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FK228 Induces G1 Arrest/apoptosis Via Microphthalmia-associated Transcription Factor Modulation in Malignant Melanoma

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ABSTRACT

Histone deacetylase (HDAC) inhibitors are novel anticancer agents that might be used to treat melanoma because their mechanisms of action are distinct from those of conventional anticancer agents. Using human melanoma cells we examined signaling mechanism(s) involved in the action of an HDAC inhibitor, FK228. The addition of FK228 elicited G1 arrest and apoptosis in all 4 cell lines tested. However, there were differences in the apoptotic effects, with cells having higher expression of microphthalmia-associated transcription factor (MITF), Bcl2, and CDK2 also having stronger apoptotic effects. The expression of MITF was markedly down-regulated at the gene and protein levels by FK228, which was followed by a significant suppression of CDK2 or CDK4. In contrast, the CDK2 inhibitor protein $p21^{WAF1}$ was markedly up-regulated and was negatively correlated with MITF, whereas Bcl2 expression was not significantly affected. Transfection of MITF short interfering RNA caused apoptosis in all 4 cell lines and its effects on CDK2/4, Bcl2 and $p21^{WAF1}$ were similar to those of FK228. These findings suggest that FK228 exerts its antimelanoma effects via the down-regulation of multiple functions of MITF.

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Key words : melanoma, histone deacetylase inhibitor, FK228, microphthalmia-associated transcription factor, cell cycle/apoptosis

INTRODUCTION

Melanoma is a potentially lethal cancer with a high resistance to conventional chemotherapy[1,2]. Recently, microphthalmia-associated transcription factor (MITF), the melanocyte master regulator, has been reported to play an important role in the mechanism of chemotherapeutic resistance in malignant melanoma[3]. MITF is a basic helix-loop-helix leucine zipper transcription factor[4] that up-regulates the pigmentation enzyme genes tyrosinase, TRP1, and TRP2[5-7]. Recently, novel roles for MITF have been reported as a regulator of the cell-cycle regulatory protein CDK2[8] as well as its inhibitory protein $p21^{WAF1}$ (p21)[9]. Furthermore, McGill et al.[3] have recently demonstrated that MITF also up-regulates the antiapoptotic protein Bcl2, which contributes to the high resistance to chemotherapy of human melanoma cell lines (HMCLs) that express MITF at high levels. A study of 116 patients with metastatic melanoma by Ugurel et al.[10] has found that MITF gene amplification in metastatic melanoma can be a prognostic marker for patient survival. On the other hand, a study by Lekmine et al.[11] in which melanoma

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cell lines were injected subcutaneously in nude mice has shown that MITF leads to a less-aggressive phenotype. The above evidence suggests controversial functions of MITF with respect to proliferation, G1 arrest, and apoptosis. This controversy prompts us to attempt to answer the question of whether appropriate drugs for the treatment of melanoma could trigger the up-regulation or down-regulation of MITF functions and lead to a successful therapy for melanoma.

Recently, novel antitumor drugs, histone deacetylase (HDAC) inhibitors, have been reported to have strong antitumor effects on malignant melanoma cells despite having weak toxic effects on normal human melanocytes[12,13]. Aberrant transcriptional repression of genes regulating cell growth and differentiation has been thought to be a hallmark of cancer[14]. The altered activation of HDAC inhibitors has been thought to underlie the transcriptional repression in malignancies[15–18]. HDAC inhibitors can restore the expression of genes that are aberrantly suppressed in cancer cells, which may result in cell-cycle arrest, differentiation, or apoptosis[19–26]. HDAC inhibitors induce apoptosis in many tumor cell lines by regulating various proteins, such as p21[15,19,27,28], and differ from conventional anticancer drugs in many respects[29–31]. Recently, Yokoyama et al.[13] have demonstrated that HDAC inhibitors induce apoptosis in human melanoma cell lines via down-regulation of MITF, although the epistatic connection between MITF and G1 arrest or apoptosis-related molecules has not been clarified. Thus, the dynamics and behavior of the above molecules following treatment with HDAC inhibitors would provide important clues for determining the functions of MITF in melanoma therapy.

In the present study, using FK228, a promising HDAC inhibitor for the treatment of malignant melanoma[12], we determined the essential role of MITF and its epistatic regulation of cell cycle/apoptosis regulatory proteins during proliferation, G1 arrest, and apoptosis in HMCLs. FK228 was isolated from *Chromobacterium violaceum* No. 968 as a compound that reversed the malignant phenotypes of H-ras-transformed fibroblasts by blocking the $p21^{ras}$-mediated signal transduction pathways[32]. Kobayashi et al.[12] have reported that FK228 suppresses the Ras-mitogen-activated protein kinase signaling pathway by up-regulating Rap1 and induces apoptosis in malignant melanoma. This drug has exhibited therapeutic effects against a diverse range of malignancies, including melanoma, in phase I and II clinical trials[33,34]. We report that FK228 exerts its antimelanoma effects through the down-regulation MITF, which results in the suppression of CDK2 and the concomitant up-regulation of p21 without any effect on Bcl2. Furthermore, we found that FK228 tended to have stronger apoptotic effects on cell lines that constitutively express MITF, CDK2, and Bcl2 at higher levels.

