The histone deacetylase inhibitor FK228 given prior to adenovirus infection can boost infection in melanoma xenograft model systems

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Abstract

A major limitation of adenovirus type 5–mediated cancer gene therapy is the inefficient infection of many cancer cells. Previously, we showed that treatment with low doses of the histone deacetylase inhibitor FK228 (FR901228, depsipeptide) increased coxsackie adenovirus receptor (CAR) levels, histone H3 acetylation, and adenovirus infection efficiencies as measured by viral transgene expression in cancer cell lines but not in cultured normal cells. To evaluate FK228 *in vivo*, the effects of FK228 therapy in athymic mice bearing LOX IMVI or UACC-62 human melanoma xenografts were examined. Groups of mice were treated with FK228 using several dosing schedules and the differences between treated and control animals were determined. In mice with LOX IMVI xenografts ($n = 6$), maximum CAR induction was observed 24 h following a single FK228 dose of 3.6 mg/kg with a 13.6 ± 4.3-fold (mean ± SD) increase in human CAR mRNA as determined by semiquantitative reverse transcription-PCR analysis. By comparison, mouse CAR levels in liver, kidney, and lung from the same animals showed little to no change. Maximum CAR protein induction of 9.2 ± 4.8-fold was achieved with these treatment conditions and was associated with increased histone H3 acetylation. Adenovirus carrying a green fluorescent protein (GFP) transgene (2×10^9 viral particles) was injected into the xenografts and GFP mRNA levels were determined. A 7.4 ± 5.2-fold increase in GFP mRNA was found 24 h following adenovirus injection into optimally FK228-treated mice ($n = 10$). A 4-fold increase in GFP protein–positive cells was found following FK228 treatment. These studies suggest that FK228 treatment prior to adenovirus infection could increase the efficiency of adenovirus gene therapy in xenograft model systems. [Mol Cancer Ther 2007;6(2):496–505]

Introduction

A major limitation of adenovirus type 5–mediated cancer gene therapy is the inability of adenovirus to efficiently infect many cancer cells. Although many malignant cells are poorly infected by adenovirus, normal tissues, especially liver, are susceptible to adenovirus infection (1). Our previous studies showed that treatment with subcytotoxic doses of the histone deacetylase inhibitor FK228 (FR901228, depsipeptide; refs. 2, 3), a drug in phase II clinical trials for the treatment of patients with peripheral and cutaneous T cell lymphoma (4, 5), can increase the efficiency of adenovirus infection *in vitro* (6). The purpose of the current study was to test our hypothesis that FK228 could increase the efficiency of adenovirus infection *in vivo* using xenograft model systems. If successful, these experiments may suggest a way to improve the efficiency of adenovirus gene therapy against solid tumors.

In vitro adenovirus serotype 5 requires the coxsackie adenovirus receptor (CAR) and the α_v integrin component of integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$ to infect most cells efficiently (7–9). *In vivo* adenovirus infection may be more complex with alternate receptors used in some cells (10). In most cell types, CAR mediates the attachment of adenovirus to cells and α_v integrin mediates the internalization of virus into cells. Many studies have reported correlations between CAR and α_v integrin levels and adenovirus infection (11–13). Low levels of CAR in tumors are thought to be one of the reasons for poor adenovirus infection (14, 15). Our previous studies showed that FK228 treatment *in vitro* prior to adenovirus infection could increase both CAR and α_v integrin mRNA levels in six cancer cell lines from a variety of tissues (6). As would be expected from treatment with a histone deacetylase inhibitor, there was an increase in histone H3 acetylation in the cell lines following FK228 treatment. These studies showed that treatment of cells *in vitro* with 1 ng/mL of FK228 prior to adenovirus

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infection resulted in a 4- to 10-fold increase in adenovirus transgene expression. The magnitude of the FK228-mediated fold increase in CAR was inversely correlated with initial CAR expression with high CAR–expressing cell lines showing only modest increases in CAR levels in response to the drug. Although our *in vitro* studies suggested that FK228 treatment could increase transgene expression when added both before and after adenovirus infection (16), the former effect was more pronounced. In the current *in vivo* studies, we have restricted FK228 treatment to before adenovirus infection in order to focus on the drug effect on the infection process.

Because FK228 increases the efficiency of adenoviral transgene expression *in vitro*, FK228 treatment might be useful in cancer gene therapy; however, to be successful, such an approach must preferentially affect cancer cells. Additional studies from our laboratory showed that low concentrations of FK228 which resulted in a marked increase in adenovirus transgene expression in cancer cell lines from breast, kidney, and liver were found to have little effect on cultured normal cells from these tissues (16). Because low concentrations of FK228 prior to adenovirus infection are capable of conferring a preferential increase in adenovirus transgene expression in cancer cells *in vitro*, the object of the current study was to investigate whether FK228 treatment prior to adenovirus infection could increase the efficacy of adenovirus therapies *in vivo* in xenograft model systems using therapeutically relevant drug dosages.

Our results in two xenograft model systems show that FK228 treatment prior to adenovirus infection can substantially increase expression from the transgene carried by the adenovirus.

Materials and Methods

Drugs

FK228, a fermentation product from *Chromobacterium violaceum*, was first isolated by the Fujisawa Pharmaceutical Company (Osaka, Japan) (2). FK228 was obtained from the Pharmaceutical Management Branch, Cancer Therapy Evaluation Program, National Cancer Institute, NIH (Bethesda, MD).

Cell Lines

The LOX IMVI and UACC-62 human melanoma cell lines were obtained from the Developmental Therapeutics Program, National Cancer Institute, NIH (Frederick, MD).

Adenovirus

Ad5.hCMV-GFP, an E1 and E3 gene–deleted, replication-defective type 5 adenovirus was produced, purified, tested, and titered by Qbiogene (Montreal, Canada).

