Histone deacetylase inhibitor FK228 enhances adenovirus-mediated p53 family gene therapy in cancer models

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Abstract

Therapeutic replacement of the wild-type *p53* gene has been pursued as a potential gene therapy strategy in a variety of cancer types; however, some cancer models are resistant to p53 *in vivo* and *in vitro*. Therefore, to improve *p53* gene therapy, it is important to overcome the resistance to p53-mediated apoptosis. Histone deacetylase inhibitors are a novel class of chemotherapeutic agents that are able to reverse the malignant phenotype of transformed cells. A natural histone deacetylase inhibitor, FK228, is reported to enhance adenovirus infection due in part to the up-regulation of coxsackievirus adenovirus receptor expression. In this study, preclinical experiments were done to establish a mechanistic rationale for the combination of adenovirus-mediated p53 family gene transfer and FK228 pretreatment in future clinical trials. Pretreatment with FK228 enhanced apoptosis in human cancer cells through enhanced transduction of Ad-p53. FK228 also induced hyperacetylation of the p53 protein and specifically enhanced p53-mediated Noxa expression. Additionally, the combination of FK228 and Ad-p53 induced Bax translocation to the mitochondria. The double knockdown of *Bax* and *Noxa* expression by small interfering RNA antagonized the synergistic effect of Ad-p53 and FK228 on apoptosis induction. In human cancer xenograft models, FK228 significantly increased the therapeutic effectiveness of *p53* as well as *p63* gene therapy. These results provide a strong rationale for combining *p53* gene therapy and FK228 pretreatment in cancer therapy. [Mol Cancer Ther 2008;7(4):779–87]

Introduction

Inactivation of the p53 pathway not only is involved in carcinogenesis but also confers chemoresistance (1, 2). Therefore, therapeutic replacement of the wild-type *p53* gene using adenoviral vectors has been pursued as a potential gene therapy strategy in several types of cancer (3). This strategy potentially relies on p53-mediated apoptosis, which in turn depends on induction of a distinct class of transcriptional target genes, including *BAX, PUMA, NOXA, p53AIP1*, etc. (4). However, in clinical trials, restoration of wild-type *p53* gene function does not always lead to tumor regression or tumor growth inhibition, suggesting that some tumors are resistant to exogenous p53 (5, 6). This resistance may result from the influence of negative regulators of p53, such as MDM2 (7, 8).

The two *p53* relatives, *p73* and *p63*, encode proteins that share considerable structural homology with p53 (9–11). p73 and p63 are expressed in different isoforms as a result of differential promoter usage and alternative splicing. The isoforms containing a transactivation domain at their NH_2-terminal end can also bind to p53-responsive elements to transactivate the p53 target genes and are termed TAp73 and TAp63. When overexpressed, exogenous TAp73 and TAp63 can induce apoptosis of cancer cells, suggesting that the p53 family members have a potential functional overlap with p53 itself. MDM2 can also bind the NH_2-terminal of p73 and p63. On the other hand, MDM2 binding does not promote p63 or p73 degradation (12, 13). Thus, to overcome resistance to p53-mediated apoptosis, p73 and p63 may be useful. For example, a *p73*-expressing recombinant adenovirus inhibited the growth of human papillomavirus-positive cervical cancer cell lines more efficiently than *p53* (14). Additionally, osteosarcoma cell lines having *MDM2* amplification undergo apoptosis in response to p63 but not p53 (15).

Histone deacetylase inhibitors (HDACI) are novel chemotherapeutic agents that are potentially effective against a broad range of human cancers. Generally, HDACIs induce accumulation of hyperacetylated nucleosome core histones and results in transcriptional activation of genes, the expression of which potentially causes inhibition of tumor cell growth (16). In addition, HDACIs induce acetylation of nonhistone proteins, raising the possibility of a histone-independent effect of HDACIs is also important for their anticancer activity (17). FK228, also known as depsipeptide (FR901228), is one of the most promising HDACIs for

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cancer treatment because of its effectiveness at low concentrations (18, 19). Clinical trials using FK228 are currently ongoing for patients with acute myelogenous leukemia, chronic lymphocytic leukemia, T-cell lymphoma, and refractory solid-tumor malignancies (20). Importantly, FK228 increases adenovirus-mediated transduction in cancer cell lines as a result of enhancing the levels of coxsackievirus adenovirus receptor (CAR) on the cell surface (21–23).

One of the major weaknesses of adenovirus-mediated gene therapy is the less-than-optimal delivery; thus, high multiplicity of infection (MOI) of adenoviral vectors may be required to achieve effective killing of tumor cells. In the present study, we investigated the combined effect of FK228 and an adenoviral vector containing the p53 family genes against human cancer cells. We found that FK228 significantly increased the therapeutic effectiveness of the p53 family adenovirus using *in vitro* and *in vivo* models. The results highlight the importance of combining FK228 with p53 family gene therapy for the treatment of human cancer.

Materials and Methods

Cell Lines, Recombinant Adenovirus, and Drugs

Human cancer cell lines SW480 and MKN45 were purchased from the American Type Culture Collection and the Japanese Collection of Research Bioresources, respectively. MKN45 cells express wild-type p53, whereas SW480 cells express mutated p53 proteins. The recombinant adenoviral vectors expressing human *p53* (Ad-p53), *TAp73β* (Ad-p73β), and *TAp63γ* (Ad-p63γ) and the bacterial *lacZ* gene (Ad-lacZ) were constructed as described previously (24, 25). Adenovirus titer in plaque-forming units was determined by plaque formation assays following infection of 293 cells. The MOI was defined as the ratio of the total number of plaque-forming units used in a particular infection to the total number of cells to be infected. FK228 was kindly provided by Fujisawa Pharmaceutical.