MATERIALS AND METHODS

1. *Cell lines and culture conditions*

A normal human epidermal melanocyte cell line (Lonza Walkersville, Inc., Walkersville, MD, USA) was maintained in melanocyte growth medium (MGM-4 BulletKit, Lonza Walkersville, Inc.). The HMCLs MM-BP and RPM-EP were kindly provided by Dr. H. Randolph Byers (Harvard Medical School, Cambridge, MA, USA). These cell lines were established from metastatic melanoma in axillary lymph nodes and a recurrent primary lesion[35], respectively. The colo679 and G361 melanoma cells were purchased from RIKEN Bioresource Center (Ibaraki, Japan). Both cell lines were established from primary lesions of the skin (refer to the Web site http://www.ecacc.org.uk/). The K562 cell line, derived from a patient with chronic myelogenous leukemia, was purchased from RIKEN Bioresource Center. The MV4-11 cell line, derived from a patient with acute monocytic leukemia carrying t(4 ; 11), was purchased from the American Type Culture Collection (Manassas, VA, USA). All melanoma cell lines and leukemia cell lines were maintained in F-12 Nutrient Mixture (Ham's F-12) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal calf serum, penicillin G (100 IU/mL), and streptomycin sulfate (0.1 mg/mL). All cells were cultured in a 5% CO_2 and 95% air humidified atmosphere at 37°C. For cell growth

assays, cell numbers were counted at various time intervals after the cells had been harvested by means of trypsinization and were stained with crystal violet. FK228 was provided by Fujisawa Pharmaceutical Co. (Osaka, Japan).

2. Western blotting analysis

For preparation of protein samples, cells were washed twice with ice-cold phosphate-buffered saline and lysed on ice in cell-lysis buffer (20 mM Tris-HCl [pH 8.0] 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodumdodecylsulfate, 50 mM sodium fluoride, and 200 μM sodium orthovanadate) containing protease inhibitors made of complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics Corp., Indianapolis, IN, USA). The extracts were centrifuged at 14,500 *g* for 15 minutes at 4°C, the supernatants were collected, and the protein contents and concentrations were measured with a spectrophotometer (Ultrospec 2100 *pro*, GE Healthcare Bioscience Corp., Piscataway, NJ). Equal amounts of protein in each sample (20-40 μg) were subjected to electrophoresis on 8% to 16% gradient sodium-dodecylsulfate-polyacrylamide gels and were then transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). The membranes were incubated in 10% non-fat dry milk and 1% bovine serum albumin in Tris-HCl-buffered saline containing 0.05% Tween-20 for 1 hour at room temperature to avoid nonspecific protein binding. The membranes were placed in a primary antibody solution for 1 hour at room temperature or overnight at 4°C, depending on the antibody. The following primary antibodies were used : anti-Bcl2 (clone 121 ; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (clone 18 ; BD Transduction Laboratories, Lexington, KY, USA), anti-p53 (BD Transduction Laboratories), anti-Cdk2 (Cell Signaling Technology, Beverley, MA, USA), anti-Cdk4 (Cell Signaling Technology), anti-cdc2 (Cell Signaling Technology), anti-MITF (clone C5 ; Cell Signaling Technology), and anti-β-actin (clone C4 ; ICN Biomedicals, Aurora, OH, USA). We used appropriate anti-rabbit or anti-mouse IgG linked to horseradish peroxidase (GE Healthcare Bioscience Corp., Piscataway, NJ) as the second antibody, and an ECL plus Enhanced chemiluminescence system (GE Healthcare Bioscience Corp) for detection. Bands were quantified with an image processing and analysis software program (ImageJ, available at http://rsb.info.nih.gov/ij/index.html), and correlation coefficients were calculated.

A strong positive correlation has been reported between the expression of MITF and CDK2 proteins in variety of HMCLs[8]. And Bcl2 and p21 have also been reported to be associated with MITF expression in variety of HMCLs[3,9]. Therefore, we examined the expression of MITF and cell cycle regulatory kinases such as CDK2/4 or cdc2, and apoptosis-related protein Bcl2 or G1-arrest related protein p21 in the condition with or without FK228 treatment. Because p21 is a transcriptional target of p53[38], we then determined whether the expression of p21 is affected by the induction of p53 protein.

3. Cell-cycle and apoptosis analysis

Cells were harvested by scraping on ice, and cell-cycle profiles were obtained by staining DNA with propidium iodide (PI) in preparation for flow cytometric analysis with the FACScan/CellQuest system (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). The sizes of the sub-G1, G0/G1, and S+G2/M fractions were calculated as percentages by analyzing the DNA histograms with a software program (ModFit LT 2.0, Verity Software House, Topsham, ME, USA). Additionally, apoptosis was analyzed by means of staining with Annexin-V 568 (Roche Diagnostics Corp.) in preparation for flow cytometric analysis with the FACScan/CellQuest system.

We examined the effect of FK228 on the cell cycle and apoptosis at 24 hours and at 48 hours using flow cytometric analysis with PI and Annexin-V staining. Cells were treated with 2 to 10 nM FK228, which corresponds to 0.2% to 1.0% of the mean maximum plasma concentrations (C max) measured in phase I clinical trials[33].

4. Analysis of mRNA with RT-PCR and real-time RT-PCR

Total RNA was isolated with an RNeasy Mini Kit according to the manufacturer's instructions

(Qiagen Inc., Valencia, CA, USA) and was quantified with a spectrophotometer (Ultrospec 2100 *pro*, GE Healthcare Bioscience). Reverse transcription (RT) was performed a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The polymerase chain reaction (PCR) with High Fidelity PCR Master (Roche Diagnostics Corp.) was run for 25 to 40 cycles under the following conditions : stage 1, 94°C, 2 minutes ; stage 2, 94°C, 30 seconds ; stage 3, 65°C, 30 seconds ; and stage 4, 72°C, 1 minute. Human MITF mRNA was detected with the forward primer 5′-ACCGTCTCTCACTGGATTGGT G-3′ and the reverse primer 5′-CGTGAATGTGTGTTCATGCCTGG-3′. Levels of MITF mRNA were normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (forward primer 5′-GAAGGTGAAGGTCGGAGTCAACG-3′, reverse primer 5′-AGTCCTTCCACGATACCAAAGTTG-3′). For real-time RT-PCR, cDNAs were synthesized with a ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan) through reverse transcription of 100 ng total RNA using oligo dT and Moloney murine leukemia virus reverse transcriptase. The sets of oligonucleotide primers used were (forward, 5′-GAAATCTTGGGCTTGATGGA-3′, and reverse, 3′-CCTCACCATCAGCAACTCCT-5′). Real-time RT-PCR with SYBR Green was per-