Xenograft Studies

Animal studies were done at the Southern Research Institute (Birmingham, AL), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The Institutional Animal Care and Use Committee of the Southern Research Institute approved all experimental procedures. The origin, development, characterization, and use of human tumor xenografts for *in vivo* efficacy evaluations were previously described (17, 18). The host animals for these tumor models were random-bred, athymic/NCr (*nu/nu*) mice (National Cancer Institute Animal Production Program, NCI Frederick). In the LOX IMVI melanoma study, male athymic *nu/nu* mice were implanted s.c. with 1×10^6 cells from ascites fluid obtained from an *in vivo* passage. Treatment began on day 8 (tumor implantation day = day 0) when the tumor weight ranged from 245 to 283 mg (tumor weight calculated from the tumor dimensions; ref. 17). In the UACC-62 melanoma study, female athymic *nu/nu* mice were implanted s.c. with 30 to 40 mg tumor fragments from an *in vivo* passage. Treatment began on day 14 when the tumor weight ranged from 279 to 352 mg. FK228 was formulated at a concentration of 5 mg/mL in 25% polyethylene glycol 400 in distilled water or 20% ethanol, 80% propylene glycol, and was administered i.v. using an injection volume of 0.1 mL/10 g body weight. Control mice were treated with the vehicle without drug. If more than a single drug dose was administered, injections were given twice (q4dx2) or thrice (q4dx3) every fourth day. Drug doses were based on previously published doses (19). At various times following drug treatment, the mice were euthanized and tissues were removed and frozen.

In experiments with adenovirus, mice bearing advanced-stage xenografts were treated with FK228 at a dose of 3.6 mg/kg. After 24 h, the tumors were injected with 2×10^9 VP Ad5.hCMV-GFP in 0.05 mL using four needle tracks. After an additional 24 h, the mice were euthanized. RNA was isolated from the entire xenograft to insure comparable samples.

Semiquantitative Reverse Transcription-PCR Analysis

RNA was isolated and semiquantitative reverse transcription-PCR (RT-PCR) was done as previously described (16). The primers used for the analysis of human CAR (GenBank accession no. NM_001338), human α_v integrin (NM_002210), 28S rRNA (M11167; ref. 20), mouse CAR (NM_009988), mouse α_v integrin (NM_008402), and green fluorescent protein (GFP; M62653) were as follows:

hCAR 5′ (sense), ^{779}GATCAGTGCCTGTTGCGTCTA^{799}
hCAR 3′ (antisense), ^{1178}TCACAGGAATCGCACCCA^{1161}
hα_v integrin 5′ (sense), ^{1792}TAAAGGCAGATGGCAAAG-GAGT^{1813}
hα_v integrin 3′ (antisense), ^{2303}CAGTGGAATGGAAAC-GATGAGC^{2282}
28S 5′ (sense), ^{1542}AAACTCTGGTGGAGGTCCGT^{1561}
28S 3′ (antisense), ^{1847}CTTACCAAAAGTGGCCCACTA^{1827}
mCAR 5′ (sense), ^{221}AGAGGATCGAAAAAGCCAAAGG^{242}
mCAR 3′ (antisense), ^{558}AACAAGAACGGTCAGCAGG-AAT^{537}
mα_v integrin 5′ (sense), ^{926}AACCAATTAGCAACACGG-ACTG^{947}
mα_v integrin 3′ (antisense), ^{1331}CAAACCTGGCAAAAA-CCTCAAA^{1310}
GFP 5′ (sense), ^{134}GCAACATACGGAAAACTTACCC^{155}
GFP 3′ (antisense), ^{649}CGAAAGGGCAGATTGTGTGG^{630}

The human CAR primers were used for the xenografts and the mouse CAR primers were used for the normal mouse tissues. The experimental conditions were chosen based on preliminary experiments which indicated that all samples would be in the exponential range of amplification to allow for more precise quantitation. All semiquantitative analyses were normalized using 28S rRNA as the control (20). Several housekeeping genes were evaluated as a control gene but 28S rRNA was chosen as the control gene because its expression, as compared with carefully measured RNA, seemed to be unaffected by FK228.

Statistical Analysis

The results are presented as the mean ± SD. The statistical significance of the difference in values between two groups of animals was determined by an exact Wilcoxon rank sum test. The Jonckheere-Terpstra test for trend was used to evaluate trends in expression over increasing dose levels (21). All P values are two-tailed and have not been adjusted for multiple comparisons.

Immunohistochemistry

Xenografts from some mice were prepared for immunohistochemistry. The xenografts were fixed in 10% formalin, embedded in paraffin and sectioned. The sections were incubated with a rabbit polyclonal antibody against GFP (Chemicon International, Temecula, CA) and stained with avidin-biotin complex and peroxide reagents (Vector Laboratories, Burlingame, CA). The number and fluorescent intensity of GFP-expressing cells in the sections were quantified using an iCys laser scanning cytometer (Compucyte Corp., Cambridge, MA) using 488 nm laser light (20 mW) absorption by the tissue and chromagen. The laser light absorption values were inverted (so chromagen directly corresponded to pixel brightness), and is shown in histograms.

Western Blot Analysis

Total cellular protein was isolated from the xenografts as previously described (16). For acetylated histone H3 analysis, the proteins were run on an Invitrogen (Carlsbad, CA) 8% E-PAGE 48 high-throughput bufferless protein gel electrophoresis system, transferred to nitrocellulose and analyzed. The blots were probed with glyceraldehyde-3-phosphate dehydrogenase antibody (American Research Products, Belmont, MA), stripped, and then probed with acetylated histone H3 antibody (Upstate, Charlottesville, VA). The acetylated histone H3 levels were normalized using glyceraldehyde-3-phosphate dehydrogenase as the control. Analysis of CAR and α_v integrin was done as previously described using CAR (Santa Cruz Biotechnology, Santa Cruz, CA), α_v integrin (Santa Cruz Biotechnology), or glyceraldehyde-3-phosphate dehydrogenase (American Research Products; control) antibody (16).