Immunoblot Analysis

The primary antibodies used for immunoblotting in this study are as follows: mouse anti-human p53 monoclonal antibody (mAb; DO-1; Santa Cruz Biotechnology), rabbit anti-human p53 polyclonal antibody (acetyl K373/382; Abcam), mouse anti-human p73 mAb (ER-15; Oncogene Research), mouse anti-human p63 mAb (4A4; Oncogene Research), mouse anti-human p21 mAb (EA10; Oncogene Research), mouse anti-human MDM2 mAb (SMP14; Santa Cruz Biotechnology), rabbit anti-human BAX polyclonal antibody (P19; Santa Cruz Biotechnology), mouse anti-human Noxa mAb (114C307; Oncogene Research), rabbit anti-human PUMA polyclonal antibody (BD Transduction Laboratory), goat anti-human histone H3 polyclonal antibody (Santa Cruz Biotechnology), rabbit anti-acetylated human histone H3 polyclonal antibody (Santa Cruz Biotechnology), mouse anti-human poly(ADP-ribose) polymerase mAb (BD PharMingen), mouse anti-human caspase-9 mAb (BD Transduction Laboratory), mouse anti-human actin mAb (Chemicon), mouse anti-human mitochondrial heat shock protein 70 mAb (JG1; Affinity Bioreagents), and mouse anti-β-galactosidase mAb (Promega). Proteins were transferred to Immobilon P membranes (Millipore) by electroblotting, and immunoblot analysis was done as described previously (15, 25). Subcellular fractionation was done using the Mitochondria Cytosol Fractionation kit (BioVision).

Detection of Apoptosis

Apoptosis was analyzed by flow cytometry and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analyses. For flow cytometry, both adherent and detached cells were combined, fixed in 90% cold ethanol, treated with RNase A, and stained with propidium iodide. Samples were analyzed on a FACSCalibur as described previously (15). The percentage of apoptotic cells was determined to be the percentage of the area of fluorescence smaller than the G_1 peak, out of the total area of fluorescence. For the TUNEL assay, cells were plated at 5×10^4 per well in a four-well chamber slide. TUNEL reactions were done using the DeadEnd Fluorometric TUNEL system (Promega) according to the manufacturer's instructions.

Immunofluorescence Microscopy

Cells were fixed in 4% paraformaldehyde and incubated for 2 h with rabbit anti-Bax polyclonal antibody. The slides were then stained with Alexa 488–conjugated goat anti-rabbit IgG (Invitrogen) for 1 h and counterstained with MitoTracker Red 580 (Invitrogen) for mitochondrial staining. The specimens were examined using a laser-scanning confocal microscope (Fluoview; Olympus).

RNA Interference

Human Bax small interfering RNAs (siRNA), si-BAX (5′-GGUGCCGGAACUGAUCAGA-3′), human Noxa siRNA, si-Noxa (5′-CUUCCGGCAGAAACUUCUG-3′), and nonspecific control siRNA (target sequence 5′-NNACTCTATCGCCAGCGTGAC-3′) were purchased from Qiagen. Cells (1×10^5) were plated per well in six-well plates with 2 mL medium in each well at day 0. Double-stranded siRNAs were transfected every 24 h for 3 consecutive days using Oligofectamine (Invitrogen). The final concentration of siRNA was 50 nmol/L. FK228 was added to the cells 8 h after the last transfection. After 24 h, the cells were incubated with a recombinant adenovirus for 48 h and subjected to flow cytometry and immunobloting. Down-regulation of the target genes by specific siRNA but not negative controls was confirmed with by semiquantitative reverse transcription-PCR. Primers are listed in Supplementary Table 1.[3]

Animal Models

All animals were maintained under a specific pathogen-free condition and treated in accordance with guidelines by the Animal Care and Use Committee of Sapporo Medical University. The efficiency of the adenovirus-mediated gene transfer *in vivo* was determined by β-galactosidase activity

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

and immunoblot analysis. For determination of β-galactosidase activity, each tumor specimen was homogenized in 100 μL per 50 mm^3 sample of lysis buffer (Roche). Protein concentration was determined and β-galactosidase activity was assayed chemiluminescently using a β-Gal Reporter Gene Assay (Roche). Activity was expressed as relative light units/mg protein. Five mice were used for each group. For lacZ immunoblotting, nude mice were injected s.c. into right and left flanks with 2×10^6 MKN45 cells. When tumor size reached 400 mm^3, mice were treated with 0 or 0.2 μg/g FK228. Ad-lacZ was injected directly into one tumor on days 3 and 5. The other tumor was used as control. Both tumors were resected on day 7 and processed for immunoblotting.

To evaluate the potential to treat established tumors, MKN45 cells were injected s.c. into the right flank of nude mice as described above. When tumor size reached 100 $mm^3 \pm 10\%$, treatment was started. FK228 (0.2 μg/g) was injected i.p. three times at days 1, 3, and 5. Mice also received direct intratumoral injection of 5×10^9 plaque-forming units of the adenoviruses at days 3 to 5. Seven mice were used for each treatment group. The tumor volume was calculated using the equation: $V\ (mm^3) = a \times b^2 / 2$, where a is the largest dimension and b is the perpendicular diameter.

Statistical Analysis

All data were evaluated using Student's t test with probability values less than 0.05 considered significant.

