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Biochemical Pharmacology 66 (2003) 897–906
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Antitumor efficacy of FK228, a novel histone deacetylase inhibitor, depends on the effect on expression of angiogenesis factors

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Received 11 December 2002; accepted 12 May 2003

Abstract

It has been recently demonstrated that histone deacetylase inhibitors inhibit angiogenesis, but their mechanism of action has not been characterized well. In this study, we examined the *in vitro* and *in vivo* effects of FK228 [(*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo-[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone; FR901228, depsipeptide], an HDAC inhibitor, on the expression of angiogenesis factors in FK228-sensitive PC-3 prostate and FK228-resistant ACHN renal cancer cells.

FK228 suppressed the expression of VEGF mRNA in PC-3 cells, but not in ACHN cells. FK228 also suppressed the expression of basic fibroblast growth factor (bFGF) mRNA in both PC-3 and ACHN cells. Under conditions of hypoxia, FK228 suppressed the expression of VEGF mRNA without modulating the expression of hypoxia-inducible factor-1α mRNA in PC-3 cells. FK228 induced the highest acetylation of histone H3 and H4 in the P2 region of the VEGF promoter, which includes the hypoxia-inducible factor-1α binding site that plays an important role in regulating the expression of VEGF gene. Moreover, FK228 reduced the amount of VEGF and bFGF protein, and their mRNA levels in PC-3 xenograft implanted in nude mice, but did not reduce them in ACHN xenograft. In conclusion: (i) FK228 showed a suppressive effect on the expression of angiogenesis factors, such as VEGF and bFGF, in PC-3 xenograft but not in ACHN xenograft, which suggests that the effect on the expression of angiogenesis factors is important for the antitumor efficacy of FK228; (ii) FK228 caused histone acetylation of the VEGF promoter regions, which may contribute to the suppression of VEGF gene expression.

Keywords: Histone deacetylase inhibitor; FK228; VEGF; bFGF; PC-3; ACHN

1. Introduction

FK228 [(*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo-[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone; FR901228, depsipeptide] is a unique bicyclic peptide containing a non-cysteine disulfide bridge, isolated from *Chromobacterium violaceum* Strain WB968 as an agent inducing morphological reversion of H-ras transformed NIH 3T3 cells [1,2] (Fig. 1). FK228 possesses potent antitumor activity against human tumor cell lines and significant inhibitory effects on the growth of human solid tumors implanted in mice [3]. FK228 was also found to exhibit significant selectivity for tumor cells. Its IC_{50} values against human tumor cells are of the order of several ng/mL and against human normal fibroblast cells >1000 ng/mL [1].

It has been suggested that the antitumor activity of HDAC inhibitors is exerted through multiple mechanisms, such as apoptosis, cell cycle arrest, and differentiation via the modulation of the gene expression [4–8]. In addition, it has been recently demonstrated that HDAC inhibitors inhibit angiogenesis by the suppression of the production of VEGF from tumor cells and the direct inhibition of endothelial cell migration and proliferation [9–11], however the mechanism of action has not been well characterized.

Interestingly, we found that FK228 showed antitumor activity with different sensitivity against Meth A fibrosarcoma inoculated intradermally in BALB/c mice using a

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Abbreviations: HDAC, histone deacetylase; HAT, histone acetyltransferase; SAHA, suberoylanilide hydroxamic acid; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HIF, hypoxia-inducible factor; VHL, Von Hippel–Lindau; TSA, trichostatin A.

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doi:10.1016/S0006-2952(03)00411-8

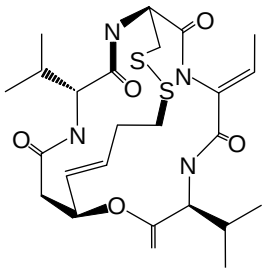

Fig. 1. The chemical structure of FK228.

treatment schedule of FK228 [3]. When FK228 was given i.v. to mice from 7 days after tumor inoculation, the growth of Meth A solid tumor was strongly suppressed by FK228. However, FK228 only marginally suppressed the growth of Meth A tumor when FK228 was given from 1 day after tumor inoculation. These results suggest that FK228 more strongly suppressed the growth of large solid tumors whose growth depends on the development of a network of capillaries, than small solid tumors whose growth depends little on capillaries. Solid tumors need angiogenesis factors to grow [12], therefore the antitumor effect of FK228 on Meth A tumor may partly depend on the suppression of angiogenesis factors.

In other experiments, FK228 showed antitumor activity with differential sensitivity against PC-3 prostate and ACHN renal cancer xenografts implanted subcutaneously (s.c.) in BALB/c *nu/nu* mice [13]. When FK228 (3.2 mg/kg) was given i.v. to mice three times at 4-day intervals, the growth of PC-3 and ACHN xenografts was suppressed by 98 and 20%, respectively, at 21 days after FK228 first administration. In *in vitro* tests, FK228 inhibited the growth of PC-3 and ACHN cells with IC_{50} values of 1.21 and 4.25 ng/mL, respectively [13]. This indicates that the antitumor activity of FK228 against cancer xenografts depends not only on direct cytotoxicity but also other factors. The speed of tumor growth of PC-3 xenograft is different from that of the ACHN xenograft, because the relative tumor volume of PC-3 and ACHN xenografts were 13.2- and 1.7-fold, respectively, at 21 days after tumor implantation [13]. We suspected that the expression of angiogenesis factors may be related to the speed of tumor growth and that the effect of FK228 on the expression of angiogenesis factors may be related to differential sensitivity against PC-3 and ACHN xenografts. In this study, we examined the *in vitro* and *in vivo* effects of FK228 on the expression of angiogenesis factors in PC-3 prostate and ACHN renal cancer cells.

