiation or etoposide treatment.

On the other hand, Nakamura et al. (21) reported that transfection of mutant p53 plasmids, of which lysine residues at 372, 373, 381, and 382, were replaced with alanines, histigines or asparagines resulted in induction of p21/Waf1, suggesting that acetylation or deacetylation of these lysine residues does not influence transcription of p53. In addition, Espinosa et al. (22), using an in vitro transcription assay of p21/Waf1, found the same results with wild-type p53 as with a mutant p53, of which lysine residue at 320, 372, 373, 381, and 382 were replaced with arginine.

Furthermore, Barlev et al. (23) found that after irradiation, H1299 cells showed no appreciable difference in the binding of mutant p53, of which lysine residues at 320, 373, 381 or 382 were replaced with arginine to the p21/Waf1 promoter, yet they did find an impairment of the p21/Waf1 inducibility of these mutated p53 as compared with that of wild-type p53.

In the present study, we therefore attempted to clarify if the p53 molecule is indeed involved in HDAC inhibitor-induced apoptosis and, if so, which acetylated lysine residues of p53 and which target genes of acetylated p53 are responsible for apoptotic cell death by the HDAC inhibitor.
MATERIALS AND METHODS

Reagents. Sodium butyrate was obtained from Wako (Osaka, Japan). TSA was from Sigma (St. Louis, MO). Z-DEVD-FMK (caspase 3 inhibitor) was purchased from KamiyBi Chemical Company (Seattle, WA).

Cell Cultures. Three cell lines derived from human gastric carcinoma were used. KATO-III (24), a human signet ring cell carcinoma cell line with deleted p53, was purchased from American Type Culture Collection. TMK-1 (24), a poorly differentiated, human adenocarcinoma cell line with mutant (V173M) p53 and HSC-39 (25), a human signet ring cell carcinoma with mutant (G24S) p53, were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). These cells were maintained in a humidified atmosphere at 37°C and 5% CO2.

Recombinant Adenoviruses and Infection. Adenovirus-p53 and adenovirus-p53ΔN24 were generated as we reported previously (26). Briefly, an E1-deleted, replication-defective, recombinant adenovirus was constructed with the use of a modified type 5 adenovirus genome. The cytomegalovirus promoter was used to drive the transcription of human wt-p53 or β-galactosidase cDNA. For adenovirus gene transfer, cells were incubated with 100 multiplicity of infection of adenovirus constructs at 37°C for 24 h.

Plasmids and Transfection. pC53-SN3 from a cytomegalovirus promoter and which expresses wild-type human p53 was kindly provided by Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). Mutant vectors, p53-K320R, p53-K373R, and p53-K382R were constructed from pC53-SN3 by replacing lysine at codon 320, codon 373 and codon 382, respectively, with arginine. The changes were confirmed by DNA sequencing analysis. In brief, the QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions to introduce a single nucleotide change from lysine to arginine using the following primers: oligo-nucleotides 5'-CTCCATCGCCGAGGAAGGGACATCTGATGAGG3'- and 5'-CTCCATCGCCGAGGAAGGGACATCTGATGAGG3'- for codon 320; 5'-ACTCCAGGCACCTGAGAAGGGGTCACTG3'- and 5'-ACT-GAC CCTTTCTTCTGCTGAGTCACTG3'- for codon 373; 5'-CTCACTCCGGCCCAATAAAAGACATCTGATGAGG3'- and 5'-CTCTTGAACA-TGAAGCTTTATGCGGAGGAT3'- for codon 382. The changes were confirmed by DNA sequencing analysis.

For transfections, 2 × 105 cells were seeded on each well of a 6-well dish and transfected with Lipofectamine 2000 (Life Technologies, Inc.). Cells were harvested 24 h after transfection.

Detection of Apoptosis. Apoptosis was detected by three independent methods: terminal deoxynucleotidyl transferase-mediated deoxyuridinethosphate nick-end labeling; Annexin V staining; and DNA ladder assays.