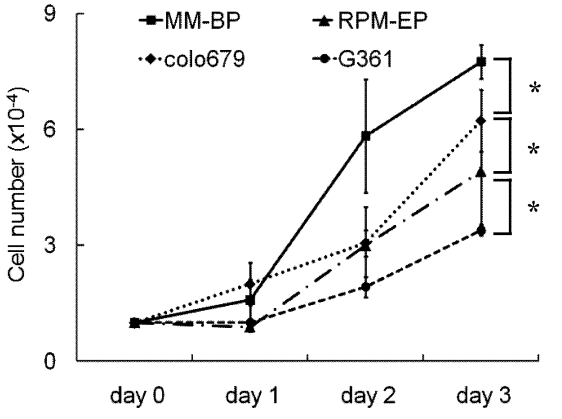

Fig. 1A.

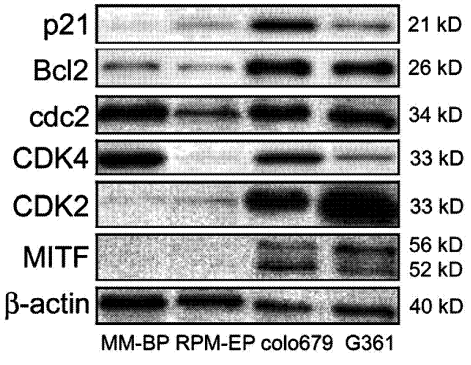

Fig. 1B.

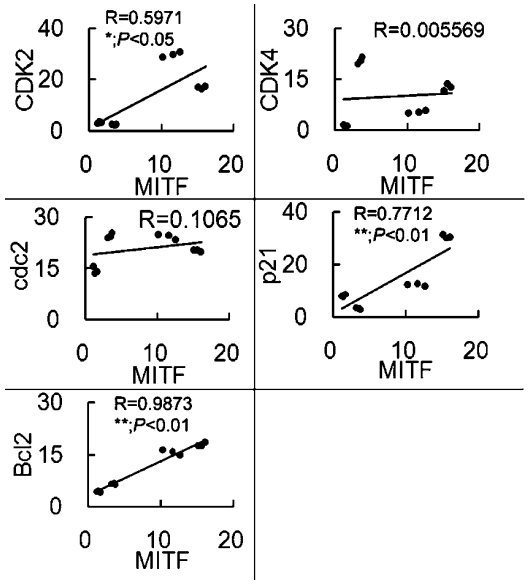

Fig. 1C.

Fig. 1. Cell growth and protein expression of each cell line. A : Cell growth curves. The means±SDs (bars) of 3 independent experiments are shown. *P* values were calculated for differences between cell lines at the last date. ANOVA, * ; $P<0.05$, ** $P<0.01$. B : Protein expression of MITF and cell-cycle regulatory kinases analyzed with Western blotting. C : Relationship between the expression of cell-cycle or apoptosis regulatory proteins and MITF. The numbers on the axes represent the relative intensity of each protein band on Western blotting. Dots on the graphs indicate the relative intensity of proteins in Figure 1B in 3 independent experiments. *P* values were calculated from correlation coefficients between 2 different proteins.

formed with Power SYBR Green PCR (Applied Biosystems Japan, Tokyo, Japan) in an Applied Biosystems 7900HT Real-Time PCR detection system (Applied Biosystems Japan). Briefly, each 25 μL reaction volume contained cDNA templates, primer pairs, and 2x Master Mix (Toyobo, Tokyo, Japan). Amplification in the Sequence Detection System (Applied Biosystems) after initial denaturation at 94°C for 15 minutes was performed for 40 cycles at 94°C for 15 seconds, 60°C for 30 seconds, and 74°C for 1 minute.

5. Transfection of MITF siRNA

Ten nanomoles of short interfering (si) RNA was transfected with X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics Corp.) and incubated for 2 days. The MITF-specific sequences used were 5′-GGUGAAUCGGAUCAUCAAG-d(TT)-3′ and 5′-CUUGAUGAUCCGAUUCACC-d(TT)-3′, and the control siRNA sequences used were 5′-UUCUCCGAACGUGUCACGU-d(TT)-3′ and 5′-ACGUGACACGUUCGGAGAA-d(TT)-3′ as described elsewhere[36]. Because the effect of MITF knockdown is maintained for an extremely short time, cell-cycle and apoptosis analyses were performed a total 72 hours later: cells had been trypsinized from dishes following siRNA treatment for 48 hours and were cultured again in another dish for 24 hours in Ham's F-12 medium without siRNA.

6. Statistical analysis

A nonparametric one-way analysis of variance (ANOVA; Kruskal-Wallis test) was used to evaluate differences between groups (Fig. 1A, 2C, and 2D). N is the number of samples used to obtain the correlation value, and R is the correlation coefficient. The associated probability was computed with the TDIST EXCEL function as: P value = TDIST(ABS(R)*SQRT((N-2)/(1-ABS(R)^2)),N-2,2) (Fig. 1C, 3C, and 4B). A P value less than 0.05 was considered to indicate statistical significance.

Results

1. Growth Properties of HMCLs

The fastest growing HMCL was MM-BP, followed in descending order by colo679, RPM-EP, and G361 (Fig. 1A).

2. Expression of MITF and CDK2 or 4/cdc2/Bcl2/p21 in HMCLs

Western blotting revealed that while MITF protein was expressed by 2 of 4 HMCLs, the 2 MITF-positive HMCLs also had increased levels of CDK2 expression (Fig. 1B). On the other hand, cdc2 and CDK4 were expressed by all 4 HMCLs regardless of the expression levels of MITF. Plotting MITF expression against each cell-cycle regulatory kinase demonstrated a strong correlation ($R=0.597$, $P<0.05$) between MITF and CDK2 (Fig. 1C).