2. Materials and methods

2.1. Chemicals

FK228 was prepared at Fujisawa Pharmaceutical Co., Ltd. The chemical structure of FK228 is shown in Fig. 1. In *in vitro* studies, FK228 was dissolved in ethanol and diluted with each experimental medium. In *in vivo* studies, FK228 was dissolved in and diluted with 10% polyoxyethylated (60 mol) hydrogenated castor oil (HCO60) in saline.

2.2. Tumor cells and animals

PC-3 prostate and ACHN renal cancer cells, obtained from ATCC, were maintained in high-glucose Dulbecco's Modified Eagle's Medium (D-MEM) (Nikken Biological Laboratories) supplemented with 10% fetal calf serum (FCS) (Moregate), 50 U/mL of penicillin and 50 μg/mL of streptomycin (ICN Biomedicals) at 37° under 5% CO_2 until the start of experiments. Male BALB/c *nu/nu* strain mice were purchased from Charles River Japan Inc.

2.3. PC-3 and ACHN cell culture for real-time quantitative polymerase chain reaction (q-PCR)

Cells were seeded at 2×10^6 per 25-cm^2 flask with 5 ng/mL of FK228 at 37° under 5% CO_2 for 1, 3, 12 and 24 hr in high-glucose D-MEM supplemented with 10% FCS, 50 U/mL of penicillin and 50 μg/mL of streptomycin. RNA was extracted from the cells using RNeasy Mini Kit (QIAGEN).

2.4. PC-3 and ACHN xenografts for real-time q-PCR

Fragments (3 mm × 3 mm × 3 mm) of human solid tumor were implanted s.c. into the right flank of BALB/c *nu/nu* mice. When the estimated tumor weight in mice had reached 100–300 mg, animals were treated i.v. once with 3.2 mg/kg of FK228. Tumor weight was calculated from the following formula: tumor weight (mg) $= L \times W^2/2$, where L and W represent the length and the width of the tumor mass, respectively. RNA was extracted from solid tumors at 1, 3, 6, and 24 hr after FK228 administration using RNeasy Mini Kit.

2.5. Estimation of VEGF and bFGF mRNA expression by real-time q-PCR

RNA was reverse-transcribed using Taq-Man reverse transcription reagents (PE Biosystems). Primers for VEGF, bFGF, and β-actin gene were designed using "primer express" software (PE Biosystems). The following primer sequences were used: 5′-GAA GTG GTG AAG TTC ATG GAT GTC-3′ (VEGF upstream), 5′-TCA GGG TAC TCC TGG AAG ATG TC-3′ (VEGF downstream), 5′-GAC CCT CAC ATC AAG CTA CAA CTT C-3′ (bFGF upstream), 5′-TAA CGG TTA GCA CAC ACT CCT TTG-3′ (bFGF downstream), 5′-CAT TGG CAA TGA GCG GTT-3′ (β-actin upstream), 5′-GGT AGT TTC GTG GAT GCC ACA-3′ (β-actin downstream). The expression of VEGF,

bFGF, and β-actin mRNA was estimated by real-time q-PCR using a SYBR green PCR master mix (PE Biosystems). The final expression value was calculated as follows: expression level of VEGF or bFGF mRNA/expression level of β-actin. Each value at 0 hr was set as 1. In the graph of basal expression level, the value at 0 hr in PC-3 cells was set as 1. Value increases of more than 200% or decreases less than 50% were judged as significant changes.

2.6. Detection of hypoxia-induced VEGF and HIF-1α mRNA expression in PC-3 cells by reverse transcription (RT)–PCR

Cells were seeded at 4×10^6 per 75-cm^2 flask and incubated overnight at 37° under 5% CO_2, in high-glucose D-MEM supplemented with 10% FCS, 50 U/mL of penicillin and 50 μg/mL of streptomycin. The following day, cells were collected without treatment at 0 hr and the remaining cells were cultured with or without 5 ng/mL of FK228 for 2, 4, 8, and 16 hr from the onset of hypoxia-culture at 37° under 2% O_2, in low-glucose D-MEM (Nikken Biological Laboratories) supplemented with 10% FCS, 50 U/mL of penicillin and 50 μg/mL of streptomycin. In another test, cells were cultured with or without 5 ng/mL of FK228 from 4 hr to 8 or 16 hr after the onset of hypoxia-culture. RNA was extracted from the cells using RNeasy Mini Kit and reverse-transcribed using M-MLV reverse transcriptase (PE Biosystems). PCR was performed using recombinant Taq DNA polymerase (Takara Shuzo Co., Ltd.). The optimal reaction conditions for PCR were determined for each primer pair. PCR cycling parameters were denaturation at 94° for 30 s, annealing at 60° for 30 s, followed by elongation at 72° for 30 s. PCR products were analyzed by 2.5% agarose/ethidium bromide gel electrophoresis. Primers for VEGF, HIF-1α, and β-actin gene were designed using "primer express" software. The following primer sequences were used: 5′-TTG CTG CTC TAC CTC CAC CAT-3′ (VEGF upstream), 5′-TGA TTC TGC CCT CCT CCT TCT-3′ (VEGF downstream), 5′-TGT TAG CTC CCT ATA TCC CAA TGG-3′ (HIF-1α upstream), 5′-GCT TGC GGA ACT GCT TTC TAA T-3′ (HIF-1α downstream), 5′-TCA ACA CCC CAG CCA TGT ACG-3′ (β-actin upstream), 5′-CAG GAA GGA AGG CTG GAA GAG-3′ (β-actin downstream).

