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Inhibition of hypoxia-induced angiogenesis by FK228, a specific histone deacetylase inhibitor, via suppression of HIF-1α activity

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Abstract

Hypoxia is generally detected in central regions of solid tumors and regulates a variety of transcription factors including hypoxia-inducible factor-1 (HIF-1). HIF-1 plays a pivotal role in cellular response to low oxygen concentration, such as angiogenesis in tumor. Here, we found that a histone deacetylase (HDAC) inhibitor, FK228, inhibits the induction and activity of HIF-1 in response to hypoxia. Moreover, FK228 significantly suppressed the induction of vascular endothelial growth factor (VEGF) under hypoxia, suggesting that FK228 contributes to the inhibition of tumor angiogenesis. In Lewis lung carcinoma model, FK228 also blocked angiogenesis induced by hypoxia. These results suggest that FK228 can downregulate hypoxia-responsive angiogenesis through suppression of HIF-1α activity.

Oxygen is essential for the life of most aerobic organisms. Limitations of oxygen have profound physiological and pathological consequences. In mammalian cells, a number of different genes can be induced by hypoxia and may play a role in oxygen-regulated cell responses. Hypoxia-inducible factor-1 (HIF-1) is a key transcriptional factor responsible for various cellular responses upon hypoxia including the increase of blood oxygen level, angiogenesis and the regulation of blood vessel tone by the induction of erythropoietin (EPO), vascular endothelial growth factor (VEGF), and nitrogen oxide (NO), respectively [1,2].

It has become increasingly apparent that acetylation of nuclear histones has a crucial role in gene expression because transcriptionally activated genes have been found to be highly associated with acetylation [3–5]. Furthermore, histone acetyl transferases (HATs) and histone deacetylase (HDACs) are identified as transcriptional coactivator and corepressor, respectively [6–8], which suggests that they modulate transcriptional activity at specific promoters by locally releasing or perturbing chromatin structure. Generally, HATs are involved in the modification of histones as well as non-histone transcriptional factors [9,10]. Interaction of HIF-1α with steroid receptor coactivator-1 (SRC-1) and cAMP response element binding protein (CBP), and the transactivation potential of HIF-1α are increased in a hypoxia-dependent manner [11]. Moreover, HIF-1α function is also repressed by some cell-type specific factors possibly harboring HDAC activity [12] as revealed by treatment of HDAC inhibitor. These observations suggest that the activation of hypoxia-regulated genes might be regulated by the chromatin structure modification of itself and other upstream genes also.

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Recently, we reported that HDAC-1 is upregulated by hypoxia and its specific inhibitor, trichostatin A (TSA), inhibits the hypoxia-induced angiogenesis [13]. FK228 (also known as FR901228 or NSC 630176) is a natural cyclic depsipeptide and a specific HDAC inhibitor [14]. It is known as an anti-tumor agent which has a function of cell cycle arrest at G1 and G2/M phase, differentiation as well as apoptosis in many tumor cell lines [15] and greatly suppresses the growth of transplanted tumors in mice [16]. Moreover, FK228 inhibits in vitro hypoxia-induced angiogenesis [17], suggesting that the acetylation of histone of specific genes by FK228 may be related to the molecular mechanism of its anti-angiogenic activity under hypoxic condition in tumor. However, it is still unknown how FK228 acts in hypoxic condition of tumor cells, which is a main stimulator of angiogenesis in many solid tumors.

Here, we show that FK228 specifically inhibited the induction of HIF-1α and functional activity of HIF-1 in hypoxic condition. FK228 effectively inhibited the hypoxia-induced mRNA and protein expression of VEGF. Moreover, we demonstrate that the anti-angiogenic activity of FK228 was specific for the hypoxia-induced angiogenesis of Lewis lung carcinoma (LLC) model.

Materials and methods

Cell culture. LLC cell line was provided by Dr. Jung-Joon Lee, the Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea. LLC and HT1080 human fibrosarcoma cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Subconfluent cells were treated with FK228 and incubated under normoxic or hypoxic condition. For the condition of hypoxia, cells were incubated at a CO_2 level of 5% with 1% O_2 balanced with N_2.

Animals. Specific pathogen-free male C57BL/6J mice (7 weeks old) were purchased from Hyochang Science, Korea. They were provided with autoclaved tap water and laboratory chow ad libitum and were kept at 23 ± 0.5 °C, 55 ± 10% humidity in 12 h light–dark cycle.