Western blotting revealed that the 2 MITF-positive HMCLs expressed Bcl2 or p21 at higher levels, although the case for p21 of G361 was somewhat weak (Fig. 1B). Plotting MITF expression against Bcl2 and p21 demonstrated high correlation coefficients ($R=0.771$, $P<0.01$, and $R=0.987$, $P<0.01$, respectively) (Fig. 1C).

3. Analysis of Cell Cycle and Apoptosis Following the Addition of FK228

At 24 hours postincubation, PI staining analysis showed that the percentage of cells arrested in the G1 phase increased, whereas the percentage of cells arrested in the S phase decreased at concentrations of FK228 greater than 5 nM (Fig. 2A). These data indicate that all cell lines were equally sensitive to FK228 (Fig. 2C). At a concentration of 2 nM, colo679 and G361 cells were more sensitive to FK228 than were MM-BP and RPM-EP cells. On the other hand, apoptosis was not substantially induced in any of the HMCLs 24 hours postincubation. At 48 hours postincubation, cells entering apoptosis increased with increasing concentrations of FK228 (Fig. 2B). At FK228 concentrations of 2 and 5 nM, colo679 and G361 cell lines underwent apoptosis, whereas MM-BP and RPM-EP did not but did undergo apoptosis at a FK228 concentration of 10 nM (Fig. 2D). On the other hand, in cultured normal human epidermal melanocytes, the effect of FK228 on apoptosis and G1 arrest was undetectable even if FK228 was added at a concentration of as high as 10 μM 48 hours postin-

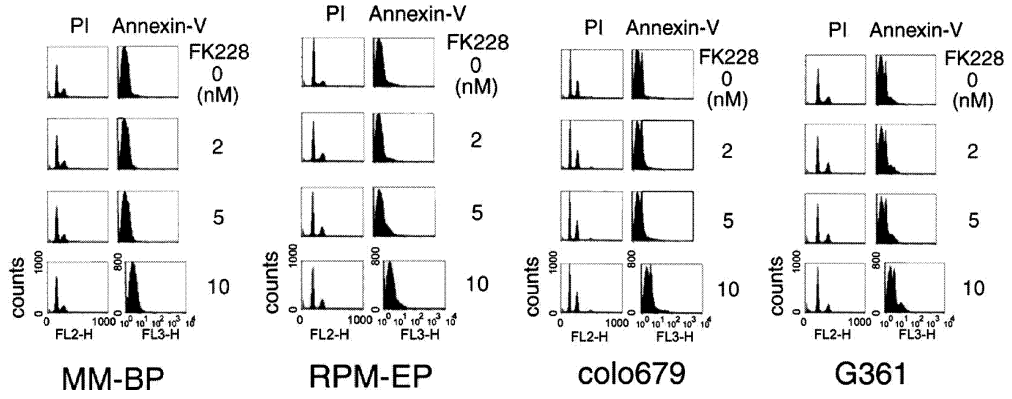

Fig. 2A.

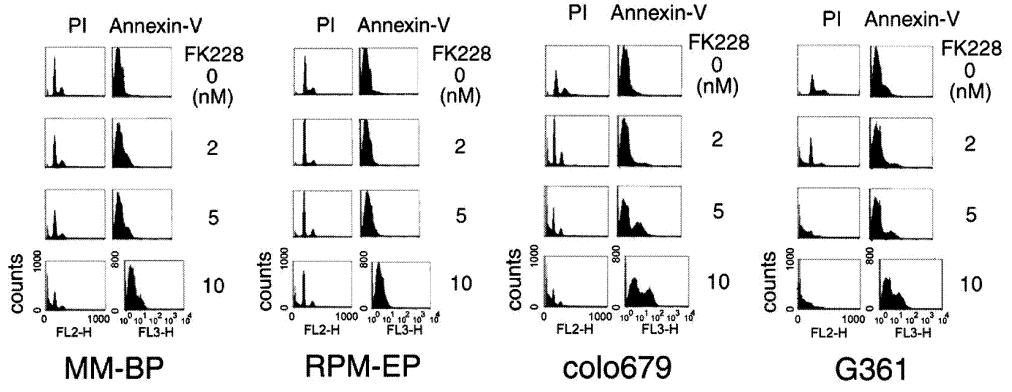

Fig. 2B.

Fig. 2. Effect of FK228 on each cell line. Cell-cycle profiles were obtained by staining DNA with PI in preparation for flow cytometric analysis. Apoptosis was analyzed by staining with Annexin-V 568 in preparation for flow cytometric analysis. A: Analysis of cell cycle and apoptosis with flow cytometry 24 hours after the addition of FK228 at concentrations of 2 to 10 nM. Vertical and horizontal axis represents cell counts and fluorescence intensity, respectively. B: Analysis of cell cycle and apoptosis with flow cytometry 48 hours after the addition of FK228 at concentrations of 2 to 10 nM. Vertical and horizontal axis represents cell counts and fluorescence intensity, respectively. C: Graphs representing the percentage of G1-phase and S-phase cells in FK228-treated versus untreated control cultures in each HMCL at 24 hours. The means±SDs (bars) of 3 independent experiments are shown. P values are for the differences between each percentage of the 2 highest cell lines versus the lowest cell line treated with 2 nM FK228. ANOVA, *; $P<0.05$, **$P<0.01$. D: Graphs represent percentages of apoptotic cells in FK228-treated versus untreated control cultures for each HMCL. The means±SDs (bars) of 3 independent experiments are shown. P values are for differences between each percentage of the 2 highest cell lines versus the lowest cell line at 2 nM FK228. ANOVA, *; $P<0.05$, **$P<0.01$. E: Analysis for cell cycle and apoptosis in NHEMs with flow cytometry 48 hours after the addition of FK228 at a concentration of 10 μM. Vertical and horizontal axis represents cell counts and fluorescence intensity, respectively.

cubation (Fig. 2E).