Results

FK228 Enhances Adenoviral Infection in Human Cancer Cells

We first examined the proapoptotic effect of FK228 in human cancer cells using flow cytometry. Human stomach

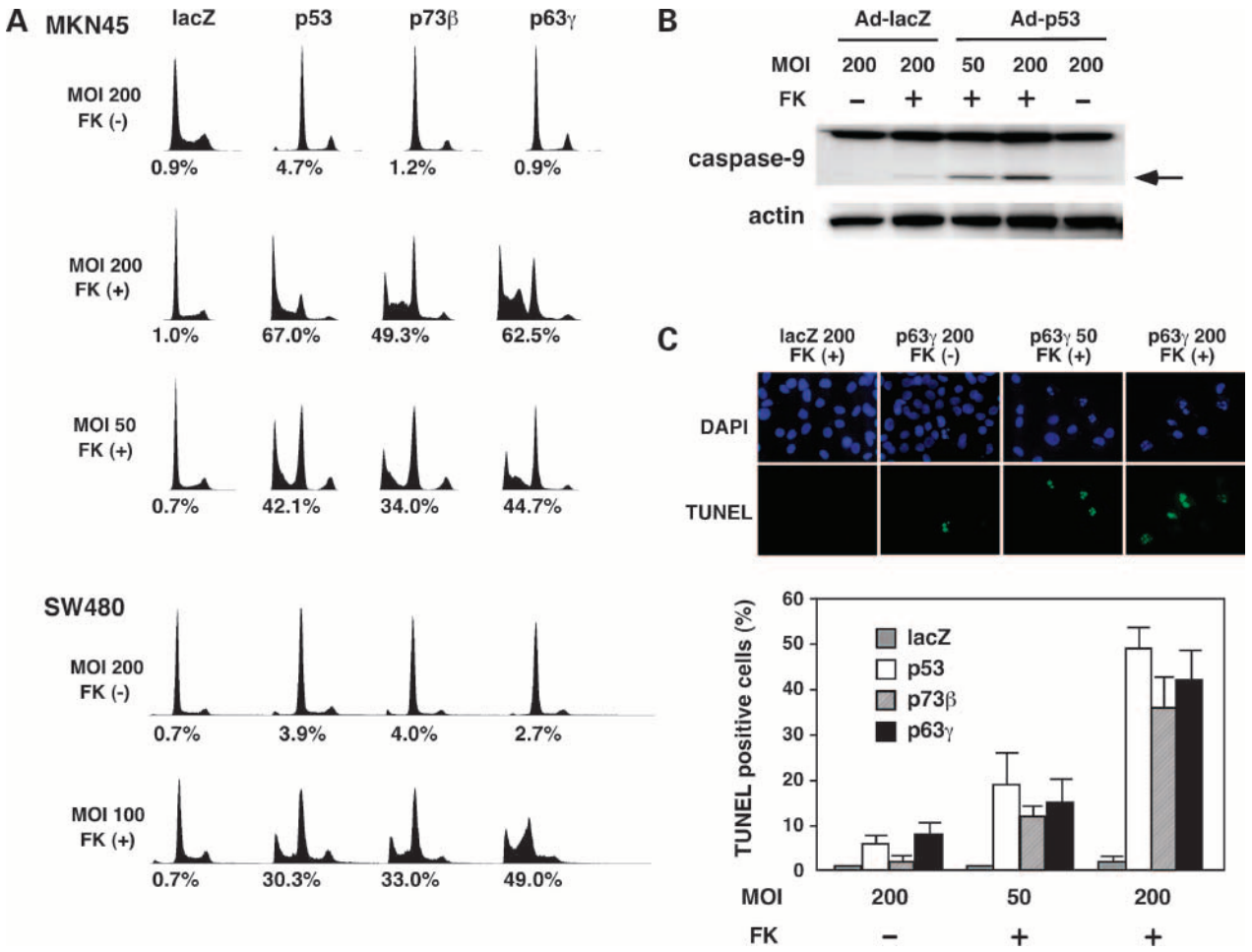

Figure 1. Combined treatment of human cancer cells with adenovirus-mediated transfer of p53 family genes and FK228. **A,** MKN45 cells were incubated with or without 5 nmol/L FK228. After 24 h, the indicated adenovirus was added to the medium at a MOI of 50 or 200. Apoptosis was examined by flow cytometry 24 h after infection. Percentage of cells in the sub-G_1 population. Experiments were repeated three times. Representative results. Synergistic effects on apoptosis were also examined in SW480 cells (*bottom*). **B,** detection of caspase-9 protein cleavage by immunoblotting. MKN45 cells were treated as described above. Immunoblot of caspase-9 and control actin proteins. *Arrow,* bands of cleaved products. **C,** TUNEL assay of MKN45 cells after the combined treatment as described in **A**. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole. *Bottom,* percentages of TUNEL-positive cells. Experiments were repeated three times. Mean percentages.

cancer cells MKN45 and colorectal cancer cells SW480 were chosen because they are relatively resistant to p53-induced apoptosis (24). Treatment of MKN45 and SW480 cells with FK228 for 48 h induced apoptosis in a dose-dependent manner (Supplementary Fig. S1A).[3] To analyze the augmentation of antitumor effect of adenovirus-mediated transfer of p53 family by combination with FK228, the optimum condition of FK228 showing no or minimal cytotoxicity by itself was determined to be 5 and 1 nmol/L for MKN45 and SW480, respectively. We also found that expression of the transgene lacZ was increased in MKN45 cells following FK228 pretreatment most likely by enhancing adenoviral infection (Supplementary Fig. S1B).[3] Similar results were observed for SW480 cells (data not shown). Efficient adenovirus infection requires CAR and integrins for attachment and internalization to enter host cells (26, 27). After incubation in FK228, increased expression of *CAR* as well as *integrin* α_V and β_3 mRNA were observed in MKN45 cells (Supplementary Fig. S1C).[3] Additionally, the cell surface expression of CAR and α_V integrin apparently increased by exposure to FK228 (Supplementary Fig. S1D),[3] suggesting that FK228 at a noncytotoxic concentration was able to significantly increase the expression of the CAR and integrins and enhance adenovirus-mediated gene transfer.