2.7. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as described previously [14], with modifications. Cells were seeded at 4×10^6 per 75-cm^2 flask and incubated overnight at 37° under 5% CO_2 in high-glucose D-MEM supplemented with 10% FCS, 50 U/mL of penicillin and 50 μg/mL of streptomycin. The following day, cells were cultured with 5 ng/mL of FK228 for 2 hr at 37° under 2% O_2, in low-glucose D-MEM supplemented with 10% FCS, 50 U/mL of penicillin and 50 μg/mL of streptomycin. Formaldehyde was then added to cells to a final concentration of 1%, and cells were incubated at 37° for 10 min. The medium was removed, and cells were suspended in 1 mL of ice-cold PBS containing the serine protease inhibitor phenylmethylsulfonyl fluoride (0.1 mM). Cells were pelletted, resuspended in 0.5 mL of SDS lysis buffer (1% SDS/10 mM EDTA/50 mM Tris–HCl, pH 8.1) and incubated on ice for 10 min. Lysates were sonicated with three sets of 10 s pulses. Debris was removed from samples by centrifugation at 15,000 *g* for 10 min at 4°. An aliquot of the chromatin preparation was set aside and designated as the "input fraction." Supernatants were diluted 5-fold in immunoprecipitation buffer (0.01% SDS/1.1% Triton X-100/1.2 mM EDTA/16.7 mM NaCl/16.7 mM Tris–HCl, pH 8.1), then 80 μL of a 50% protein A Sepharose slurry containing 20 μg of sonicated salmon sperm DNA and 1 mg/mL of BSA in TE buffer (10 mM Tris–HCl/1 mM EDTA, pH 8.0) was added and incubated for 30 min at 4° with rocking. Sepharose beads were pelletted by centrifugation at 12,000 *g* for 1 min at 4°, and supernatants were placed in fresh tubes with 5 μg of anti-acetylhistone H3 or anti-acetylhistone H4 antibody (Upstate Biotechnology, Inc.) and incubated overnight at 4°. A protein A Sepharose slurry (60 μL) was added, and samples were rocked for 1 hr at 4°. The protein A complexes were centrifuged at 12,000 *g* for 1 min at 4° and washed five times for 5 min each. Immune complexes were eluted twice with 250 μL of elution buffer (1% SDS/0.1 M $NaHCO_3$) for 15 min at room temperature. Twenty microliters of 5 M NaCl was added to the combined eluates, then samples were incubated for 4 hr at 65°. EDTA, Tris–HCl, pH 6.5, and proteinase K were then added to the samples at a final concentration of 10 mM, 40 mM, and 0.04 μg/mL, respectively, then samples were incubated for 1 hr at 45°. Immunoprecipitated DNA (both immunoprecipitation samples and input) were recovered using phenol/chloroform extraction and ethanol precipitation then analyzed by PCR using recombinant Taq DNA polymerase. VEGF-specific primers were used to carry out PCR from DNA isolated from ChIP experiments and input samples. The optimal reaction conditions for PCR were determined for each primer pair. PCR cycling parameters were: denaturation at 94° for 30 s, annealing at 60° for 30 s, followed by elongation at 72° for 30 s. PCR products were analyzed by 2.5% agarose/ethidium bromide gel electrophoresis. Primers for VEGF ChIP analysis were designed using "primer express" software. The primer pairs used for VEGF ChIP analysis were: 5′-CTT CGA GAG TGA GGA CGT GTG T-3′ (P1 upstream), 5′-GGA GCA GGA AAG TGAGGT TAC G-3′ (P1 downstream), 5′-CCA GAC TCC ACA GTG CAT ACG T-3′ (P2 upstream), 5′-TGG GAC TGG AGT TGC TTC ATG-3′ (P2 downstream), 5′-TGC TGC ATT CCC ATT CTC AGT-3′ (P3 upstream), 5′-ATC TTC CCT AAG TGC TCC CAA AG-3′ (P3 downstream), 5′-CAG GGA AAG GAT GAT CAC TGT CA-3′

(I1 upstream), 5′-TGC CTT TCA CCA GGA CAA AGT-3′ (I1 downstream), 5′-ATG GAT GTC TAT CAG CGC AGC T-3′ (E3 upstream), 5′-TGG TGA TGT TGG ACT CCT CAG T-3′ (E3 downstream).

2.8. Histological analysis of PC-3 and ACHN xenografts with antibodies to VEGF and bFGF

Fragments (3 mm × 3 mm × 3 mm) of human solid tumor were implanted s.c. into the right flank of BALB/c *nu/nu* mice. When the estimated tumor weight in mice had reached 100–300 mg, the animals were treated i.v. with 3.2 mg/kg of FK228 or 10% HCO60 in saline (control), every 4 days for a total of three times. After final administration, frozen slices of solid tumors were immunostained with antibodies to VEGF (Upstate Biotechnology) and bFGF (Sigma), using dilutions of 1:50 and 1:400, respectively. Histological changes of cells were photographed at 40× magnification.

3. Results

3.1. Effect of FK228 on the expression of VEGF and bFGF mRNA in cultured PC-3 and ACHN cells

VEGF and bFGF are critical angiogenesis factors [15]. To determine the effect of FK228 on the expression of angiogenesis factors, we examined the effect of FK228 on the expression of VEGF and bFGF mRNA in cultured PC-3 prostate and ACHN renal cancer cells by real-time q-PCR. PC-3 and ACHN cells were treated with 5 ng/mL of FK228 for 1, 3, 12, and 24 hr. In PC-3 cells, the expression level of VEGF mRNA was decreased by 51% at 12 hr (Fig. 2A). However, FK228 did not sufficiently modulate the expression level of VEGF mRNA in ACHN cells. The expression level of bFGF mRNA was markedly decreased in both PC-3 and ACHN cells at 12 hr by 94 and 81%, respectively (Fig. 2A). The basal expression level of VEGF mRNA showed a similarity