4. Time Course of MITF and CDK2/4 or cdc2 Expression Following the Addition of FK228

Following treatment with FK228, the expression of MITF protein decreased 12 to 24 hours postincubation and completely disappeared after 48 hours in the 2 cell lines that expressed MITF under untreated conditions (Fig. 3A). Although there is a cleaved form of MITF (at 45 kDa), treatment with FK228 did not increase the level of cleaved MITF (arrows in Fig. 3A), despite the complete disappearance of MITF

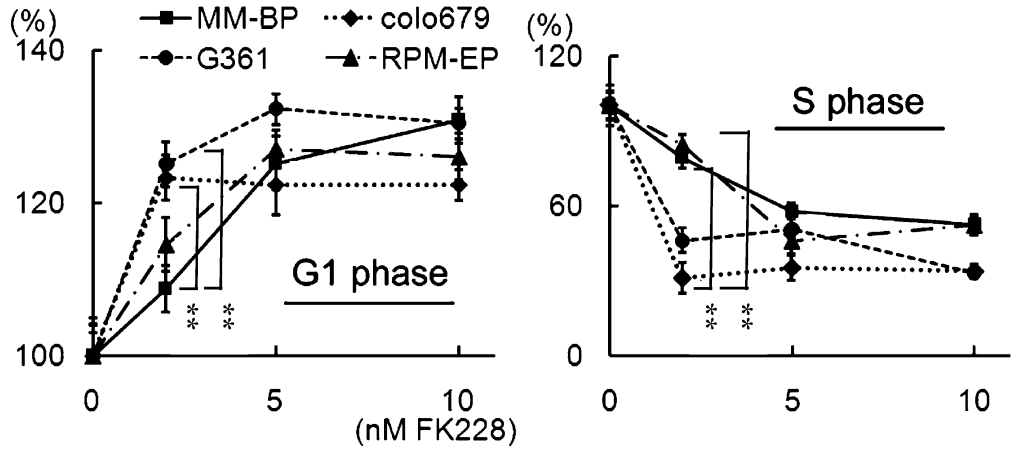

Fig. 2C.

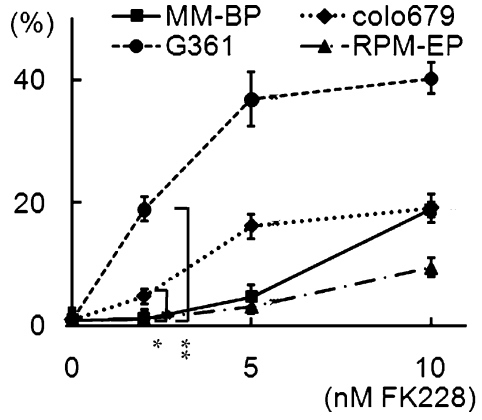

Fig. 2D.

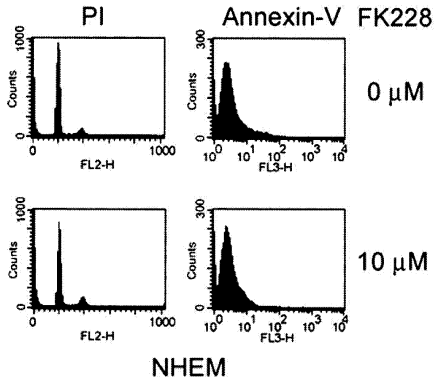

Fig. 2E.

Fig. 2. Continued.

protein. This decreased expression of MITF protein also involved a reduction of MITF mRNA (Fig. 3B). Interestingly, the expression of CDK2 was also correspondingly reduced in the 2 cell lines ($R=0.658$, $P<0.01$), which suggests a possible close epistatic connection between CDK2 and MITF. We also found that CDK4 and cdc2 proteins were markedly down-regulated in patterns similar to that of CDK2. Expression of MITF and these cell-cycle-related enzymes were closely related and were strongly correlated with CDK4 expression ($R=0.715$, $P<0.01$) (Fig. 3C). On the other hand, cdc2 expression was weakly correlated ($R=0.194$), because cdc2 protein levels were up-regulated 12 hours before the subsequent down-regulation.

Simultaneous measurements of protein levels and MITF mRNA levels in cell lines not expressing MITF protein showed that MITF mRNA was expressed in these cell lines but that expression levels were reduced following the addition of FK228 (Fig. 3D). These results suggest some problem in the synthesis of MITF protein from mRNA. We could not detect the expression of CDK2 protein because the expression level was extremely low in these cell lines. The expression of cdc2 protein was also reduced, although there was not an early up-regulation toward the reduction.

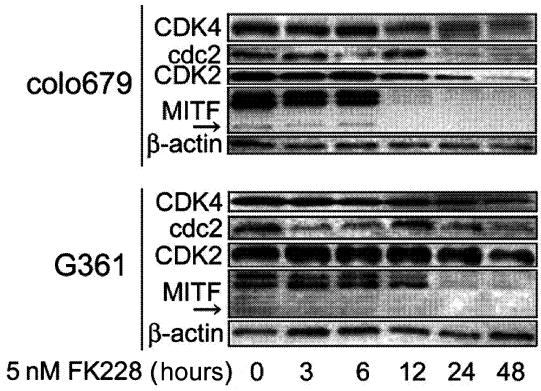

Fig. 3A.

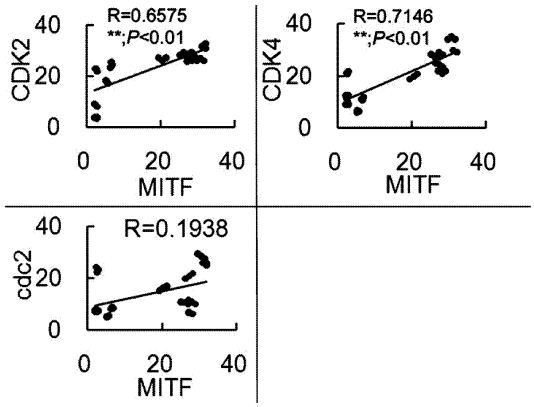

Fig. 3C.