Results

Our *in vitro* results showing higher adenovirus transgene expression levels in FK228-treated cancer cell lines compared with cultured normal cells prompted an investigation of the *in vivo* susceptibility of FK228-treated human tumor xenografts to CAR-mediated adenovirus infection. Mice with LOX IMVI melanoma xenografts were injected once, twice, or thrice with FK228 at a dose of 3.6 mg/kg, and xenografts and normal tissues were obtained for analysis. Figure 1A shows semiquantitative RT-PCR analysis of CAR mRNA levels (normalized to 28S rRNA) in xenografts from vehicle-treated (control) mice and FK228-treated mice euthanized 6 h following drug treatment. CAR mRNA levels in the control xenografts were low. The levels in the samples obtained from treated mice were considerably higher but showed animal-to-animal differences. Xenografts from mice ($n = 6$) treated with a single dose of FK228 had a mean ± SD increase in CAR mRNA levels of 10.7 ± 4.9-fold ($P = 0.0022$) at 6 h following drug treatment compared with control animals ($n = 6$) with mean CAR mRNA levels of 1.0 ± 0.6-fold. Despite the noted increase in CAR levels in the xenografts, the levels of α_v integrin mRNA were unchanged, however, it should be noted that the threshold levels of α_v integrin mRNA were high (data not shown). Because xenografts from mice treated with two or three doses of FK228 did not have higher CAR mRNA levels, we concluded that there was no measurable advantage to using multiple drug doses. Therefore, our subsequent experiments used a single drug dose. In addition, published studies using FK228 for tumor regression used multiple drug doses and we wanted to minimize tumor shrinkage (19, 22).

The mechanism responsible for FK228 activity probably relates to its function as a histone deacetylase inhibitor. Our previous *in vitro* results showed that FK228 increased histone H3 acetylation and that the histone deacetylase inhibitors sodium butyrate and trichostatin A also increased CAR mRNA levels (6). Figure 1B shows the results of Western blot analysis of xenograft proteins from FK228-treated and control mice ($n = 6$) and indicated that FK228 treatment increased histone H3 acetylation *in vivo*. Using this analysis at 6 h, there was a mean 28.2 ± 22.9-fold ($P = 0.0022$) increase in acetylated histone H3 in xenografts from animals treated with a single 3.6 mg/kg dose of FK228 compared with untreated controls with a mean 1.0 ± 0.1 level. By 24 h, the average increase had dropped to 20.4 ± 12.6-fold ($P = 0.0022$) compared with 1.0 ± 1.4 for controls. Inter-animal differences were responsible for the large standard deviations. These results suggest that FK228 may work through the acetylation of histones or other proteins.

Next, LOX IMVI xenograft-bearing mice were treated with different doses of FK228 and euthanized at different times following drug administration. The analysis shown in Fig. 1C indicated that the highest induction of CAR mRNA in the LOX IMVI xenografts was obtained in animals ($n = 6$) euthanized 24 h following treatment with FK228 at a dose of 3.6 mg/kg. Trend tests show the strength of the association with $P < 0.0005$ for the significance of the trend at 6 h, and $P < 0.0001$ for the trend at 24 h. Inter-animal variability was responsible for the large error bars. CAR mRNA levels had begun to decrease by 48 h following FK228 administration (data not shown).