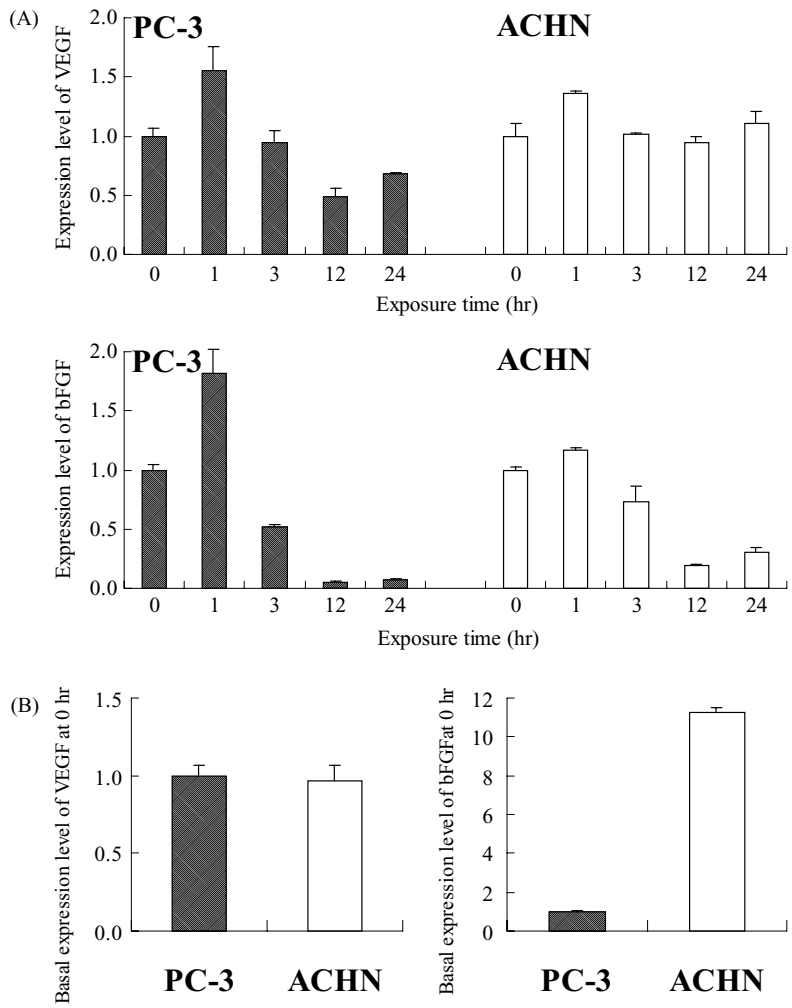

Fig. 2. Changes in the expression of VEGF and bFGF (A) mRNA in cultured PC-3 and ACHN cells after treatment with FK228. Quantitative changes in the expression levels of VEGF and bFGF mRNA after treatment with 5 ng/mL of FK228 and basal expression levels of VEGF and bFGF mRNA (B) in PC-3 and ACHN cells were detected by real-time q-PCR. Each value at 0 hr was set as 1. In the graph of the basal expression level, the value at 0 hr in PC-3 cells was set as 1. Results are shown as means ± SEM of three independent experiments.

between PC-3 and ACHN cells and the basal expression level of bFGF mRNA in ACHN cells was higher than that in PC-3 cells (Fig. 2B). These results suggest that the basal expression level of VEGF and bFGF mRNA did not influence FK228-induced suppression of the expression of these mRNAs. Thus, FK228 suppressed the expression of VEGF and bFGF mRNA in PC-3 cells, while FK228 suppressed the expression of bFGF mRNA, but did not suppress the expression of VEGF mRNA in ACHN cells *in vitro*.

3.2. Effect of FK228 on hypoxia-induced expression of VEGF and HIF-1α mRNA in PC-3 cells

The transcription of VEGF gene is mainly induced by hypoxia and tumor angiogenesis extends under conditions of hypoxia [16,17]. The effect of FK228 on the expression of VEGF mRNA was examined using PC-3 cells under conditions of hypoxia, which mimicked *in vivo* angiogenesis conditions. HIF-1 is the transcription activator of VEGF gene under hypoxia and its activity is primarily determined by hypoxia-induced stabilization of HIF-1α, which is a component subunit of HIF-1 [16,18]. Therefore, we also examined whether or not the effect of FK228 on

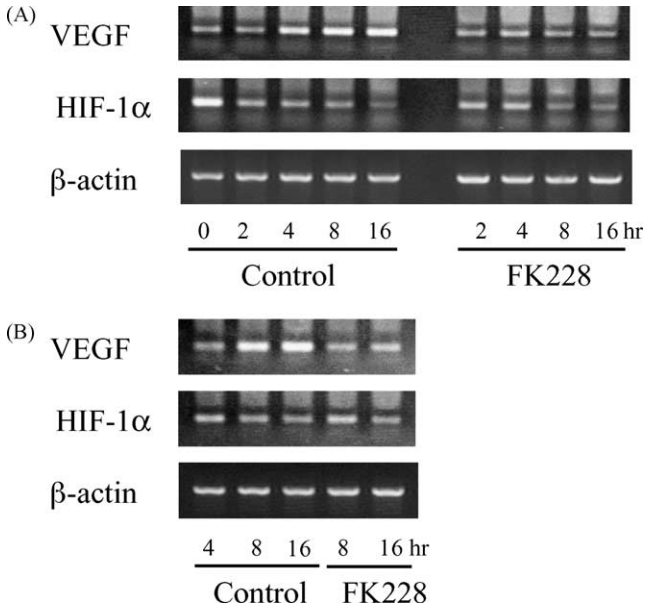

Fig. 3. Detection of hypoxia-induced VEGF and HIF-1α mRNA expression in PC-3 cells after treatment with FK228. Expression of VEGF and HIF-1α mRNA in PC-3 cells after treatment with or without 5 ng/mL of FK228 under hypoxia-culture were detected by RT–PCR. (A) Cells were cultured with or without FK228 from the onset of hypoxia-culture. (B) Cells were cultured with or without FK228 from 4 hr after the onset of hypoxia-culture.