Terminal deoxynucleotidyl transferase-mediated deoxyuridinethosphate nick-end labeling assay (in situ terminal transferase-mediated dUTP nick end labeling) was performed using an in situ Apoptosis Detection kit (TakaRa) as suggested by the manufacturers. The DNA ladders from cells, purified by the DNeasy Tissue System (Qiagen, Valencia, CA), were analyzed by electrophoresis on 2.0% agarose gels containing ethidium bromide (1 μg/ml).

Phosphotyrosine positive cells were detected by an Annexin-V FITC Apoptosis Detection kit (Medical Biological Laboratories Co. Nagoya, Japan) according to the manufacturer’s protocol. Cells were analyzed by flow cytometry on a FACScan using CellQuest software (Becton Dickinson, San Jose, CA).

Immunofluorescent Staining. Transiently transfected KATO-III cells with wild-type p53, p53-K320R, p53-K372R, or p53-K382R were plated on poly-L-lysine-coated multiwell chamber slides (Becton Dickinson). Then cells were fixed with 4% paraformaldehyde in PBS at ambient temperature, permeabilized with 0.1% Triton X-100 in PBS for 3 min at 4°C. Cells were covered with blocking solution (10% BSA in PBS) for 60 min at room temperature. The slides were then treated with p53 monoclonal antibodies (pAb1801; Oncogene Science) and with a secondary antibody conjugated to rhodamine and viewed on a confocal microscope MRC1024ES (Bio-Rad).

Western Analyses. Adherent and detached cells were collected and suspended in chilled lysis buffer: 50 mM HEPES (pH 7.0), 2 mM MgCl2, 150 mM NaCl, 0.1 mM EDTA, 2 mM Na2VO4, 10 mM Na3P04, 10 mM NaF, 1.0% NP40, 0.5 mM p-amidinophenyl methanesulfonyl fluoride hydrochloride and a protease inhibitor mixture consisting of 2.5 μg/ml pepstatin A, 2.5 μg/ml antipain, 2.5 μg/ml chymostatin, and 0.25 μg/ml leupeptin. Collected cells were allowed to lyse by sonication on ice. The homogenate was centrifuged for 15 min in a microcentrifuge at 4°C, and the supernatants were collected and boiled in SDS sample buffer. Each 30 μg of SDS sample was loaded onto 4 to 20% gradient SDS-PAGE gels and blotted onto an Optiprep BA-S 85 membrane (Schlischer and Schuell, Dassel, Germany) and blocked with 5% (w/v) nonfat dry milk in PBS (100 mM sodium phosphate (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20). Membranes were incubated with the following primary antibodies: anti-p53 DO-1 (Oncogene Research Products, Cambridge, MA); anti-p21/Wan antibody (Transduction Laboratories); anti-acetylated p53 (Lys389) antibody (Upstate Biotechnology, Lake Placid, NY); anti-acetylated p53 (Lys389) antibody (Upstate Biotechnology); and anti-acetylated p53 (Lys389) antibody (Oncogene Research Products). Subsequently, membranes were incubated with goat antimouse- or goat antirabbit-horseradish peroxidase (Amersham) and visualized by enhanced chemiluminescence (Amersham). Band intensities were evaluated by NIH Imaging.

Semiquantitative Reverse Transcription-PCR Analysis. Isolation of total RNA from cells was performed using RNeasy spin column kits (Qiagen) according to the manufacturer’s instructions. cDNAs were synthesized from 5 μg of total RNAs with the SuperScript Preamplification System (Life Technologies, Inc.). The reverse transcription-PCR exponential phase was determined on 18–36 cycles to allow semiquantitative comparisons among cDNAs developed from identical reactions. Each PCR regime involved an initial denaturation step at 94°C for 5 min followed by a number of cycles predetermined for each type of cDNA: 20 cycles for p53 and GAPDH; 27 cycles for PIG3; 28 cycles for NOXA; 32 cycles for p53AIP1; 31 cycles for PUMA; 30 cycles for Bax; 25 cycles for Killer/DR5; 26 cycles for Fas; and 22 cycles for p21/Waf1. All samples were then processed on a Gene Amp PCR system 2400 (Perkin-Elmer) at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min.