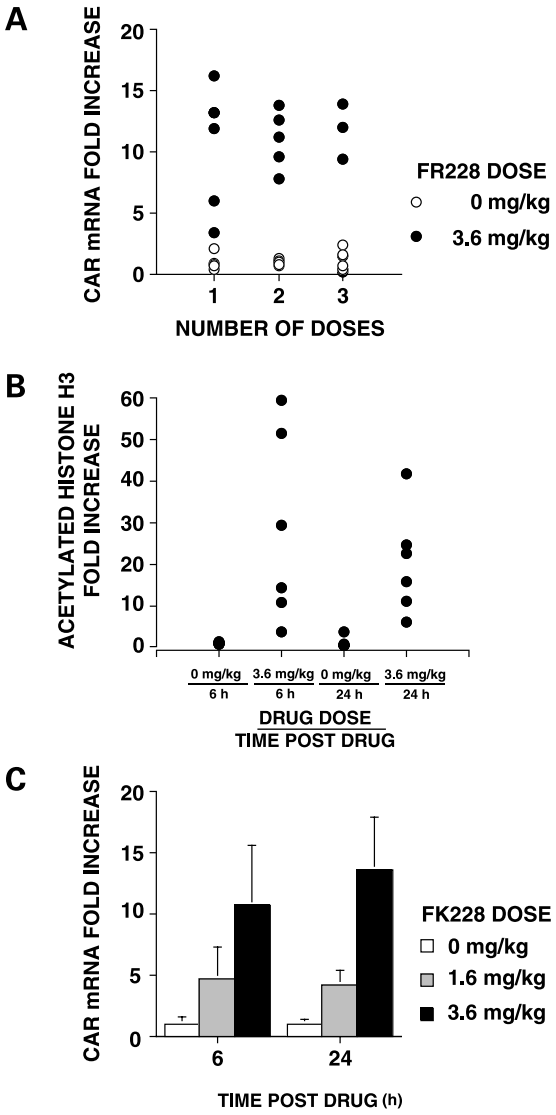

Figure 1. FK228 treatment increases CAR mRNA and acetylated histone H3 protein in LOX IMVI melanoma xenografts. Athymic mice with LOX IMVI xenografts (n = 6) were treated with different doses of FK228 and the mice were euthanized at different times following treatment. **A,** FK228 increases CAR mRNA. Mice were treated with one, two (q4dx2), or three (q4dx3) doses of FK228 or vehicle (control) and were euthanized 6 h following drug administration. Semiquantitative RT-PCR analysis of CAR mRNA in the xenografts was done. The normalized mean values for control and treated samples were 1.0 ± 0.6-fold and 10.7 ± 4.9-fold (P = 0.0022) for one dose, 1.0 ± 0.2-fold and 9.4 ± 4.5-fold (P = 0.0043) for two doses, and 1.0 ± 0.9-fold and 7.4 ± 6.2-fold (P = 0.23) for three doses. **B,** FK228 increases acetylated histone H3 protein. Mice were treated with a single dose of FK228. Western blot analysis for acetylated histone H3 in the xenografts was done. The normalized mean values for control and treated samples were 1.0 ± 0.1-fold and 28.2 ± 22.9-fold (P = 0.0022) for 6 h, and 1.0 ± 1.4-fold and 20.4 ± 12.6-fold (P = 0.0022) for 24 h. **C,** FK228 increases CAR mRNA. Mice were treated with a single dose of FK228. Semiquantitative RT-PCR analysis of CAR mRNA in the xenografts was done. The normalized mean values were 1.0 ± 0.6-fold, 4.7 ± 2.6-fold, and 10.7 ± 4.9-fold for control, 1.6, and 3.6 mg/kg samples after 6 h (P < 0.0005 for significance of trend), and 1.0 ± 0.4-fold, 4.2 ± 1.2-fold, and 13.6 ± 4.3-fold for samples after 24 h (P < 0.0001 for significance of trend).

Compared with the average 13.6 ± 4.3-fold (P = 0.0022) increase in human CAR mRNA levels in the xenografts following FK228 treatment versus 1.0 ± 0.4 for controls, FK228 had little effect on mouse CAR mRNA levels in normal mouse tissues. Figure 2A shows an analysis of mouse CAR mRNA levels in liver, kidney, and lung tissue obtained from the same mice as the tumor xenograft material 24 h following a 3.6 mg/kg dose of FK228. The individual P values for the comparisons are as follows: P = 0.015 for liver, P = 0.078 for kidney, P = 0.37 for lung, and P = 0.0022 for xenograft. This analysis typically indicated little effect of FK228 on normal mouse tissues despite maximal induction in the xenografts. Thus, treatment of mice with a single FK228 dose of 3.6 mg/kg selectively affected the human cancer cells in the xenografts. The digital images of some of the RT-PCR analyses that were used to derive the graph are also shown in Fig. 2A. Because different primers were used for the human and mouse analyses, a direct cross-species comparison cannot be made, however, the FK228-induced CAR mRNAs were detected at a similar number of cycles in the two species. This observation suggests that the FK228-treated human and mouse CAR levels may be in the same range. The levels of CAR in the liver may not be critical because recent studies have suggested that adenovirus uptake into the liver may be CAR-independent (23). Regardless of the mechanism of adenovirus uptake in the liver, boosting virus infection of the target tissue would be beneficial.

The effect of FK228 on CAR protein levels was similar to that on CAR mRNA levels. The analysis of CAR proteins in the xenografts is shown in Fig. 2B. Immunoblot analysis of CAR protein from xenografts indicated that compared with control mice with a mean 1.0 ± 0.7-fold increase, there was a mean 9.2 ± 4.8-fold (P = 0.0022) increase in CAR protein levels in xenografts from mice (n = 6) 24 h following treatment with FK228 at a dose of 3.6 mg/kg. A lower dose or shorter posttreatment sampling time did not result in increased CAR protein levels (P = 0.85 and P = 0.94, respectively). There was little change in α_v integrin protein levels in the xenografts following drug treatment. Thus, FK228 treatment increased both CAR protein and mRNA levels in tumor xenograft tissues.

At the beginning of these studies, we evaluated several xenograft model systems to determine the one that would be used for further analysis. We evaluated two melanoma xenograft model systems, UACC-62 and LOX IMVI. Figure 2C shows that CAR mRNA levels were increased in both model systems following treatment with FK228. In a dose-response analysis comparing untreated controls with mice treated thrice with different doses of FK228 (q4dx3), CAR mRNA levels were increased by up to 5.1 ± 0.3-fold at the highest level, with P < 0.0001 for a trend over all doses in UACC-62 xenografts (n = 3), whereas CAR mRNA levels were increased by up to 8.0 ± 0.8-fold (P = 0.0019 by a trend test) in LOX IMVI xenografts (n = 3) at the highest dose level. Thus, the FK228-mediated induction in CAR mRNA was not unique to a single melanoma xenograft