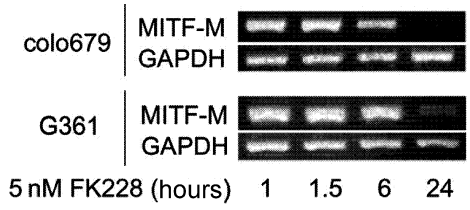

Fig. 3B.

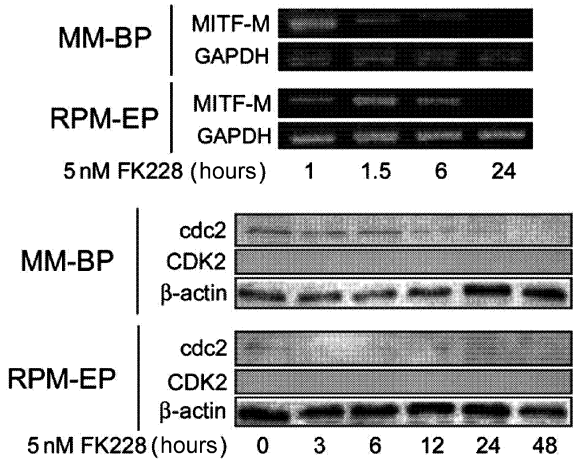

Fig. 3D.

Fig. 3. Time course of cell cycle regulatory protein expression after the addition of FK228. A: Time course of CDK4, cdc2, CDK2, and MITF expression in the cell lines expressing MITF protein following the addition of FK228 as analyzed with Western blotting. The arrows show the cleaved form of MITF. B: Time course of mRNA expression following the addition of FK228. MITF-M is an isoform of MITF mainly expressed in melanocytes and melanoma cells. PCR was run for 30 cell cycles for each experiment. C: Relationship between the expression of cell-cycle regulatory kinases and MITF as analyzed with the Western blotting of Figure 3A. The numbers on the axes represent the relative intensity of each protein band on Western blotting. Dots on the graphs indicate the relative intensity of proteins at each time period in Figure 3A in each of 3 independent experiments. *P* values were calculated from correlation coefficient between 2 different proteins. D: Time course of cdc2 and CDK2 proteins and mRNA of MITF-M expression in the cell lines not expressing MITF protein following the addition of FK228, analyzed with Western blotting and RT-PCR, respectively. PCR was run for 25 and 40 cell cycles for GAPDH and MITF-M, respectively.

5. Time Course of Bcl2 Expression Following the Addition of FK228

Although Bcl2 expression corresponded to levels of MITF expression in untreated HMCLs (Fig. 1B), the addition of 5 nM FK228 slightly reduced Bcl2 protein levels by 48 hours in RPM-EP cells but not in the other 3 HMCLs tested, indicating that MITF expression and Bcl2 expression are not connected (Fig. 4A).

6. Time Course of p21 Protein Expression Following the Addition of FK228

Although p21 protein levels differed among untreated HMCLs, p21 protein expression levels

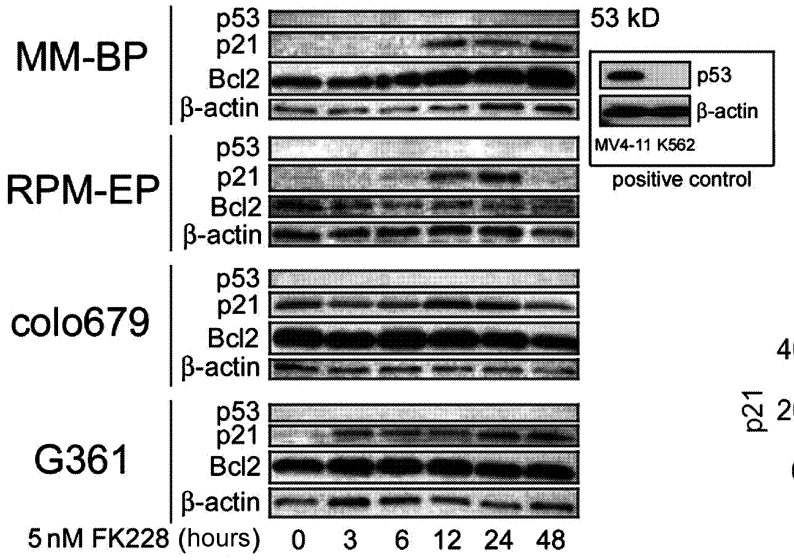

Fig. 4A.

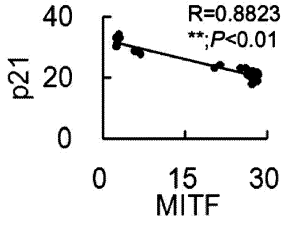

Fig. 4B.

Fig. 4. Time course of apoptosis regulatory protein expression after the addition of FK228. A : Time course of Bcl2, p21, and p53 protein expression following the addition of FK228 and analyzed with Western blotting. MV4-11 and K562 were positive and negative controls, respectively, each for p53. B : Relationship between the expression of p21 and MITF protein 2 to 3 hours postincubation with FK228. The numbers on the axes represent the relative intensities of each protein band on Western blotting. Dots on the graphs indicate the relative intensities of proteins at each time period in Figure 4A in each of 3 independent experiments. *P* values were calculated from correlation coefficients between 2 different proteins.

peaked 24 to 48 hours after the addition of FK228 in all cell lines (Fig. 4A), a result that is consistent with the negative correlation between p21 and MITF protein expression 3 to 24 hours postincubation with FK228 (Fig. 4B).

p53 could not be detected within 0 to 48 hours of treatment with FK228 in any of the HMCLs tested ; the p53 protein expression level was not affected by FK228, and the expression of p21 increased independently of p53.