Northern blot analysis. Total RNA was isolated from LLC cells by a single-step procedure with Trizol Reagent (Invitrogen) [18] and used for Northern blot analysis as described [19]. ^{32}P-labeled DNA probes were prepared by using random primer oligonucleotides (*redi*prime DNA Labeling System, Amersham Pharmacia Biotech).

Western blotting. Cell extracts were prepared and aliquots (20–30 μg) were loaded into 6–12% SDS–PAGE gel and transferred to protein nitrocellulose membrane (Sigma–Aldrich). The membrane was rinsed in TBS-T solution (0.1% Tween 20 in TBS, pH 7.5) and incubated in blocking buffer (5% skim milk in TBS-T) at room temperature (RT). Then the filter was incubated with specific primary antibody at RT for 1 h or overnight at 4 °C and washed with TBS-T three times every 15 min, followed by incubation with secondary antibody at RT for 1 h [20]. The blocked membranes were incubated with HIF-1α or [21] VEGF (Santa Cruz Biotech.) antibodies, and the immunoreactive bands were visualized using chemiluminescent reagent as recommended by Amersham Pharmacia Biotechnology.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts prepared from HT1080 treated with or without FK228 exposed to 20% or 1% O_2 were incubated with ^{32}P-labeled double-stranded oligonucleotides for the VEGF-specific HIF-1α-binding sequences (5′-TGCATACGTGGGCTCCAACAG-3′) [22] and was analyzed by non-denaturing 6% polyacrylamide gel at 200 V in TBE buffer. The gels were dried and exposed to Fuji RX X-ray film. HIF-1α binding to the probe was confirmed by competition assays using 50- and 100-fold oligonucleotides without α-^{32}P-labeling.

Tumor generation and FK228 treatment. Tumor development for detection of hypoxia and angiogenesis inside tumor is previously described [13]. Briefly, C57BL/6J mice were anesthetized with diethyl ether and implanted with LLC about 1×10^6 cells/0.1 ml of media at the flank of mouse. When tumors were about 20 mm^3 in size, approximately 7 days after implant, FK228 (0.5 mg/kg body weight) or the same amount of saline (containing 10% DMSO) was treated daily intraperitoneally. Tumor length (L), width (W), and height (H) were monitored and tumor volume was calculated as $L \times W \times H$ (mm^3). Ten days after treatment of FK228, control and treated mice were injected with pimonidazole (Natural Pharmacia International, 60 mg/kg body weight) for detection of tumor hypoxia [23]. Two hours later mice were sacrificed and tumor masses were removed from the flank and fixed with 4% paraformaldehyde solution (pH 7.4).

Immunohistochemistry. Fixed tumor masses were frozen in OCT compound (Miles) and serially sectioned at 10 μm of thickness in the sagittal and coronal directions at −20 °C. Sections were quenched with hydrogen peroxide, blocked with 3% bovine serum albumin (Sigma–Aldrich), and processed for immunohistochemistry as described [13]. Briefly, application of the antibodies against pimonidazole-adduct (Natural Pharmacia International), PECAM-1 (CD31/MEK13.3, Pharmingen), and HIF-1α (Santa Cruz Biotech.) at a proper dilution concentration was performed. Negative controls were performed using PBS as well as isotype matched antibodies instead of primary antibodies. Biotinylated SP-conjugated F(ab′)2 fragment of a goat anti-mouse IgG (Jackson) was treated for pimonidazole adduct, for PECAM-1 biotinylated anti-rat IgG (DAKO), for HDAC-1 biotinylated anti-goat IgG (DAKO), and for VEGF biotinylated anti-rabbit IgG (Sigma–Aldrich) as a secondary antibody. Immunoreactivity was detected with streptavidin-conjugated peroxidase (DAKO) and diaminobenzidine (DAB) as a chromogen. The sections were counterstained with Mayer's Hematoxylin (Sigma–Aldrich).

Statistical analysis. All data are expressed as means ± SE. Student's t test was used to establish which groups differed from the control group. P value < 0.05 was considered statistically significant.