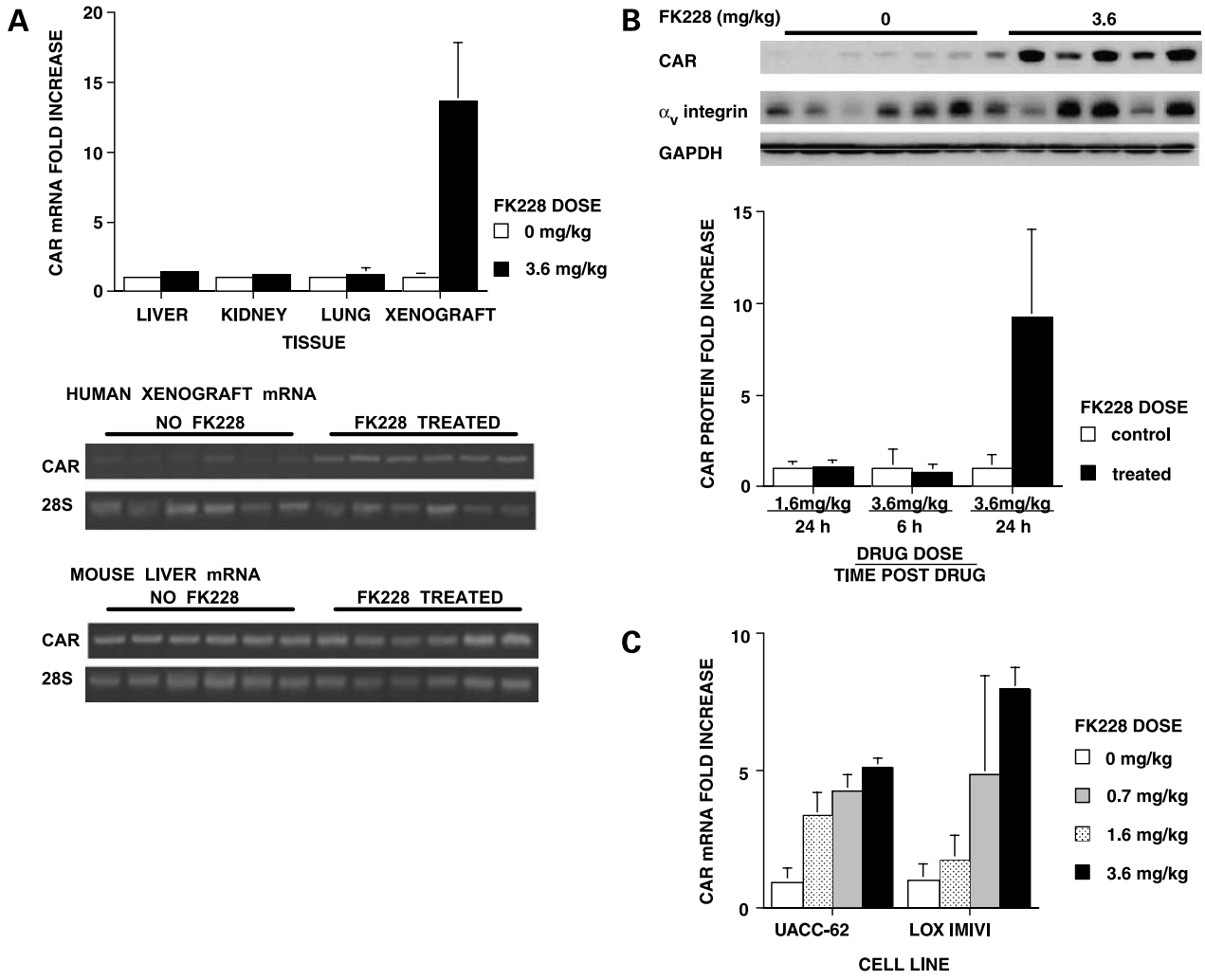

Figure 2. FK228 treatment increases CAR mRNA and protein in two xenograft models but not in normal mouse tissues. **A,** FK228 does not increase mouse CAR mRNA in normal mouse tissues. Athymic mice with LOX IMVI xenografts ($n = 6$) were treated with a single dose of FK228 or vehicle (control) and were euthanized 24 h following drug treatment. Semiquantitative RT-PCR analysis of CAR mRNA in the xenografts and normal mouse tissues was done. Digital images show FK228-treated and control human xenograft mRNA and mouse liver mRNA. The human CAR is shown at 29 cycles and the corresponding 28S rRNA at 10 cycles whereas the mouse CAR is shown at 28 cycles and the 28S rRNA at 11 cycles. The normalized mean values of control and treated samples were 1.0 ± 0.3-fold and 1.4 ± 0.1-fold ($P = 0.015$) for liver, 1.0 ± 0.1-fold and 1.3 ± 0.2-fold ($P = 0.078$) for kidney, 1.0 ± 0.2-fold and 1.2 ± 0.5-fold ($P = 0.37$) for lung, and 1.0 ± 0.4-fold and 13.6 ± 4.3-fold ($P = 0.0022$) for the xenograft. **B,** FK228 increases CAR protein in LOX IMVI xenografts. Western blot analysis of protein from xenografts from athymic mice with LOX IMVI xenografts ($n = 6$) treated with different doses of FK228 and euthanized at different times. The digital images were from xenografts from mice euthanized 24 h following treatment with FK228. *Bottom,* graph summarizing Western blot data of proteins from xenografts from mice treated with the indicated conditions. The normalized mean values for control and treated samples were 1.0 ± 0.4-fold and 1.1 ± 0.4-fold ($P = 0.85$) for animals treated with 1.6 mg/kg for 24 h, 1.0 ± 1.0-fold and 0.8 ± 0.4-fold ($P = 0.93$) for animals treated with 3.6 mg/kg for 6 h, and 1.0 ± 0.7-fold and 9.2 ± 4.8-fold ($P = 0.0022$) for animals treated with 3.6 mg/kg for 24 h. **C,** FK228 increases CAR mRNA in two melanoma xenograft models. Semiquantitative RT-PCR analysis was done on RNA from UACC-62 or LOX IMVI xenografts grown in athymic mice ($n = 3$) treated with three doses of FK228 (q4dx3) or vehicle (control). The mice were euthanized 6 h following the last drug treatment. The normalized mean values were 1.0 ± 0.5-fold, 3.4 ± 0.8-fold, 4.3 ± 0.6-fold, and 5.1 ± 0.3-fold for control, 0.7, 1.6, and 3.6 mg/kg–treated mice with UACC-62 xenografts ($P < 0.0001$ for trend) and 1.0 ± 0.6-fold, 1.7 ± 0.9-fold, 4.9 ± 3.6-fold, and 8.0 ± 0.8-fold, respectively, for mice with LOX IMVI xenografts ($P = 0.0019$ for trend).

model. These initial studies suggested that the LOX IMVI xenograft model was the more promising to pursue.