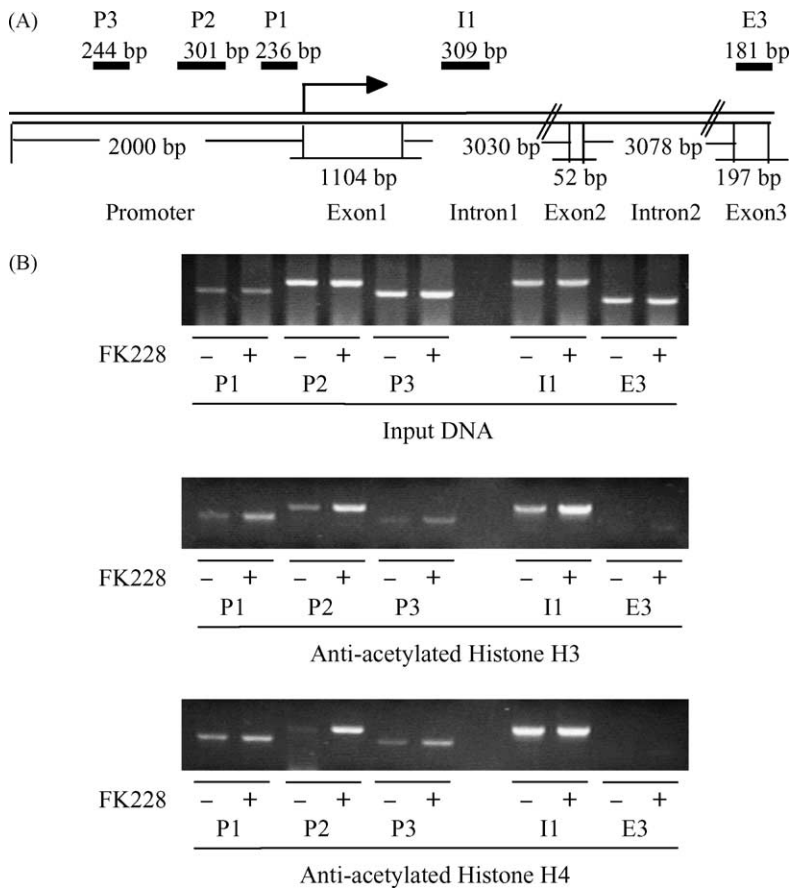

Fig. 4. FK228 acetylated histones associated with the VEGF gene promoter. (A) Position of primers in VEGF gene. (B) Treatment of PC-3 cells with or without 5 ng/mL of FK228 for 2 hr and immunoprecipitation of chromatin extracts with input DNA and antibodies to acetylated histone H3 and H4.

VEGF could be explained by the decreased expression of HIF-1α in PC-3 cells. At first, PC-3 cells were treated with or without 5 ng/mL of FK228 from the onset of hypoxia. The expression of VEGF mRNA in control cells was time-dependently increased from 4 to 16 hr, but the increase in expression of VEGF mRNA was completely inhibited by FK228 treatment (Fig. 3A). To determine the effect of FK228 on the high expression of VEGF mRNA, cells were treated with or without 5 ng/mL of FK228 from 4 hr after the onset of hypoxia. Under these conditions, FK228 also inhibited the expression of VEGF mRNA at 8 and 16 hr (Fig. 3B). In both treatment schedules for FK228, FK228 did not have any effect on the expression of HIF-1α mRNA (Fig. 3A and B). These results suggest that FK228 suppressed the expression of VEGF gene without modulating the expression of HIF-1α gene in PC-3 cells.

3.3. FK228 induces accumulation of acetylated histones in chromatin associated with VEGF gene promoter

It has been reported that histone acetylation of the $p21^{WAF1/Cip1}$ gene promoter may contribute to the up-regulation of $p21^{WAF1/Cip1}$ gene expression by HDAC inhibitors, such as FK228, SAHA, and trapoxin [14,19,20]. However, the effect of HDAC inhibitors on histone acetylation associated with the VEGF gene promoter has not been observed. Here, we examined the effect of FK228 on acetylation of histone H4 or H3 associated with the VEGF promoter in PC-3 cells using the ChIP assay. Cells were cultured with or without 5 ng/mL of FK228 for 2 hr under conditions of hypoxia, then chromatin fragments were extracted from cells and immunoprecipitated with antibodies to acetylated histone H3 or H4. The isolated DNA was amplified by using primers to the VEGF promoter, intron 1