Combined Treatment with Adenovirus-Mediated Transfer of p53 Family and FK228

We then determined whether the combination of FK228 and adenovirus-mediated transfer of the p53 family could enhance apoptosis. Cells were pretreated with or without FK228 at a noncytotoxic concentration for 24 h, and recombinant adenovirus was added to medium. Apoptosis was analyzed by flow cytometry 24 h later. In the absence of FK228, transduction of Ad-p53, Ad-p73β, and Ad-p63γ at a MOI of 200 induced a significant cell cycle arrest but no apoptosis in MKN45 cells (Fig. 1A, *row 1*). Ad-lacZ-infected MKN45 cells pretreated with FK228 also displayed no apoptosis. In contrast, either Ad-p53-, Ad-p73β-, or Ad-p63γ-infected MKN45 cells underwent apoptosis when pretreated with FK228 (Fig. 1A, *row 2*). Importantly, apoptosis was seen even when infected virus was decreased to a MOI of 50 (Fig. 1A, *row 3*). This synergic effect of FK228 on apoptosis in combination with adenovirus-mediated transfer of p53 family genes was independently confirmed by caspase-9 activation (Fig. 1B) and TUNEL assay (Fig. 1C). Furthermore, this synergic effect was observed in SW480 cells (Fig. 1A, compare *row 4* with *row 5*). These results suggest that FK228 pretreatment increases the sensitivity of cancer cells to adenovirus-mediated p53 family gene therapy *in vitro*.

Accumulation of Acetylated p53 and p63 Proteins following FK228 Treatment

Next, we measured the expression level of p53 protein in Ad-p53 infected cells following the FK228 pretreatment. Following exposure to 5 nmol/L FK228 for 24 h, MKN45 cells were infected with Ad-p53 at various MOIs. An additional 24 h later, p53 protein was detected by immunoblot analysis. In control cells, the endogenous p53 protein was detected at a low level. FK228 pretreatment alone did not alter the expression level of endogenous p53 protein (Fig. 2A, compare *lane 1* with *lane 5*). In contrast, the exogenous p53 protein following Ad-p53 infection was increased in MKN45 cells pretreated with FK228 (Fig. 2A, *lanes 6-8*) compared with untreated cells (*lanes 2-4*). The p53 protein level in the FK228-treated cells after infection with Ad-p53 at a MOI of 50 is similar to that cells without FK228 treatment after infection with Ad-p53 at a MOI of 200 (Fig. 2A, compare *lane 4* with *lane 7*). This result is consistent with that of β-galactosidase gene transfer (Supplementary Fig. S1B)[3] in which adenoviral tranduction

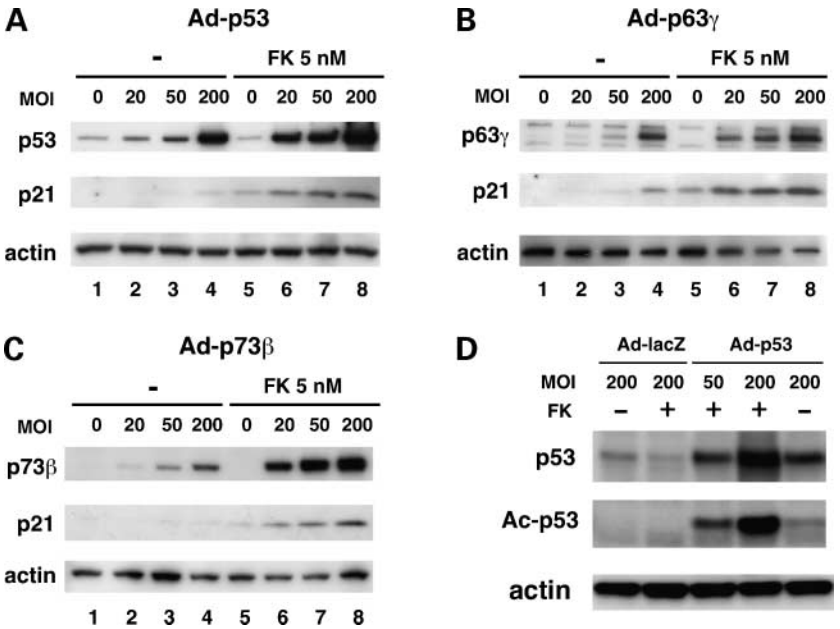

Figure 2. Accumulation of acetylated p53 protein by FK228. **A** to **C,** immunoblot analysis of p53, p73β, p63γ, and p21 proteins. MKN45 cells were treated with or without 5 nmol/L FK228. After 24 h, Ad-p53 (**A**), Ad-p63γ (**B**), or Ad-p73β (**C**) was added to the medium at the indicated MOI. Cells were harvested 24 h after infection and the amounts of p53, p73, p63, p21, and actin were determined by immunoblotting. **D,** levels of acetylated p53 proteins were determined by immunoblot analysis.

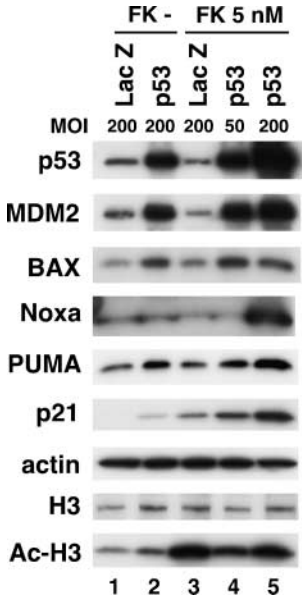

Figure 3. Endogenous expression of p53 target genes in control and FK228-treated cells overexpressing p53. MKN45 cells were incubated with or without 5 nmol/L FK228. After 24 h, Ad-lacZ or Ad-p53 was added to the medium at the indicated MOI. Cells were harvested 24 h after infection and the amount of p53, MDM2, Bax, Noxa, Puma, p21, actin, histone H3, and acetylated histone H3 was determined by immunoblotting.

efficiency was increased 4-fold by the FK228 pretreatment. Similar transgene enhancement was found when Ad-p63γ was infected (Fig. 2B). Interestingly, the exogenous p73β protein was remarkably increased by the FK228 pretreatment (Fig. 2C). FK228 treatment alone induced p21 expression as reported previously (19, 20). In MKN45 cells, adenovirus-mediated transfer of the p53 family alone had a slight effect on p21 induction, but the combination greatly increased p21 expression (Fig. 2A-C).