To determine whether these increases in CAR mRNA and protein levels were associated with an increase in the efficiency of adenovirus infection, xenograft-bearing mice were treated with FK228 at a dose of 3.6 mg/kg, and 24 h later, the xenografts were injected with an adenovirus carrying a GFP transgene. GFP mRNA levels in the xenografts were determined by semiquantitative RT-PCR analysis normalized to control 28S rRNA. The data in Fig. 3A shows that in xenografts from FK228-treated mice ($n = 10$), there was a mean 7.4 ± 5.2-fold ($P = 0.0002$) increase in GFP mRNA levels compared with control xenografts with a 1.0 ± 0.7-fold increase. Thus, as we

observed in our *in vitro* studies, the increases in CAR levels in response to FK228 treatment were associated with an increase in adenovirus transgene expression *in vivo*. Because adenovirus type 5 can infect mouse cells using the mouse CAR receptor (8) and because systemically administered adenovirus is sequestered in the liver (1), we analyzed the livers of the xenograft-injected animals to determine if any adenovirus had escaped from the xenografts into the mouse circulatory system. We found very little GFP expression in the livers of FK228-treated or control mice. As shown in Fig. 3B, at 30 cycles, no GFP expression was detected in the liver samples. At 40 cycles, the level of GFP in several of the liver samples approached that in the untreated xenograft sample (lane 14) at 30 cycles, indicating a fold difference in the expression levels of >1,000-fold between the xenograft and the liver. These studies suggest that only a small fraction of the xenograft-injected adenovirus entered the mouse circulatory system.

Mice (n = 3) were evaluated to determine the effect of FK228 treatment on mouse and xenograft weight during the 48 h treatment period. These results are shown in Table 1. Although based on a very small number of animals, the magnitudes of the changes were small, and none of the differences were statistically significant (P > 0.05), suggesting that FK228 treatment had little adverse effect on the mice during the experiments and modest growth delay in the xenografts.

Table 1. Comparison of FK228-treated and control mice

FK228	t = 0, xenograft weight (mg)	SD	t = 48, xenograft weight (mg)	SD
+	734	187	734	187
−	651	204	994	278
FK228	**t = 0, mouse weight**	**SD**	**t = 48, mouse weight**	**SD**
+	23.7	2.7	20.2	2.6
−	21.5	1.5	20.4	3.6

NOTE: Measurements were taken just prior to the start (t = 0) and at the completion (t = 48) of the experiment. The xenograft weight was calculated from the dimensions. The values are the mean and SD. +, FK228-treated. −, control.

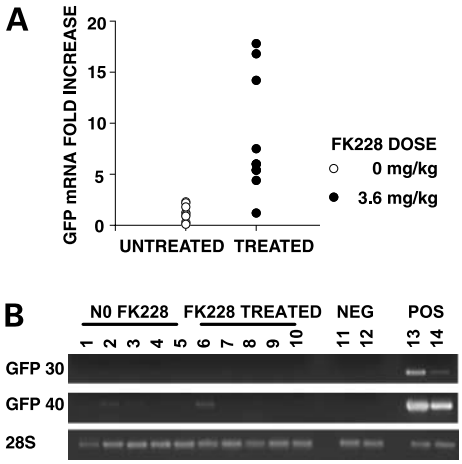

Figure 3. FK228 treatment increases adenovirus GFP transgene mRNA expression in xenografts but not in mouse liver. Mice with LOX IMVI xenografts (n = 10) were treated with a single dose of FK228 or vehicle (control). After 24 h, xenografts were injected with Ad5.hCMV-GFP. **A,** FK228 increases GFP expression in Ad5.hCMV-GFP–injected xenografts. GFP mRNA levels were determined by semiquantitative RT-PCR analysis. The normalized mean values for control and treated samples were 1.0 ± 0.7-fold and 7.4 ± 5.2-fold (P = 0.0002). **B,** little GFP expression in mouse livers from mice with Ad5.hCMV-GFP–injected xenografts. Digital images show semiquantitative RT-PCR analysis of mouse liver RNA for GFP and 28S rRNA. *Lanes 1* to *5,* RNA from livers of mice not treated with FK228 prior to infection; *lanes 6* to *10,* RNA from livers of mice treated with FK228 prior to infection; *lane 11,* RNA from the liver of a mouse treated with FK228 and not infected; *lane 12,* RNA from the liver of a control mouse not treated with FK228 and not infected; *lane 13,* RNA from the xenograft of a mouse treated with FK228 prior to infection; *lane 14,* RNA from the xenograft of a mouse not treated with FK228 prior to infection. Samples 1 and 14, as well as, 6 and 13 came from the same animals. The GFP was run for 30 or 40 cycles. The control 28S rRNA was run for 10 cycles.

The xenografts of some mice treated similarly to those shown in Fig. 3 were assayed for GFP protein expression by immunohistochemistry analysis. Figure 4A to C show representative fields from xenograft sections at high-power magnification. The entire xenograft from which the field was taken is shown immediately below at a lower magnification in Fig. 4D to F. The FK228-treated adenovirus-infected xenograft analyzed 24 h following virus infection had many more brown-stained cells indicative of GFP protein than the untreated virus-infected section. Although not all cells seem to be infected, the FK228-treated xenograft had considerably more GFP transgene expression. A similar pattern was seen in xenografts analyzed 18 h after adenovirus infection (data not shown). We used a laser scanning cytometer to quantify the GFP protein–expressing cells and found 4.0-fold more expression in the FK228-treated compared with the untreated adenovirus-infected section. These studies show that FK228 treatment increased adenovirus transgene protein expression in the treated xenografts.