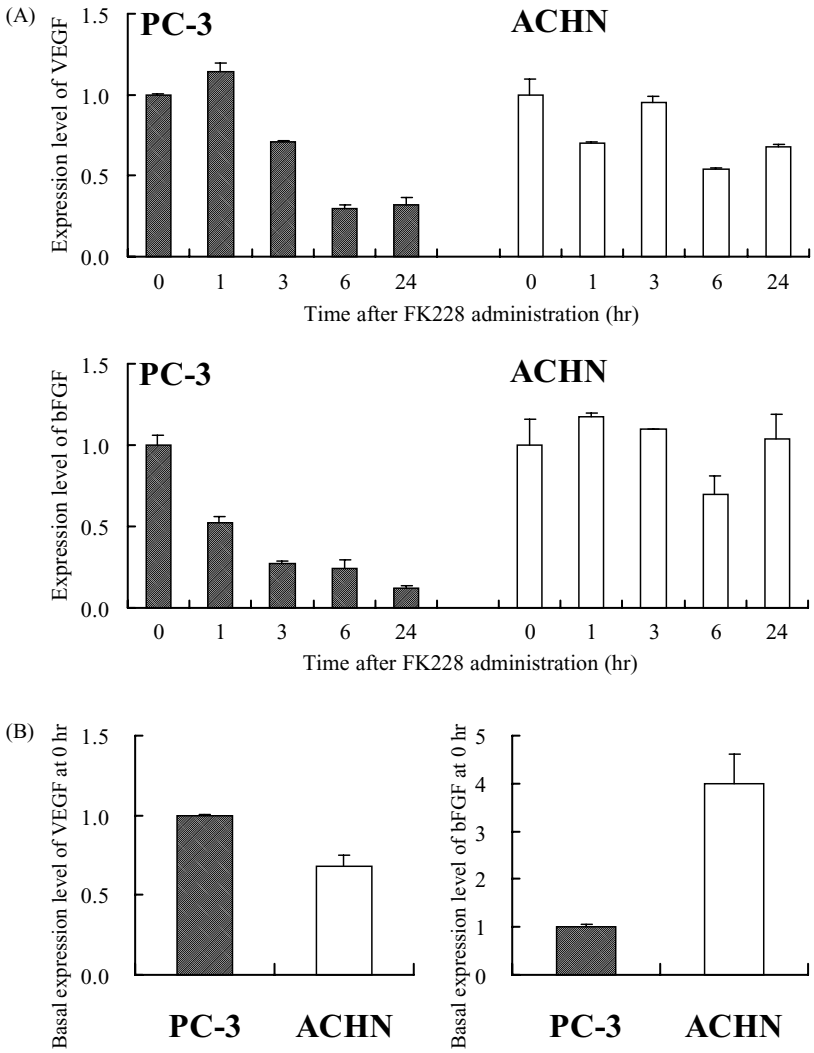

Fig. 5. Changes in the expression of VEGF and bFGF mRNA in PC-3 and ACHN xenografts after FK228 administration. Tumor cells were inoculated s.c. into nude mice. FK228 (3.2 mg/kg) was given i.v. to the mice once. Quantitative changes in the expression levels of VEGF and bFGF (A) mRNA after FK228 administration and basal expression levels of VEGF and bFGF mRNA (B) in PC-3 and ACHN xenografts were detected by real-time q-PCR. Each value at 0 hr was set as 1. In the graph of the basal expression level, the value at 0 hr in PC-3 cells was set as 1. Results are shown as means ± SEM of three independent experiments.

and exon 3 of VEGF gene, as shown in Fig. 4A. After treatment with FK228, the acetylation of histone H3 and H4 for all regions of the VEGF promoter increased (Fig. 4B). FK228 induced the highest acetylation of histone H3 and H4 in the P2 region of the VEGF promoter, a region including the HIF-1 binding site that plays an important role in regulating the expression of VEGF mRNA. In the I1 region, the acetylation of histone H3 was increased, but acetylation of histone H4 was not increased after treatment with FK228. The acetylation of histone H3 and H4 in the E3 region was not observed after treatment with FK228. These findings suggest that FK228 causes histone acetylation of VEGF promoter regions, especially in the P2 region.

3.4. Effect of FK228 on the expression of VEGF and bFGF mRNA in PC-3 and ACHN xenografts

To determine the effect of FK228 on the expression of angiogenesis factors *in vivo*, we estimated the expression level of VEGF and bFGF mRNA in PC-3 and ACHN xenografts implanted s.c. into BALB/c *nu/nu* mice after FK228 administration by real-time q-PCR. FK228