Primers sequence design were as follows: forward, 5'-CACAGCTGCTG-GATCAATAC-3'; reverse, 5'-GCTGATGTCCTTGTGGG-3'; forward, 5'-AGATGCCTGGAGAAGCGAGCG-3'; and reverse, 5'-AGTCCAGCTCAGCAAGT-3'. The DNA ladders from cells, purified by the DNeasy Tissue System (Qiagen, Valencia, CA), were analyzed by electrophoresis on 2.0% agarose gels containing ethidium bromide (1 μg/ml).

Phosphatidylserine positive cells were detected by an Annexin-V FITC Apoptosis Detection kit (Medical Biological Laboratories Co. Nagoya, Japan) according to the manufacturer’s protocol. Cells were analyzed by flow cytometry on a FACScan using CellQuest software (Becton Dickinson, San Jose, CA).

RESULTS

Induction of p53-Dependent Apoptosis in KATO-III Cells by Sodium Butyrate (SB) and TSA. KATO-III cells were transfected in vitro with human wild-type p53 cDNA by exposure to adenovirus-p53. Western blotting analysis (inset in Fig. 1A) showed a high level of p53 protein expression as early as 24 h after infection of adenovirus p53 (KATO-III/p53), but no p53 was detected in parental (uninfected) cells or control cells infected with adenovirus nLacZ (KATO-III/nLacZ) cells. Concurrent immunohistochemical examination demonstrated wild-p53 protein in >90% of KATO-III/p53 cells, suggesting that the transfer and expression of p53 by adenovirus p53 was highly efficient (data not shown).

We incubated KATO-III cells, KATO-III/nLacZ cells, or KATO-

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II/p53 cells with various concentrations of SB or TSA for 48 h and counted their cell numbers. As shown in Fig. 1A, the number of parental KATO-III cells very mildly decreased as the concentration of SB increased. KATO-III/nLacZ cells also showed mild decrement but with a slightly greater degree than parental KATO-III cells. KATO-III/p53 cells were clearly more sensitive to SB than KATO-III or KATO-III/nLacZ cells, showing an 80% reduction of cell number at 2.5 mM SB. When we treated three types of cells with TSA, the pattern of cell number reduction (35% reduction for parental KATO-III, 50% for KATO-III/nLacZ, and 95% for KATO-III/p53 with 25 mM TSA) was similar to that observed with SB-treated cells, although the former agent was apparently more potent than the latter.

We then performed a DNA ladder assay to analyze all of the cell types treated for 2 days with either 2.5 mM SB or 25 mM TSA for their apoptotic cellular events as determined by Annexin V assay. As shown in Fig. 2, p53 expression indeed increased to ~110% of the original level after 6 h of SB treatment and to 140% after TSA treatment, and these levels were closely maintained until 18 h. However, the apoptotic cellular event as determined by Annexin V assay showed a substantial increment of up to 500% until 18 h, nonparallel with p53 increment during this period.

Fig. 1. Effect of HDAC inhibitors (SB or TSA) on survival (A, B, and D) and DNA ladder formation (C) of KATO-III/p53 cells. A and B, parental KATO-III cells (●), KATO-III cells infected with adenovirus-coding nLacZ (KATO-III/nLacZ, □), and adenovirus-coding wild-type p53 (KATO-III/p53, ■) were treated with either SB (4) or TSA (B) for 48 h. Insets, Western blots for p53 in parental, KATO-III/nLacZ, and KATO-III/p53 cells. C, parental KATO-III, KATO-III/nLacZ, and KATO-III/p53 treated for 48 h with either 2.5 mM SB or 25 mM TSA were examined for their apoptotic cell death by DNA ladder assay as described in “Materials and Methods.” D, parental KATO-III (○), KATO-III treated with 2.5 mM SB (●), KATO-III treated with 25 mM TSA (□), KATO-III/nLacZ (■), KATO-III/nLacZ with 2.5 mM SB (△), KATO-III/nLacZ with 25 mM TSA (△), KATO-III/p53 with 2.5 mM SB (●), and KATO-III/p53 with 25 mM TSA (□) were plated at 5 × 10^4/well and cultivated for 4 days. Viable cell numbers were determined as described in “Materials and Methods.”