7. Effect of MITF siRNA on Apoptosis

Real-time RT-PCR analysis revealed that although MITF mRNAs was present in MM-BP and RPM-EP cells at negligible levels, MITF mRNAs were present at significant levels in colo679 cells and were present at even higher levels in G361 cells (Fig. 5A). Levels of MITF mRNAs paralleled protein expression levels among the 4 HMCLs. On the other hand, MITF mRNA levels decreased in MM-BP and RPM-EP after the addition of FK228 (Fig. 3D), indicating mRNA expression in the cells not expressing the protein.

Western blotting analysis demonstrated that MITF siRNA effectively decreased the expression of MITF protein in colo679 and G361 cells, whereas a control siRNA had no effect (Fig. 5B). Expression of CDK2/4 proteins was down-regulated by MITF siRNA transfection in colo679 and G361 cells, but expression of p21 was significantly increased (Fig. 5B), an effect resembling that of FK228 on these signaling molecules (see Fig. 3A, and Fig. 4A). The expression of Bcl2 was slightly decreased in colo679 cells but remained unchanged in G361 cells (Fig. 5B) ; these differential effects that were similar to the effects of FK228 on the same cell lines (see Fig. 4).

Annexin-V staining analysis revealed that MITF silencing elicited distinct levels of apoptosis compared with the negative control in the 2 cells constitutively expressing MITF (Fig. 5C), similar to the apoptotic effect of FK228.

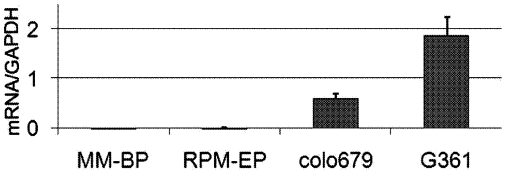

Fig. 5A.

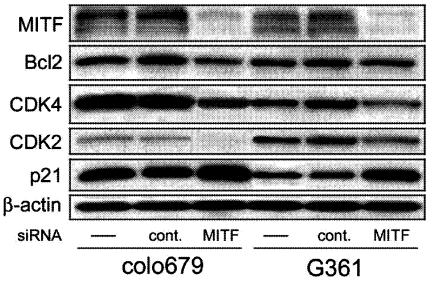

Fig. 5B.

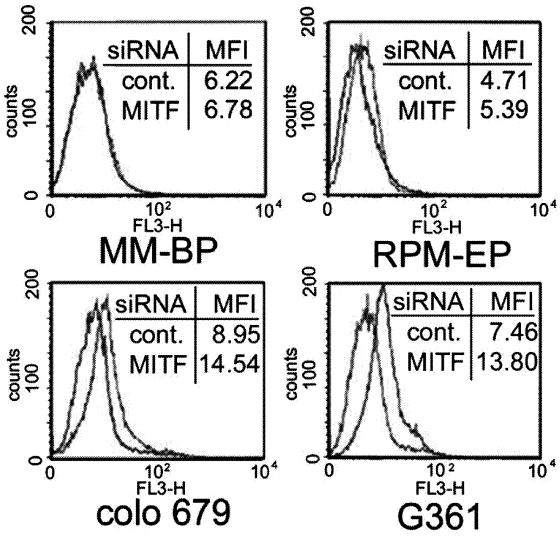

Fig. 5C.

Fig. 5. Effect of MITF siRNA. A: Comparison of MITF mRNA levels with real time RT-PCR in the 4 cell lines under untreated conditions. B: Effect of MITF siRNA transfection at 72 hours posttreatment on the expression of MITF analyzed with Western blotting. C: Effect of MITF siRNA on apoptosis, measured with flow cytometry using Annexin-V staining.

DISCUSSION

The present study has demonstrated that FK228 effectively induces G1 arrest and apoptosis in the 4 HMCLs tested. However, the apoptotic effects differed significantly among the cell lines. Cell lines with constitutively higher expression levels of MITF, CDK2, and Bcl2 tended to show stronger apoptotic effects. CDK2 is a major regulator of the cell-cycle transition from the G1 phase to the S phase[40], whereas Bcl2 is an important antiapoptotic molecule of the mitochondrial membrane[41-43]. These proteins are abnormally expressed in many kinds of malignant cell, including malignant melanoma cells[44-46]. That CDK2 and Bc12 are targets of MITF[3,8] suggests a novel role for MITF as a survival oncogene in malignant melanoma[47]. However, our results concerning the effects of FK228 contrast with the results of previous studies concerning the relationships among CDK2, Bcl2, and MITF and resistance to G1 arrest or apoptosis[3,8].

Western blotting analysis following FK228 treatment of HMCLs expressing MITF revealed that MITF protein expression was markedly down-regulated by FK228 and corresponded to the observed G1 arrest and apoptosis. These effects on MITF expression and apoptosis are consistent with the results reported by Fisher et al.[13], although they used different HDAC inhibitors and human melanoma cell lines. RT-PCR analysis demonstrated that the suppression of MITF protein by FK228 is accompanied by reduced levels of MITF mRNA, although altered signaling mechanisms upstream of MITF gene expression remain unclear. Regarding the epistatic connection between MITF and apoptosis, Larribere et al.[36] have shown that a cleaved fragment of MITF (due to degradation by caspase-3), but not the total amount of MITF, is caused by apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand. Because levels of cleaved MITF protein did not increase when levels of MITF decreased after treatment with FK228 in colo679 and G361 cells in the present study (Fig. 3A), another mechanism likely underlies the association between MITF expression and apoptotic susceptibility. In contrast to a previous report[8] that Bcl2 is a target gene of MITF, we found that the expression of Bcl2 was not affected by treatment with FK228, a finding that suggests that

factors other than Bcl2 exert apoptotic effects on treated HMCLs. This result was similar to that of a study of the effects of FK228 on human uveal malignant melanoma cells[37] in which the expression level of Bcl2 protein was not affected by treatment with FK228. A reasonable assumption is that Bcl2 is regulated by transcription factors other than MITF during FK228-induced apoptosis. Consistent with our results, Hoek et al. have demonstrated that whereas Bcl2 expression is closely associated with that of MITF, transfection of MITF in a melanoma cell line does not up-regulate Bcl2 mRNA[48].