Discussion

The goal of cancer gene therapy is to target tumor cells for destruction but leave normal tissue unharmed. Adenoviral vectors are good delivery vehicles for cancer gene therapy because the virus can infect both quiescent and replicating cells, and viral DNA does not integrate into the host genome and is eventually eliminated (24). Introducing viral DNA with tumor-specific or tissue-specific promoters into cancer cells to direct the synthesis of toxic gene products or the production of oncolytic viruses can target tumor cells (25–27). Ideally, these promoters would not function in normal cells or, alternatively, the normal tissue might be expendable (such as the normal adrenal gland; ref. 28). Adenovirus preparations of high titer and purity can be made that are suitable for patient treatment (29).

Replicating or nonreplicating adenovirus can be safely administered to patients (30). However, adenoviruses are susceptible to host immune defenses and researchers are working to lessen this problem (31). The liver sequesters systemically administered adenoviruses and several approaches are being used to overcome this problem (10). Investigators are also working to address the problem of poor adenovirus infection of tumor cells by altering the adenovirus so that it binds to a receptor other than CAR in order to gain entry into cells (32, 33). The studies presented in this article suggest a different approach to overcoming the problem of low CAR levels in some tumors by increasing the number of CAR receptors and thereby increasing the level of adenovirus infection.

We previously reported that *in vitro* FK228 treatment resulted in an increase in CAR and α_v integrin mRNA levels in cancer cells, and was associated with an increase in adenovirus infection as measured by viral transgene expression (6). In the studies presented here, we show that the effect of FK228 administration on CAR expression and adenovirus infection in cancer cells is similar *in vivo* and *in vitro. In vivo* FK228 increased CAR mRNA levels as much as 13.6-fold in human LOX IMVI melanoma xenografts in athymic mice. In this system, the threshold levels of α_v integrin mRNA were high and were unchanged following treatment. CAR protein levels increased 9.2-fold, whereas α_v integrin protein levels remained unchanged. FK228 had little to no effect on the levels of mouse CAR mRNA in the normal liver, lung, or kidney from mice in which xenografts exhibited high induction of human CAR. When an adenovirus carrying a GFP transgene was injected into the tumors of FK228-treated mice, GFP mRNA expression was 7.4-fold higher than in control mice. GFP protein expression was also increased. Thus, FK228 treatment before infection increased adenovirus infection as measured by transgene expression *in vivo*.