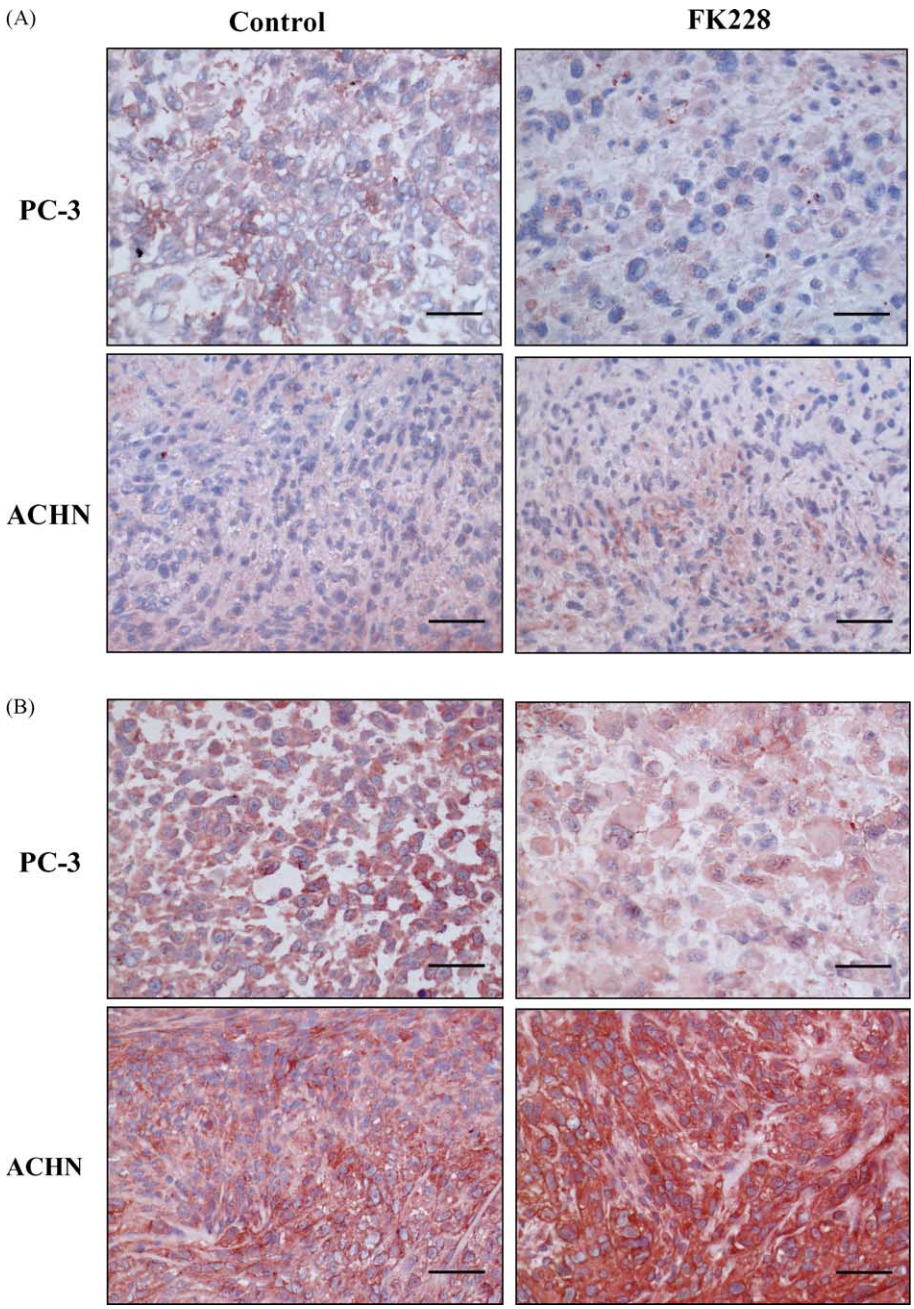

Fig. 6. Effect of FK228 on the amounts of VEGF and bFGF protein in PC-3 and ACHN xenografts. Tumor cells were inoculated s.c. into nude mice. FK228 (3.2 mg/kg) or 10% HCO60 in saline (control) was given i.v. to mice three times at 4-day intervals. After the final administration, frozen slices of solid tumors were immunostained with antibodies to VEGF (A) and bFGF (B). Histological changes of cells were photographed at 40× magnification. Scale bars: 50 μm.

(3.2 mg/kg) was administered i.v. to nude mice with tumor xenografts and RNA was extracted from tumor samples at 1, 3, 6, and 24 hr after FK228 administration. In PC-3 xenograft, the expression levels of VEGF and bFGF mRNA were decreased by 68 and 88%, respectively, 24 hr after FK228 administration (Fig. 5A). However, the expression levels of VEGF and bFGF mRNA in ACHN xenograft were not sufficiently modulated after FK228 administration. The basal expression level of VEGF mRNA was a little different between PC-3 and ACHN xenografts (Fig. 5B). The basal expression level of bFGF mRNA in ACHN xenograft was higher than the level in PC-3 xenograft. These results demonstrate that FK228 suppresses the expression of angiogenesis factors in PC-3 xenograft, but not in ACHN xenograft.

3.5. Effect of FK228 on the amount of VEGF and bFGF protein in PC-3 and ACHN xenografts

Finally, histological analysis was performed in PC-3 and ACHN xenografts after i.v. administration with FK228 (3.2 mg/kg) for three times at 4-day intervals, which was the same schedule of FK228 administration for estimating antitumor activity. In PC-3 xenograft, FK228 suppressed the amount of VEGF and bFGF protein (Fig. 6A and B). In contrast to PC-3 xenograft, the amount of VEGF protein was not modulated and bFGF protein was increased in ACHN xenograft after FK228 administration (Fig. 6A and B). These results suggest that FK228-induced suppression of the expression of VEGF and bFGF mRNA resulted in the suppression of VEGF and bFGF protein and that the suppressed expression of angiogenesis factors plays an important role in the antitumor activity of FK228.

4. Discussion

It has been recently demonstrated that HDAC inhibitors inhibit angiogenesis, however the mechanism of action has not been well characterized. In this study, we examined the *in vitro* and *in vivo* effects of FK228 on the expression of angiogenesis factors in PC-3 prostate cancer cells which are sensitive to FK228, and ACHN renal cancer cells, which are less sensitive to FK228.

As we had expected, FK228 suppressed the expression of VEGF mRNA in cultured PC-3 cells (Figs. 2A and 3). Moreover, FK228 suppressed the expression of bFGF mRNA in PC-3 and ACHN cells (Fig. 2A). This is the first report demonstrating that an HDAC inhibitor suppressed the expression of the bFGF gene. Neoplastic growth and progression to tumor malignancy are dependent on the formation of new blood vessels [21], and clinical studies have revealed that high vessel density often correlates with poor prognosis [22]. Angiogenesis is regulated by a fine balance between inducers and inhibitors of this process and VEGF is a critical inducer of angiogenesis [17]. Members of the FGF family, in particular bFGF, are also potent inducers of angiogenesis [23]. FGFs can synergize with VEGF in the induction of angiogenesis, probably by up-regulating VEGF and VEGF receptors in endothelial cells [24–27]. Soluble FGF receptor dramatically repressed tumor growth in a transgenic mouse model of pancreatic β cell carcinogenesis [28]. As with the *in vitro* results, FK228 suppressed the expression of VEGF and bFGF mRNAs and proteins in PC-3 xenograft (Figs. 5A and 6). However, FK228 did not suppress the expression of these mRNAs and proteins in ACHN xenograft. There is the discrepancy between *in vitro* and *in vivo* results for the effect of FK228 on the expression of bFGF mRNA in ACHN cells. The reason for this has not been determined, but the direct effect of FK228 on expression of bFGF mRNA in ACHN cells may somehow be disturbed *in vivo*. Taking these results together, FK228 showed suppressive effects on the expression of angiogenesis factors, such as VEGF and bFGF, in PC-3 xenograft, but not in ACHN xenograft. These suppressive effects may play an important role in the antitumor activity of FK228.