Increase of Annexin V Positivity, Nonparalleling with p53 Increment in KATO-III/p53 Cells Treated with HDAC Inhibitor. We then examined if the p53 protein level was somehow increased by HDAC inhibitor treatment, thereby inducing apoptosis in KATO-III/p53 cells. As shown in Fig. 2, p53 expression indeed increased to ~110% of the original level after 6 h of SB treatment and to 140% after TSA treatment, and these levels were closely maintained until 18 h. However, the apoptotic cellular event as determined by Annexin V assay showed a substantial increment of up to 500% until 18 h, nonparallel with p53 increment during this period.

SB and TSA-Enhanced Acetylation Status of 320, 373, and 382 Lysine Residues of p53 in KATO-III/p53 Cells. Because residues at 320, 373, and 382 are able to undergo acetylation, each by its respective enzyme PCAF or CBP/p300 (17, 27, 28), we examined the acetylation status of these lysine residues by Western blotting using a specific antibody for each residue. Both SB and TSA treatment of KATO-III/p53 cells for 60 or 120 min resulted in significant increment of acetylation at all of the residues (Fig. 3, A and B).

KATO-III Cells Transfected with K320R p53 or K373R p53 but not K382R p53 Are Insensitive to SB-Induced Apoptosis. To explore which acetylated residue(s) is responsible for induction of apoptosis by the HDAC inhibitor, we constructed mutated p53 plasmids by replacing lysine at 320, 373, or 382 with arginine and transfected them into KATO-III cells. As shown in Fig. 4, the K382R transfectant (KATO-III/K382R) but not those of K320R (KATO-III/K320R) or K373R (KATO-III/K373R) showed apoptotic change by SB treatment as revealed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining, indicating the necessity of 320 lysine and 373 lysine in SB-induced apoptosis.

Induction of PIG3 and NOXA by Acetylated p53 in KATO-III/p53 Cells Treated with SB. We next investigated the target gene(s) of acetylated p53, which may be responsible for apoptosis in KATO-III/p53 cells treated with SB. As shown in Fig. 5, among the mRNA

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of various proapoptotic molecules, including PIG3, NOXA, p33AIP1, PUMA, Bax, Killer/DR5, and Fas, only the former two were increased with SB treatment, although the mRNA expression of all other molecules were up-regulated in KATO-III/p53 cells. Incidentally, in parental KATO-III cells, expression of p21/Waf-1 was induced by a p53-independent mechanism, and the Bax gene was also expressed, even without SB treatment.

**Induction of PIG3 and NOXA Expression by Acetylation of p53 at 320 and 373 Lysine Residues.** To validate the role of 320 lysine and 373 lysine in induction of PIG3 and/or NOXA mRNA by SB, we analyzed both mRNAs in KATO-III/K320R and KATO-III/K373R transfectants treated with SB by RT-PCR (Fig. 6). As a positive control, KATO-III/K382R and KATO-III/wild p53 were also analyzed. In KATO-III/wild p53 and KATO-III/K382R cells, expression of PIG3 and NOXA mRNA were increased by SB treatment, whereas in KATO-III/K320R and KATO-III/K373R cells, increment of neither mRNA was observed.

**Effect of Caspase-3 Inhibitor on Induction of PIG3 and NOXA Expression by SB.** To confirm that induction of PIG3 and NOXA expression by SB are not the result of apoptosis, we treated KATO-III/p53 cells with a caspase-3 inhibitor (Z-DEVD-FMK). First we verified that Z-DEVD-FMK effectively inhibited Annexin V positivity of cells treated with SB (Table 1). Then we examined the effect of

<table>
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<th>Z-DEVD-FMK (+)</th>
<th>(% inhibition)</th>
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Table 1 Effect of caspase 3 inhibitor on sodium butyrate (SB)-induced apoptosis in KATO-III/p53 cells

SB treatment

**Fig. 5.** Effect of SB on mRNA expression of p53-related proapoptotic genes in KATO-III/p53 cells. KATO-III cells were infected with adenovirus wild-type p53 for 24 h, then treated with SB for 120 min. RNA were isolated, cDNA were synthesized, and mRNA expression was examined by semiquantitative reverse transcription-PCR as described in “Materials and Methods.”

Fig. 6. Effect of SB on the expression of PIG3 and NOXA mRNA in KATO-III cells transfected with K-R mutant p53. Reverse transcription-PCR analyses were performed as described in “Materials and Methods.”