Acetylation of p53 protein was reported to be important for activation of p53-targeted gene expression and apoptosis induction (28). Thus, we examined whether the FK228 pretreatment resulted in an increase in acetylation of p53 and p63 proteins by immunoblot analysis. The level of acetylated p63 as well as p53 proteins increased after the administration of FK228 (Fig. 2D; Supplementary Fig. S2).[3] These results revealed that the concentrations of FK228 used in this study can also result in accumulation of the acetylated p53 and p63 proteins.

FK228 Enhances p53-Induced Noxa Expression

p53-dependent apoptosis is regulated, in large part, by transcriptional activation of its target genes. Thus, we measured the expression level of proapoptotic p53 targets after the combined treatment. MKN45 cells were infected with Ad-p53 or Ad-lacZ in the presence or absence of FK228, and the protein levels of p53 and endogenous MDM2, Bax, Noxa, and PUMA were examined by immunoblot analysis. The exogenous p53 protein was enhanced by FK228 pretreatment (Fig. 3, *row 1*, compare *lane 2* with *lane 5*). The MDM2 protein was also induced by Ad-p53 alone, whereas the FK228 pretreatment further enhanced MDM2 induction (Fig. 3, *row 2*, compare *lane 2* with *lane 5*). Interestingly, the combined treatment with Ad-p53 and FK228 specifically induced Noxa in MKN45 cells, but the expression levels of Bax and Puma were not significantly enhanced by the combined treatment.

FK228 Promotes the Translocation of Bax to Mitochondria

Bax protein resides normally in the cytosol and translocates to mitochondria in response to a variety of apoptotic stimuli (29, 30). Thus, the mitochondrial localization of Bax protein was examined using subcellular fractionation and immunocytochemical analysis. Under a control condition, a small portion of Bax protein could be detected in the mitochondrial fraction (Fig. 4A, *lane 1*). Based on densitometric analysis, the Bax protein in mitochondria following a single treatment with Ad-p53 or FK228 was 4.0- or 3.5-fold greater than the control level, respectively (Fig. 4A). Importantly, the combined treatment facilitates the translocation of Bax from the cytosol to the mitochondria by more than 10-fold (Fig. 4A, *lanes 4* and *5*). Furthermore, these findings were confirmed by immunofluorescence staining. In control cells, Bax was diffusely distributed and did not colocalize with MitoTracker Red, consistent with cytoplasmic localization of Bax protein. As shown in Fig. 4B, the Bax protein was predominantly accumulated in the mitochondria after the combined treatment. Because a similar level of Bax protein was observed in the whole-cell lysates of Ad-p53-infected cells regardless of the FK228 pretreatment (Fig. 3), the combined therapy enhances mitochondria translocation of Bax protein and subsequent activation of the mitochondrial pathway of apoptosis.

Bax and Noxa siRNAs Antagonize the Synergistic Effect of Ad-p53 and FK228 on Apoptosis

To evaluate the significance of Bax and Noxa on the synergistic effect of Ad-p53 and FK228 on apoptosis, we used siRNAs to knockdown *Bax* (si-Bax) and *Noxa* (si-Noxa) in MKN45 cells. An siRNA lacking sequence homology with any human gene was used as a control siRNA, and cells were transfected with siRNAs every 24 h for 3 consecutive days. As shown in Fig. 5A, semiquantitative reverse transcription-PCR revealed that the specific reduction in mRNA level of *Bax* and *Noxa* in cells transfected with si-Bax and si-Noxa, respectively. Transfection of both siRNAs was also effective in knocking down the targeted mRNAs simultaneously (Fig. 5A, *si-Bax + si-Noxa*). At 8 h after the last transfection, cells were incubated with or without 5 nmol/L FK228 for 24 h. Cells were then infected with Ad-lacZ or Ad-p53 and subjected to flow cytometric analysis. Little effect of Bax siRNA or Noxa siRNA was observed when MKN45 cells were subsequently treated with Ad-lacZ, Ad-lacZ plus FK228, or Ad-p53 (Fig. 5B, *top*, *rows 2* and *3*). On the other hand, the control siRNA-transfected cells underwent apoptosis after the combined treatment (Fig. 5B, *row 4*). A small effect on apoptosis was observed when Bax siRNA or Noxa siRNA was transfected in MKN45 cells with the combined treatment (Fig. 5B, *row 4*, *si-Bax* or *si-Noxa*). In contrast, the double knockdown of *Bax* and *Noxa* by siRNA clearly

antagonizes the proapoptotic effect of Ad-p53 and FK228 (Fig. 5A, *row 4, si-Bax + si-Noxa*). We also showed that the double knockdown of *Bax* and *Noxa* almost completely blocked poly(ADP-ribose) polymerase and caspase-9 cleavage after the combined treatment (Fig. 5C, *lanes 7* and *9*). These results suggest that the synergic effect on apoptosis is at least in part mediated through Bax and Noxa activities.

Combined Therapy of Ad-p53 Family Gene and FK228 *In vivo*

We examined whether the FK228 pretreatment could enhance the therapeutic effect of Ad-p53 *in vivo* xenograft model. We first found that FK228 improved the efficiency of adenovirus-mediated gene transfer *in vivo*. MKN45 cells were injected s.c. into nude mice. When tumor size reached 400 mm^3, FK228 was injected i.p. three times at days 1, 3, and 5. To examine the efficiency of the adenovirus-mediated gene transfer *in vivo*, each mouse received direct intratumoral injection of 5×10^9 plaque-forming units of Ad-lacZ at days 3 and 5. We then sacrificed the mice at day 7 and measured β-galactosidase activity in tumor cells. We observed that β-galactosidase activity in cell extracts was increased after the FK228 pretreatment in a dose-dependent manner (Fig. 6A). In a second approach, the efficiency of the adenovirus-mediated gene transfer *in vivo* was determined by immunoblot using cell extracts from control and FK228-treated tumors after Ad-lacZ infection. Consistent with the results of β-galactosidase activity assay, the increased expression of β-galactosidase protein was detected in tumors pretreated with FK228 (Fig. 6A, compare mice 1, 4, and 5 with mice 2, 3, and 6).