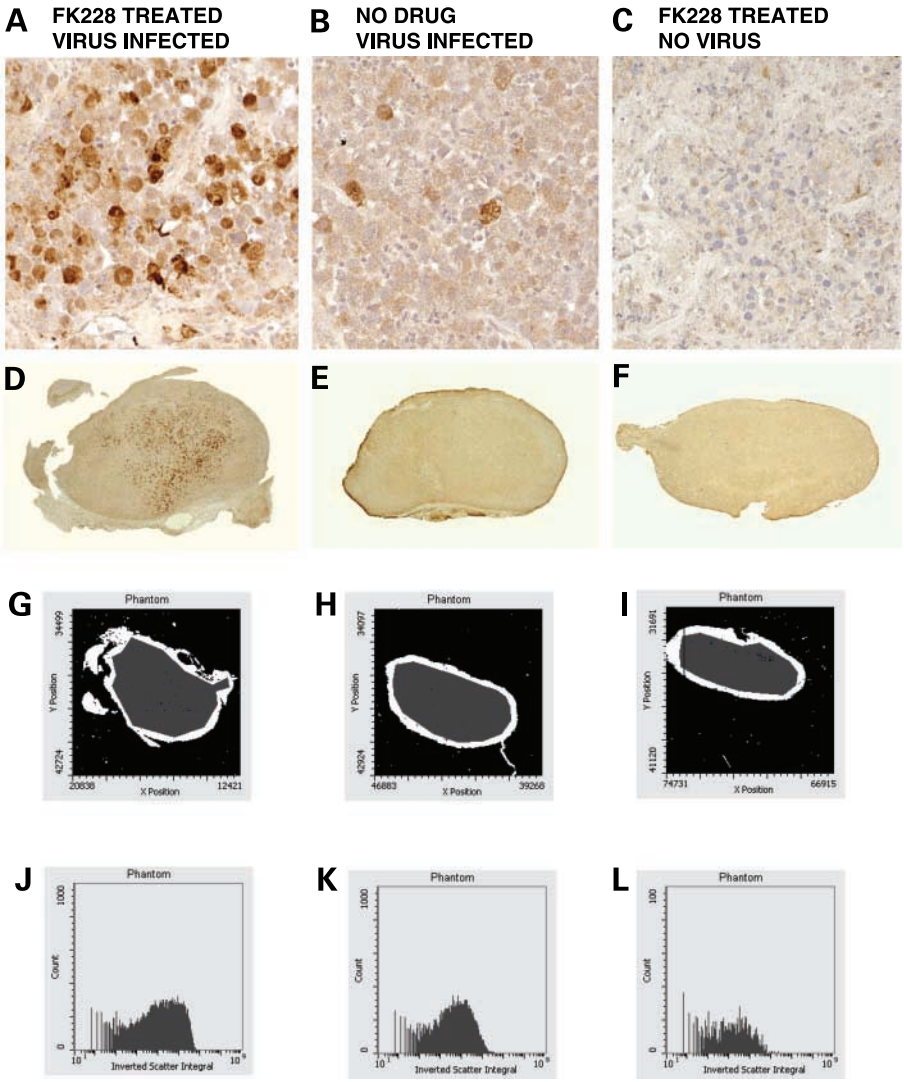

Figure 4. FK228 treatment increases adenovirus transgene protein expression in LOX IMVI xenografts. Immunohistochemistry of formalin-fixed paraffin-embedded xenografts incubated with an antibody against GFP. **A, B,** and **C,** representative fields taken from the xenografts in **D, E**, and **F**, respectively. **A** and **D,** treated with a single dose of FK228 of 3.6 mg/kg 24 h prior to adenovirus infection; **B** and **E,** not treated with FK228 prior to infection; **C** and **F,** treated with FK228 but were not infected (**A**–**C,** original magnification, ×100; **D**–**F,** original magnification, ×6.5). The GFP-expressing cells were quantified using a laser scanning cytometer. *Gray areas,* the regions quantified (**G**–**I**). The quantification is shown in histograms; the mean fluorescence intensity values were 296,954 (**J**), 75,322 (**K**), and 2,228 (**L**).

Although FK228 and other histone deacetylase inhibitors affect only a small fraction of cellular genes (34, 35), they could have multiple effects on adenovirus-infected cells. The increased levels of acetylated histone H3 in xenografts following FK228 treatment suggest that the drug functions as a histone deacetylase inhibitor in our *in vivo* LOX IMVI model system. Because our data indicated that the levels of acetylated histone H3 dropped almost 30% between 6 and 24 h following FK228 administration, the half-life of FK228 in mice is probably not long. The FK228 half-life in mice is probably similar to the half-life of 8.1 h found in patients (5), and of 3.1 h found in rats (36).

Our previous *in vitro* studies showed that 24 h following adenovirus infection, the presence of FK228 prior to adenovirus infection caused an 8- to 12-fold increase in transgene expression compared with a 2- to 3-fold increase when the drug was added following infection (16). In these studies, the maximum increase in transgene expression was obtained by including FK228 both before and after infection (16). Prior to adenovirus infection, the addition of FK228 could increase CAR levels, and in turn, the number of virus particles entering the cells. The important role of CAR is supported by experiments with cells in which FR901228 treatment caused only slightly increased CAR levels and did not cause increased viral transgene expression (37). These studies implicate CAR in the increase in transgene expression. In addition, studies using CAR promoter constructs suggest that the gene is responsive to FK228 (38) and that transcriptional modulation may be the basis for the induction of CAR mRNA expression and the increased efficiency of adenovirus infection in our studies. In contrast, FK228 added after infection must influence transgene expression by other mechanisms such as stimulating the CMV promoter that drives the adenovirus transgene. The addition of histone deacetylase inhibitors following adenovirus infection has been shown to increase adenoviral proteins and transgene expression *in vitro* (39, 40). *In vivo*, the addition of a histone deacetylase inhibitor following intratumor adenovirus injection was shown to be effective in increased tumor regression (41). Adding histone deacetylase inhibitors following adenovirus infection may be useful in cells with high CAR levels but may have little effect on difficult to infect cells with low CAR levels.

A number of different *in vitro* treatments have been reported to increase CAR and adenovirus transgene expression levels. In addition to FK228 (6, 41, 42), treatments have included other histone deacetylase inhibitors (42–46), as well as other drugs (42, 47–50). In addition to these studies which used nonreplicating adenoviruses, histone deacetylase inhibitors have been shown to enhance the antitumor effect of replication-selective adenoviruses *in vitro* (46, 51). Studies by Hemminki et al. validated the use of increased transgene expression as a surrogate marker for increased viral infection because FR901228 treatment prior to infection resulted in increased adenoviral DNA in infected cells (42).

Although two *in vivo* studies with a histone deacetylase inhibitor have been reported which suggest that these drugs might improve CAR-mediated adenovirus infection efficiency in xenograft model systems (42, 45), the studies presented here provide more convincing evidence. In our *in vivo* studies, using two xenograft models, we showed that FK228 administered before adenovirus infection increased CAR receptor levels and increased expression of the reporter transgene carried by the adenovirus. Our observations should be extendable to other xenograft models with poor infection because of low levels of the primary adenovirus receptor CAR.

Although we have shown that FK228 treatment can increase the efficiency of transgene expression in two xenograft model systems, this phenomenon may not be universal. As was shown *in vitro*, FR901228 did not cause an increase in CAR levels or transgene expression in some cells (37). Alternative infection methods may be required for cells that have adequate levels of CAR but lack α_v integrin. In addition, histone deacetylase inhibitors may positively or negatively modulate adenoviral vectors. Thus, each adenoviral vector and each viral target will need to be analyzed for drug responsiveness *in vitro* and *in vivo*. Responsive model systems will most likely then need to be optimized to obtain maximum infection efficiency.

Our studies show that FK228 treatment prior to adenovirus infection is able to increase adenovirus infection efficiency *in vitro* and *in vivo. In vitro*, we found that cell lines that express very low levels of CAR have the greatest increase in response to FK228 treatment, but cell lines that express higher levels of CAR also show some augmentation (6). The present *in vivo* studies show that FK228 treatment of xenograft-bearing mice prior to adenovirus infection increases the efficiency of adenovirus infection as measured by transgene expression. Although we have used a first-generation adenovirus carrying a reporter transgene in this study, other therapeutic adenoviral vectors that use CAR for attachment to cells could potentially be made more effective in systems in which low CAR levels on target cells prevent efficient adenovirus infection. These studies suggest that FK228 or other histone deacetylase inhibitors could increase the efficiency of adenovirus-mediated gene therapy in some xenograft model systems and support further investigation of this treatment strategy.

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