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this agent on PI3G and NOXA expression. As shown in Fig. 7, expression of PI3G and NOXA were unchanged by Z-DEVD-FMK treatment. Thus, induction of PI3G and NOXA were not ascribed to the result of apoptosis.

Effect of SB on Cell Viability of TMK-1 and HSC-39 Cells. To examine if SB is effective even on tumor cells that have p53 mutated at residues other than 320 lysine or 373 lysine, we treated TMK-1 cells, which have mutations at 173 (valine to methionine), and HSC-39 cells, which have mutations at 245 (glycine to serine), with SB. As shown in Fig. 8, SB dose dependently reduced the numbers of both types of cell. To confirm that this effect was evoked through the activity of p53, we transfected antisense p53 into these cells. Anti-sense transfectants of both types of cells, which express much less p53 than do the control neomycin-resistant (neo) gene transfectants, exhibited poor sensitivity to SB as compared with parental cells or neo-gene transfectants.

Acetylation of Lysine Residues in SB-Treated TMK-1 and HSC-39 Cells. To elucidate whether SB indeed inhibits the deacetylation of the lysine residue of p53 in TMK-1 and HSC-39 cells as was found in KATO-III/p53, we conducted Western blot analyses using specific antibodies to acetylated lysine of the p53 molecule. As shown in Fig. 9A, enhanced acetylated lysine at 320, 373, and 382 residues was clearly observed in these cells after SB treatment.

Induction of PI3G and NOXA mRNA Expression in SB-Treated TMK-1 and HSC-39 Cells. We then examined PI3G and NOXA mRNA expression in TMK-1 and HSC-39 cells treated with SB (Fig. 9B). In these cells, both messages were markedly increased after SB treatment.

DISCUSSION

In the present study, we used the p53-defective cell line KATO-III because it does not undergo apoptosis by forced expression of p53 alone and allows us to introduce p53 and elucidate the effect of the HDAC inhibitor per se. We thus confirmed that when the cell was transduced with p53 and treated with an HDAC inhibitor, p53 was indeed involved in HDAC inhibitor induced apoptosis, although a p53-independent mechanism was indicated by a mild but distinct apoptosis in parental or mock-transfected KATO-III cells (3–8).

Two possible mechanisms for induction of apoptosis by the HDAC inhibitor via p53 are increased p53 levels and activation of p53 functions. p53 may increase by stabilization after acetylation and subsequent detachment from the MDM-2 protein, which accelerates the ubiquitination and degradation of p53. In our study, the p53 level was increased by the HDAC inhibitor but only 1.3–1.5 fold, whereas apoptosis increased ~5-fold, as shown by Annexin V positivity, which suggests that increased levels of p53 had little, if any, effect on apoptosis induction.

Lysines 320, 373, and 382 have been shown to be target residues for histone acetylases such as PCAF (17, 27) and p300/CBP (28) and HDACs such as hSir2 (19, 20) and HDAC-1 (21), and we found that all three of these lysine residues were acetylated by both SB and TSA treatment, indicating that they block the activity of the HDAC-1, HDAC-2, and HDAC-3 deacetylases. This agrees, in part, with findings by Luo et al. (15), who showed acetylation of 373 and 382 residues, but not the 320 residue, by TSA treatment of p53-transfected SAOS2 and H1299 cells.