Subsequently, we tested the effect of the combined therapy on the progression of established MKN45 tumors. As shown in Fig. 6C, whereas treatment with Ad-lacZ plus FK228 had a marginal antitumor effect, single treatment with Ad-p53 or Ad-p63γ retarded tumor growth in MKN45 tumor-bearing mice compared with Ad-lacZ treatment alone. Remarkably, Ad-p53 in combination with FK228 synergistically suppressed tumor growth in tumor-bearing mice compared with either Ad-p53 alone ($P = 0.0002$ for tumor volume) or FK228 alone ($P = 0.0001$ for tumor volume). A similar synergistic effect was seen with the FK228 pretreatment in tumors with Ad-p63γ infection (Fig. 6C). Furthermore, there was no significant difference on tumor volume or survival between mice receiving Ad-p53 plus FK228 and those receiving Ad-p63γ plus FK228. Notably, Ad-p53 and Ad-p63γ in combination with FK228 induced complete tumor regression in 50% (4 of 8) and 25% (2 of 8) of tumor-bearing mice, respectively. This *in vivo* result correlates with the specificity observed *in vitro*.

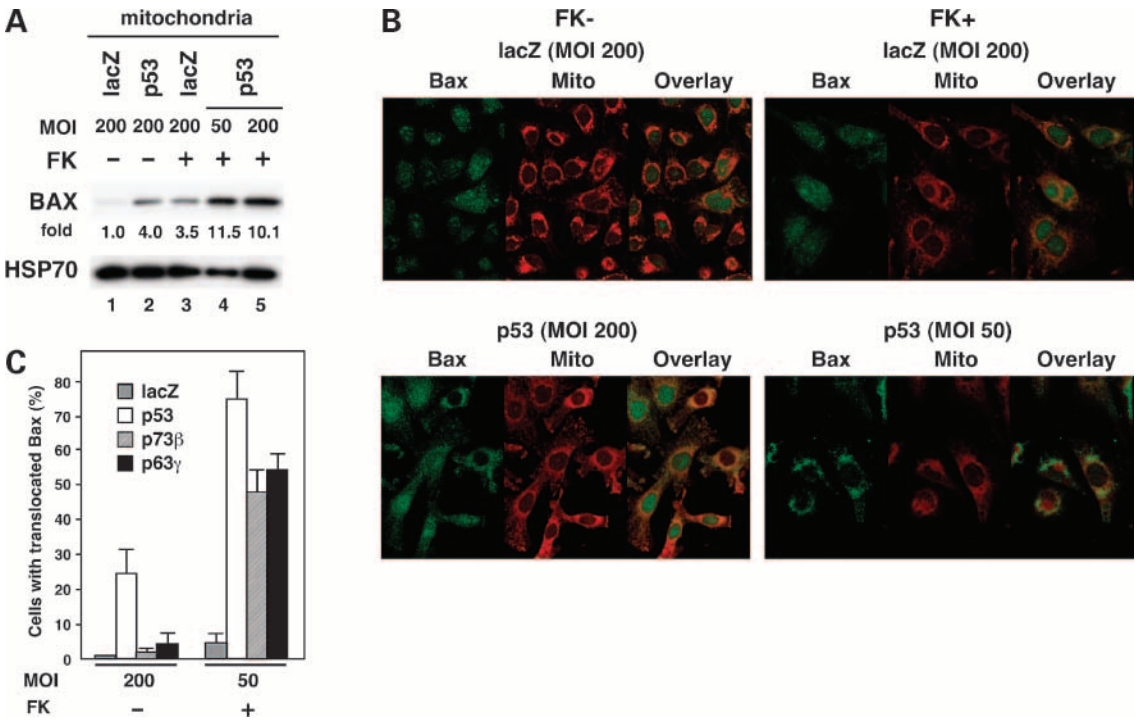

Figure 4. Combined treatment of adenovirus-mediated transfer of p53 family genes and FK228 promotes the translocation of Bax to mitochondria. **A,** equal amounts of the mitochondrial proteins isolated from control and FK228-treated MKN45 cells were separated on SDS-polyacrylamide gels and then subjected to immunoblot analysis for Bax. Mitochondrial HSP70 was used as loading control for the mitochondrial protein. The Bax protein bands were quantified and normalized against the corresponding HSP70 bands to obtain relative Bax expression levels. **B** and **C,** immunostaining of Bax subcellular distribution following FK228 treatment. Control and FK228-treated MKN45 cells were incubated with the indicated adenoviruses in a four-well slide chamber. After 16 h, cells were fixed, immunostained for Bax (*green*), and double stained with MitoTracker Red for mitochondria (*red*). Mitochondrial and Bax localization was imaged by fluorescence microscopy. Colocalization of Bax in mitochondria was shown (*yellow*) in merged images. A minimum of 200 cells were assessed for each sample. **C**, percentages of cells having Bax localized to mitochondria. Three independent experiments were done. Mean ± SD.