We examined the mechanism of the suppressive effect of FK228 on the expression of VEGF gene in PC-3 cells under conditions of hypoxia, which mimicked *in vivo* angiogenesis conditions [16,17]. As shown in Fig. 3, FK228 suppressed the expression of VEGF gene without modulating the expression of HIF-1α gene in PC-3 cells. HIF-1 is a heterodimer composed of α and β subunits and it activates the transcription of VEGF gene under hypoxia [16,18]. HIF-1 activity is not only regulated at the level of transcription but also determined by hypoxia-induced stabilization of HIF-1α [18]. Kim *et al*. suggested that HDAC inhibitors, such as TSA and FK228, decrease the stability of HIF-1α [9,29]. We did not examine the stability of HIF-1α, but there is the possibility that the suppression of HIF-1α activity contributes to the downregulation of VEGF gene by FK228. The stability of HIF-1α is negatively regulated by p53 and VHL, as the loss of p53 function amplifies HIF-1α-dependent responses to hypoxia and VHL induces the oxygen-dependent proteolysis of HIF-1α [30,31]. It has been reported that TSA, an HDAC inhibitor, may suppress VEGF gene in an indirect manner by down-regulating HIF-1α with increasing p53 and VHL in HepG2 cells under conditions of hypoxia [9]. In contrast to their results, it has been demonstrated that p53 is not necessary for FK228-induced down-regulation of VEGF gene in PC-3 cells (PC-3 cells have a deletion mutation of p53 gene) [32]. We did not investigate the role of VHL in the down-regulation of VEGF gene by FK228 and VHL status in PC-3 and ACHN cells has not been observed. It has been reported that the expression level of VEGF mRNA in tumor with a mutation of VHL is higher than that in tumor with a wild type VHL [33–35]. In this study, there was little difference in the basal expression levels of VEGF mRNA between PC-3 and ACHN xenografts (Fig. 5B). From our results, VHL status in PC-3 cells

may not be different from that in ACHN cells, and VHL may not play a role in the down-regulation of VEGF gene by FK228. If so, HDAC inhibitors may control the stability of HIF-1α without the modulation of p53 and VHL.

There is also the possibility that FK228 directly modulates the expression of VEGF gene. The effect of HDAC inhibitors on histone acetylation associated with the VEGF gene promoter has not been determined. We found that FK228 caused histone acetylation in the VEGF gene promoter in PC-3 cells (Fig. 4B). In particular, FK228 induced the highest acetylation of histone H3 and H4 in the P2 region of the VEGF promoter. The HIF-1 binding site, which is included in the P2 region, is located between 975 and 968 bp 5′ to the VEGF transcription initiation site and plays an important role in regulating the expression of VEGF mRNA in hypoxic cells [16]. The other potential HIF-1 binding site, which is included in the P1 region, is located between 313 and 306 bp 5′ to the VEGF transcription initiation site, but plays no role in the expression of VEGF mRNA in response to hypoxia and/or recombinant HIF-1 [16]. In the P3 region, there are no known binding sites of the transcription modulators [36]. Taken together, these results indicate that FK228 induced the highest acetylation of histone in the HIF-1 binding site which is important for modulating VEGF gene expression in the VEGF promoter.

It has been reported that histone acetylation of $p21^{WAF1/Cip1}$ promoter may contribute to the up-regulation of $p21^{WAF1/Cip1}$ gene expression by HDAC inhibitors, such as FK228, SAHA, and trapoxin [14,19,20]. These studies and our results suggest the possibility that HDAC inhibitors may modulate gene expression in the both directions by histone acetylation in the gene promoter. Histone acetylation levels in cells are determined by a dynamic equilibrium between competing HATs and HDACs [37]. HDACs and HATs do not bind directly to DNA but are recruited to specific locations by forming complexes with sequence-specific transcription factors [38–41]. It has been suggested that histone acetylation is mainly related to the induction of gene transcription [42–44]. The relationship between histone acetylation and gene suppression remains unclear, but we propose two hypotheses as follows: (i) Patterns of histone acetylation for down-regulating gene expression may be different from those for up-regulating gene expression. Histone has a lot of acetylation sites on lysine residues in their amino-terminal tails, such as lysines 9, 14, 18, and 23 in H3 [45], and there is a possibility that the acetylation state of each lysine is different, depending on the state of chromatin modification. Strahl and Allis demonstrated that the patterns of histone acetylation show the sign of transcription and named those patterns as "histone code" [46]. (ii) Histone acetylation by HDAC inhibitors induces some alteration of chromatin structure, which may fail to make transcription factors bind to gene promoters. Several lines of evidence suggest that histone acetylation plays a role in transcriptional regulation, probably by altering chromatin structure [39,44]. The alteration of chromatin structure by histone acetylation has not been clarified, but it may be important to explain the mechanism of regulation of gene expression by HDAC inhibitors.

In conclusion: (i) FK228 showed suppressive effects on the expression of angiogenesis factors, such as VEGF and bFGF, in PC-3 xenograft but not in ACHN xenograft; (ii) FK228 caused histone acetylation of VEGF promoter regions, which may induce suppression of VEGF gene expression. These results suggest that the effect on expression of angiogenesis factors is important for antitumor activity of FK228.

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