We then transfected lysine (K)-arginine(R) mutants of these residues into KATO-III cells. Because arginine cannot be acetylated (15, 17, 21, 22, 23), these transfected cells resist apoptosis induced by the HDAC inhibitor, and in fact, we found impaired sensitivity in K320R and K373R transfectants but not in K382R. This finding apparently contradicts previous studies that transfectants of K-R mutant residues, including that of 382, showed impaired expression of p21/Waf1 mRNA on irradiation (23) or TSA treatment (15). However, as p21/Waf1 is generally accepted as a molecule that causes cell cycle arrest, we believe that involvement of the 382 residue in apoptotic cell death is unlikely. The relevance of acetylation of p53 in p21/Waf1 induction is also controversial. Unlike the above studies, Espinosa et al. (22) and Nakamura et al. (21) both showed no increment of p21/Waf1 in transfectants of the K-R mutants of 370, 372, 373, and 382 residues. We also found no enhancement of p21/Waf1 expression by SB treatment in KATO-III/p53, although induction of p21/Waf1 by p53 transfection alone was observed in parental KATO-III cells. Of interest is that in parental KATO-III cells, Sp1 site-mediated induction of p21/Waf1 by the HDAC inhibitor alone is generally accepted occurrence (29, 30). These discrepant results may be explained by assuming that for full expression of p21/Waf1, such a highly acetylated state of p53, as occurs with HDAC inhibitor, may be unnecessary because a basal
acetylation state as evoked by an endogenous acetylation enzyme may be sufficient. In other words, p21/Waf1 induction of p53 may quickly plateau. Nevertheless, target genes of acetylated p53, which are truly responsible for apoptotic death induced by the HDAC inhibitor, have not yet been identified.

Seven proapoptotic genes, P53AIP1 (31), PUMA (32, 33), Bax (34), Killer/DR5 (35), Fas (36), PIG3 (37), and NOXA (38), are reported to be targets of p53 of which only two, PIG3 and NOXA, were induced in KATO-III/p53 by the HDAC inhibitor. p53AIP1, PUMA, Fas, and Killer/DR5 were induced by p53 transfection alone, but their expression was not enhanced by the HDAC inhibitor. Bax was expressed even in untreated parental KATO-III cells. Thus, we consider the increment of PIG3 and NOXA to be an acetylated p53-specific event, indicating that these two genes are strong candidates as targets for acetylated p53 to induce apoptosis. The results of reverse transcription-PCR of PIG3 and NOXA in KATO-III/K320R and KATO-III/K373R clearly support this concept.

The fact that KATO-III did not undergo apoptosis by transfection with p53 alone despite induction of p53AIP1, PUMA, and Bax may be explained by postulating that these molecules have different modes of action from PIG3 and NOXA, although all of the factors are known to function in mitochondria (29, 32–35, 38). A detailed elucidation of the functional differences between these molecules is an important future study.

Given that residues 320 and 373 are crucial to the induction of apoptosis, cells with other p53 mutations should be sensitive to the HDAC inhibitor. Accordingly, we additionally examined the effect of the HDAC inhibitor on TMK-1 and HSC-39 cells, having mutations at 173 and 245, respectively. Because acetylation of 320 and 373 residues occurred in response to HDAC inhibitor treatment and antisense p53 reduced the sensitivity of these cells to the HDAC inhibitor, it is reasonable to assume that mutated p53 functions as a target for the inhibitor to induce apoptosis, probably through acetylation of the 320 and 373 residues. In this respect, our results are compatible with previous studies (39, 40) that some p53 mutants retain the ability to transactivate a subset of p53 target genes. We still cannot exclude a p53-independent mechanism for inducing apoptosis by the HDAC inhibitor as the reduction in sensitivity to it by antisense p53 was incomplete. Our results are also compatible with another report of HDAC inhibitor-induced apoptosis in tumor cells with mutated p53 (41), although the authors of that study suggested it was a p53-independent event.

Recently, Oda et al. (32) demonstrated that phosphorylation of Ser46 on p53 specifically induced the transcriptional activation of proapoptotic gene p53AIP1. This is an analogous regulatory system of a specific activation of p53 and selective induction of its downstream gene(s). Results of this study and our data imply that the fate of a cell, i.e., arrest or death, partly depends on the selection of downstream target genes to be activated and moreover that the type of modification (phosphorylation or acetylation) of p53 protein is likely to be associated with this selectivity.

In conclusion, we suggest that HDAC inhibitor activates p53 by acetylation of 320 and 373 lysine residues, up-regulates PIG3 and NOXA expression, and can induce apoptosis in cancer cells with both wild and pseudo-wild-type p53 genes.

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REFERENCES

HDAC INHIBITORS INDUCE APOPTOSIS VIA ACETYLATION OF p53


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Induction of PIG3 and NOXA through Acetylation of p53 at 320 and 373 Lysine Residues as a Mechanism for Apoptotic Cell Death by Histone Deacetylase Inhibitors

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