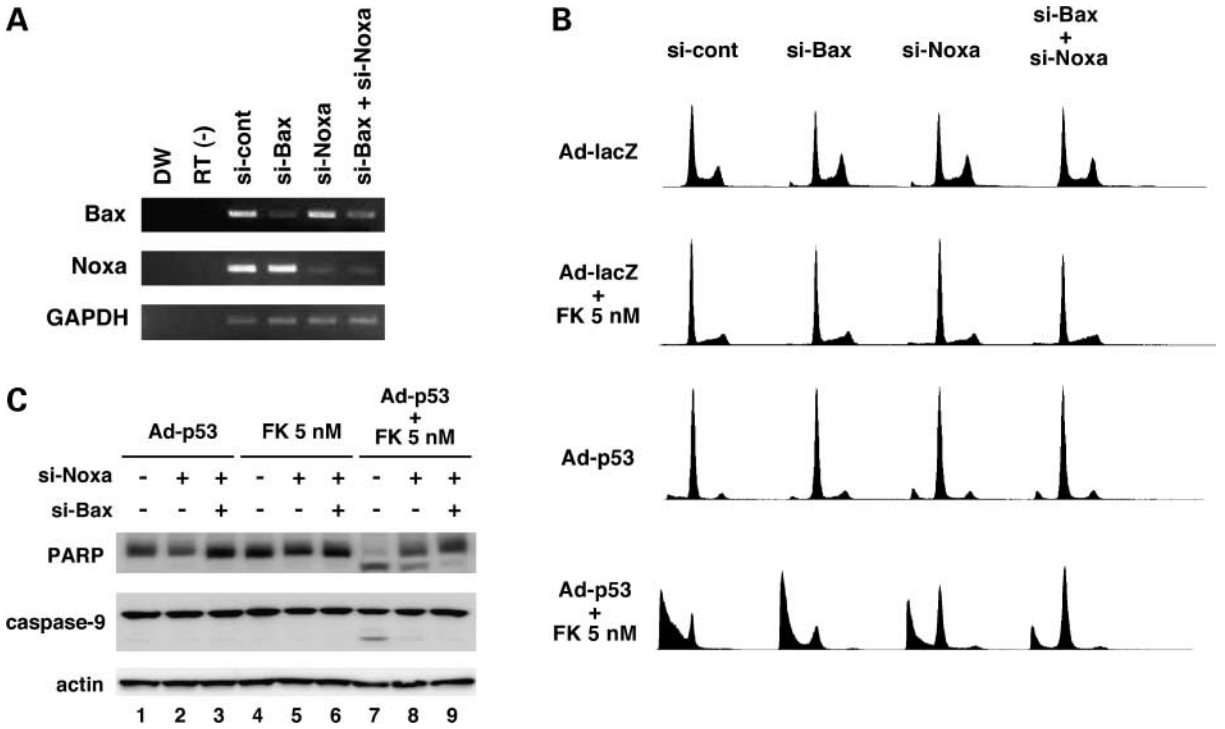

Figure 5. Bax and Noxa siRNAs antagonize the synergistic effect of Ad-p53 and FK228 on apoptosis induction. **A,** inhibition of *Bax* and *Noxa* mRNA expression by siRNAs. MKN45 cells were transfected with control siRNA (*si-cont*), Bax, and/or Noxa siRNAs every 24 h for 3 consecutive days. Twenty-four hours after the last transfection, suppression of the corresponding mRNA was determined by semiquantitative reverse transcription-PCR. **B** and **C,** siRNA was done as described above. At 8 h after the last transfection, cells were incubated with or without 5 nmol/L FK228 for 24 h. Cells were then infected with Ad-lacZ or Ad-p53 at a MOI of 200, and apoptosis was examined by flow cytometry (**B**) and poly(ADP-ribose) polymerase cleavage (**C**) 24 h after infection. Experiments were repeated three times. Representative results.

Discussion

The combination of HDACIs and Ad-p53 has been tested previously. *In vitro* studies have shown synergy between *p53* gene transduction and FK228 in thyroid carcinoma cell lines (31); however, these studies did not determine the mechanisms for synergy with Ad-p53 and FK228. In stomach and liver cancer cell lines, a synergistic effect of *p53* gene transduction and another HDACI, sodium butyrate, was observed by Takimoto et al. (32). In the present study, we show that FK228 in combination with adenovirus-mediated transfer of p53 family significantly enhanced the transgene expression as well as therapeutic effect against human cancer cells, which are resistant to apoptosis by *p53* gene transfer alone. It has been reported that adenoviral transduction was enhanced in some human carcinoma cells after FK228 treatment, which was associated with increased expression of CAR and integrin (21–23). Our results *in vitro* are consistent with previous reports. Moreover, when a recombinant adenovirus carrying the *lacZ* gene was injected into tumors in nude mice, β-galactosidase activity was significantly higher than in tumors of untreated mice (Fig. 6). Recent studies showed that FK228 can increase CAR mRNA and protein in xenograft models (33). Thus, enhancement of CAR expression may be one of the mechanisms contributing to the higher cytotoxic and tumoricidal effects of Ad-p53 on FK228-treated MKN45 cells *in vitro* and *in vivo*. In the clinical setting, administration of FK228 at a clinically acceptable dosage may improve the low efficiency of adenovirus-mediated gene transfer into cancer cells.

Lysine residues in the p53 protein can be acetylated by histone acetyltransferases such as p300/CREB-binding protein and p300/CREB-binding protein–associated factor (17, 34). FK228 may also have inhibitory activity against non-histone deacetylases (19). We showed that FK228 treatment can lead to acetylation of p53 and p63 protein. Other potential mechanisms to explain the synergistic effect of the combined treatment presented here are involved in Noxa induction and Bax translocation. Several p53 targets have been reported as candidates for the p53-mediated apoptotic pathway (4). We found that Noxa expression was considerably and specifically increased in MKN45 cells following the combined treatment (Fig. 3). Pretreatment of MKN45 cells with FK228 could enhance p53-depedent transactivation of a subset of target genes probably due to increased p53-DNA binding activity through the modulation of histone acetylation and chromatin composition or through acetylation of lysine residues in the p53 protein. The *Bax* gene product is known to translocate from the cytosol to mitochondria in the process of apoptosis (29, 30). As shown in Fig. 5, the combined treatment did not induce Bax expression but promoted mitochondrial translocation