Melanoma cells have cell-cycle characteristics that differ from those of cells of other origin. Whereas in many other cancer cell lines knockout/knockdown of CDK2 does not affect cell proliferation, and even CDK2-knockout mice can grow normally[49,50], in melanoma cells knockout/knockdown of CDK2 suppresses growth. Time-course analysis of cell-cycle regulatory enzymes demonstrates significant suppression of CDK2 or CDK4, with levels of both proteins being well correlated with each other ($R=0.66$, $P<0.01$ or $R=0.71$, $P<0.01$), which followed the observed MITF regression. This finding suggests that FK228-induced G1 arrest is due to the down-regulation of CDK2 or CDK4, probably attributable to the concomitantly induced MITF regression. The observed CDK2-associated G1 arrest is consistent with a report by Du et al.[8] that CDK2 siRNA induces G1 arrest in HMCLs. Interestingly, we found a distinct up-regulation of cdc2 protein levels after 12 hours in MITF-expressing cell lines but not in non-MITF-expressing cell lines. Clarifying this mechanism will help further clarify the epistatic connection between cdc2 and MITF in melanocytic cells.

Interestingly, we found that when G1 arrest occurred 24 hours postincubation with FK228, CDK2 expression was not completely abolished, a finding that suggests an additional factor is responsible for the induction of G1 arrest. Generally, p21 is inducible by FK228 in various nonmelanocytic cancer cell lines[20,22,37], and the appearance of p21 can be regarded as a major trigger of G1 arrest elicited by FK228. As in report by Carreira et al.[9] which showed that MITF activates p21 expression, p21 expression in the 4 HMCLs tested in the present study was strongly correlated with the constitutive expression of MITF protein under untreated conditions, suggesting that p21 can induce G1 arrest in FK228-treated melanoma cells. However, our finding that colo679 melanoma cells had the second highest growth rate and the highest p21 expression level (Fig. 1A and B) suggests that p21 expression levels alone cannot affect cell growth under untreated conditions. Western blotting revealed that all cell lines treated with FK228 had increased expression of p21, although the levels of constitutively expressed p21 in control cells differed among cell lines. Our finding that MITF was down-regulated as p21 was up-regulated in FK228-treated melanoma cell lines contrasts with the report by Carreira et al.[9]. Nevertheless, how the down-regulated expression of MITF triggers p21 expression remains unknown.

Another candidate for a transcription factor regulating p21 is p53[38]. We have found that p53 is not present at significant levels in the 4 HMCLs examined under untreated or treated conditions, a findings that suggests p53 is not responsible for the FK228-induced expression of p21. Similarly, Sandor et al.[22] have shown that the FK228-induced expression of p21 is independent of p53 in MCF-10A human breast cancer cells. On the other hand, Zhao et al.[51] have reported that FK228-induced acetylation of p53 is mainly responsible for p21 transcriptional activity in A549 human lung carcinoma cells. Because we could not detect p53 in the present study, its contribution to the induction of p21 remains unclear.

Taken together, the above results suggest that the HDAC inhibitors may trigger a decrease in MITF expression in melanoma cells which causes G1 arrest and apoptosis via a combination of the down-regulation of CDK2 or CDK4 or the up-regulation of p21 or both. However, this proposed mechanism for HDAC-inhibitor-induced G1 arrest and apoptosis has several contradictions with respect to the previously reported relationship between MITF expression and apoptosis. To resolve this issue, we performed MITF siRNA analysis of HMCLs and examined the sensitivity of MITF silencing according to their susceptibility to apoptosis under untreated conditions.

In these MITF-down-regulated melanoma cell lines, CDK2 protein was concomitantly decreased, a finding consistent with the report by Du et al.[8]. Our results regarding the effects of MITF siRNA on CDK2 expression are similar to those for the effect of FK228 ; this similarity strongly suggests that MITF down-regulation by FK228 is responsible for the decrease in CDK2 expression. In the MITF-down-regulated HMCLs, CDK4 was also down-regulated by MITF siRNA, an effect that resembles the effect of FK228 on CDK4 expression. Therefore, MITF might also be a regulatory molecule for CDK4, although further study will be required to determine whether this is true. On the other hand, although a study in the 501mel melanoma cell line has found that p21 is a positive target of MITF[9], in our MITF-down-regulated HMCLs, p21 protein was up-regulated, an effect similar to that of FK228 on p21. This finding suggests that p21 is negatively regulated by MITF in some HMCLs. As for another known target of MITF, Bcl2, our findings in MITF-down-regulated HMCLs indicate differential effects on Bcl2, with colo679 or G361 cells being down-regulated or unchanged, respectively. These differential effects also resemble the effects of FK228 on Bcl2 in the 2 HMCLs, which suggests that the slight down-regulation of Bcl2 elicited by FK228 can also be ascribed to MITF regression, although such a regression may be melanoma cell line-dependent. The above effects of MITF silencing on cell cycle/apoptosis regulatory molecules are similar to those of FK228, which strongly suggests that down-regulation or up-regulation of these cell cycle/apoptosis regulatory molecules by FK228 is mainly attributable to MITF function. Furthermore, the effects of MITF siRNA on apoptosis resemble those of FK228 on the same cell lines.

Taken together, our results strongly suggest that MITF plays a central role in regulating G1 arrest and apoptosis in melanoma cells by regulating the expression of CDK2/CDK4 as well as their inhibitory protein p21, although whether other unknown factors are triggered by MITF to induce G1 arrest or apoptosis remains unclear.

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