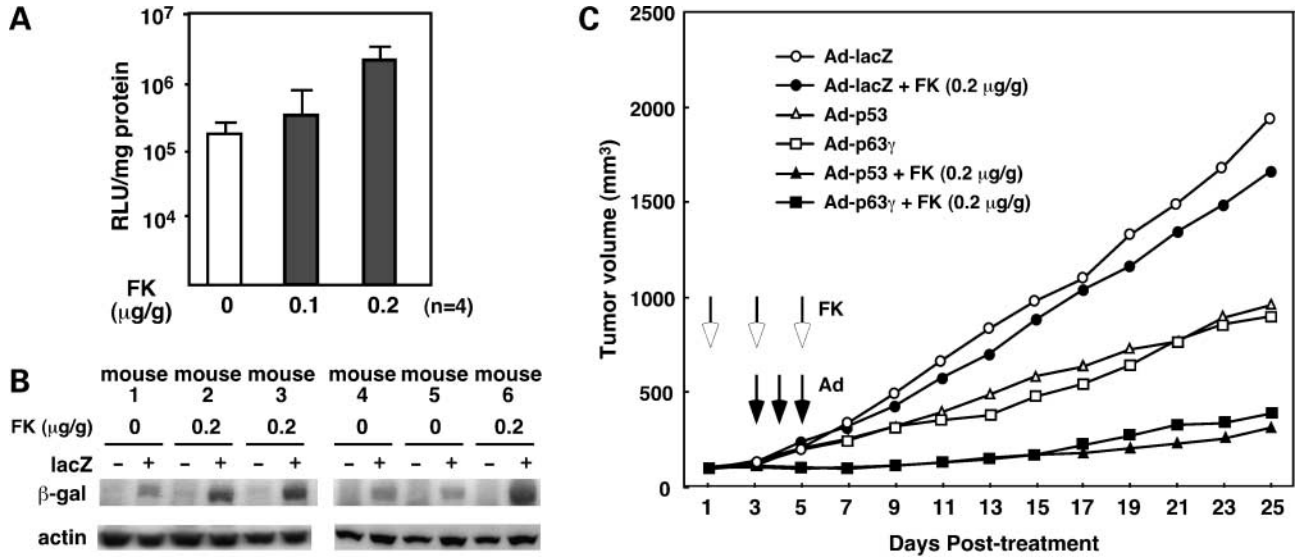

Figure 6. FK228 increases the efficacy of adenovirus-mediated p53 family gene therapy in an *in vivo* xenograft model. **A,** FK228 enhances the efficiency of the adenovirus-mediated gene transfer in an *in vivo* xenograft model. MKN45 cells were injected s.c. into nude mice. When the tumor size reached 400 mm^3, 0, 0.1, or 0.2 μg/g (body weight) of FK228 was injected i.p. at days 1, 3, and 5. Each mouse received direct intratumoral injection of 5×10^9 plaque-forming units of Ad-lacZ at days 3 and 5. Mice were killed on day 7, and the efficiency of the adenovirus-mediated gene transfer *in vivo* was determined by β-galactosidase. The mean values were 2.1×10^5 and 3.1×10^6 relative light units/mg for control and 0.2 μg/g treated mice with MKN45 xenografts ($P_{trend} < 0.0001$). **B,** MKN45 cells were injected into the right and left flanks of nude mice. When tumor size reached 400 mm^3, mice were treated with (mice 2, 3, and 6) or without FK228 (mice 1, 4, and 5). Ad-lacZ was injected directly into one tumor on days 3 and 5. The other tumor was used as control (*lacZ* -). Both tumors were resected on day 7 and processed for lacZ immunoblot. **C,** therapeutic effect of adenovirus-mediated transfer of p53 family genes on s.c. xenografts of human cancer cells. MKN45 cells were injected s.c. into nude mice. When the tumor size reached 100 mm^3, the mice were randomly divided into six treatment groups: single treatments with Ad-p53, Ad-p63γ, or Ad-LacZ alone and combined treatment with Ad-p53 + FK228, Ad-p63γ + FK228, and Ad-LacZ + FK228. Each treatment group contained eight mice, and experiments were repeated twice. Mean tumor volumes.

of the Bax protein. The double knockdown of *Bax* and *Noxa* expression by siRNA antagonizes the synergistic effect of Ad-p53 and FK228 on apoptosis induction (Fig. 5), suggesting that the two molecules play a central role in the apoptotic pathway in MNK45 cells.

In this study, we showed that FK228 enhances the proapoptotic effect of Ad-p73 and Ad-p63 in addition to Ad-p53. Exogenous expression of p53 inhibits tumor growth by inducing apoptosis. However, *p53*-based gene therapy is not effective in certain conditions, suggesting many tumors could be resistant to exogenous p53 (5, 6). For example, amplification of *MDM2*, mutational inactivation of *p14ARF*, or presence of the HPV E6 oncogene renders the p53 pathway of growth suppression inactive by degrading of p53 protein through ubiquitination (7, 8, 35–37). Thus, inhibition of several alternate candidate genes or other methodologies has been attempted for treatment of these types of cancer. We showed here that Ad-p73β and Ad-p63γ induced extensive apoptosis similar to Ad-p53 when combined with FK228 pretreatment in MKN45 and SW480 cells (Fig. 1). Moreover, Ad-p63γ in combination with FK228 synergistically suppressed tumor growth *in vivo* (Fig. 6). Earlier work by our laboratory and others has shown that some of the cancer cell lines resistant to p53-mediated apoptosis undergo apoptosis after transduction of p73β or p63γ (14, 15, 24, 38). Thus, *p73* and *p63* could be a suitable alternate candidate for gene therapy of human cancers, particularly under conditions where *p53* gene therapy is not effective.

In summary, we have shown that a noncytotoxic dose of FK228 can enhance transgene expression as well as therapeutic effect of adenovirus-mediated p53 family gene transfer. These studies suggest a simple, clinically practical method for increasing the sensitivity of cancer cells to adenoviral gene therapy vectors. By avoiding the need for high adenoviral titers, the likelihood of viral toxicity is reduced. Hence, this study should be considered in planning future clinical trials combining adenovirus-mediated p53 family gene transfer and FK228.

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