Results

Inhibition of hypoxia-induced expression of HIF-1α and HIF-1 binding activity by FK228

To investigate whether FK228 could regulate hypoxia-induced responses in carcinoma cells, we determined the HIF-1α protein levels in hypoxia-exposed LLC cells treated with FK228. Hypoxia induces HIF-1α protein expression level as early as 4 h and maximizes HIF-1α level at 16–24 h. Sixteen hours of FK228 treatment significantly inhibited the protein level of HIF-1α induced by hypoxia (Fig. 1A). Moreover, there is no significant time-dependent effect of FK228 on the hypoxia-induced HIF-1α protein level (data not shown). As shown in Fig. 1B, FK228 gradually inhibited HIF-1α protein expression level induced by hypoxia in a dose-dependent manner. When we performed EMSA with FK228-treated nuclear extracts under hypoxia, FK228 reduced the hypoxic increase of DNA binding activity of HIF-1α (Fig. 1C). Accordingly, hypoxia-induced

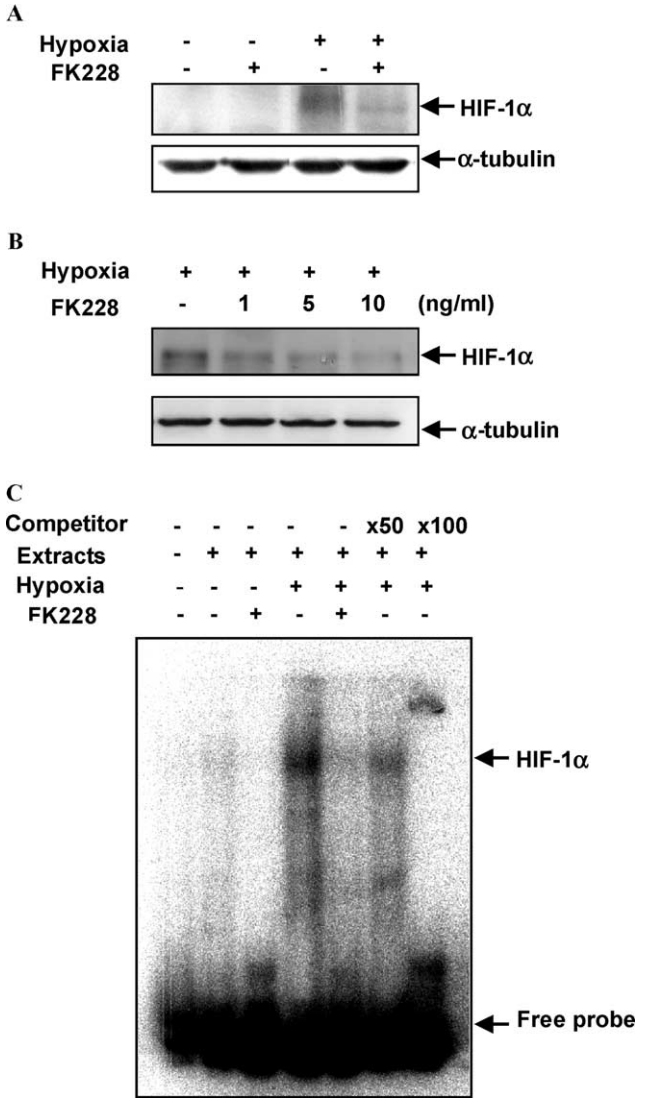

Fig. 1. Effect of FK228 on the HIF-1α expression and DNA binding activity. (A) LLC cells under hypoxia for 16 h with treatment of FK228 10 ng/ml were isolated. (B) LLC cells were cultured in 20% (normoxia) or 1% O_2 (hypoxia) concentrations with different doses of FK228 (1, 5, and 10 ng/ml) for 16 h. Protein extracts (20 μg) were examined by 6% SDS–PAGE and analyzed by Western blotting with HIF-1α-specific antibody as described in 'Materials and methods.' (C) Nuclear extracts (15 μg) from HT1080 cells cultured in normoxia or hypoxia with FK228 for 4 h were analyzed by 6% undenatured polyacrylamide gels and DNA binding activities of HIF-1 were determined by electrophoretic mobility shift assay using specific oligomer as described in 'Materials and methods'.

expression and the DNA binding activity of HIF-1α were significantly inhibited by FK228, showing that FK228 decreases the activity of HIF-1α.

Inhibition of hypoxia-induced VEGF expression by FK228

To confirm whether the reduction of HIF-1α activity is related with the inhibition of hypoxia-induced angiogenesis by FK228, we determined the expression of VEGF mRNA and protein by the treatment of FK228 under hypoxia. From the Northern blot analysis, hypoxia increased VEGF mRNA level by 6-fold, but it was significantly inhibited by FK228 (Fig. 2A).

When LLC cells were exposed to hypoxia for 16 h, the protein expression of VEGF in the cell lysate was also increased and the induction of VEGF had almost completely disappeared by treatment of FK228 (Fig. 2B).

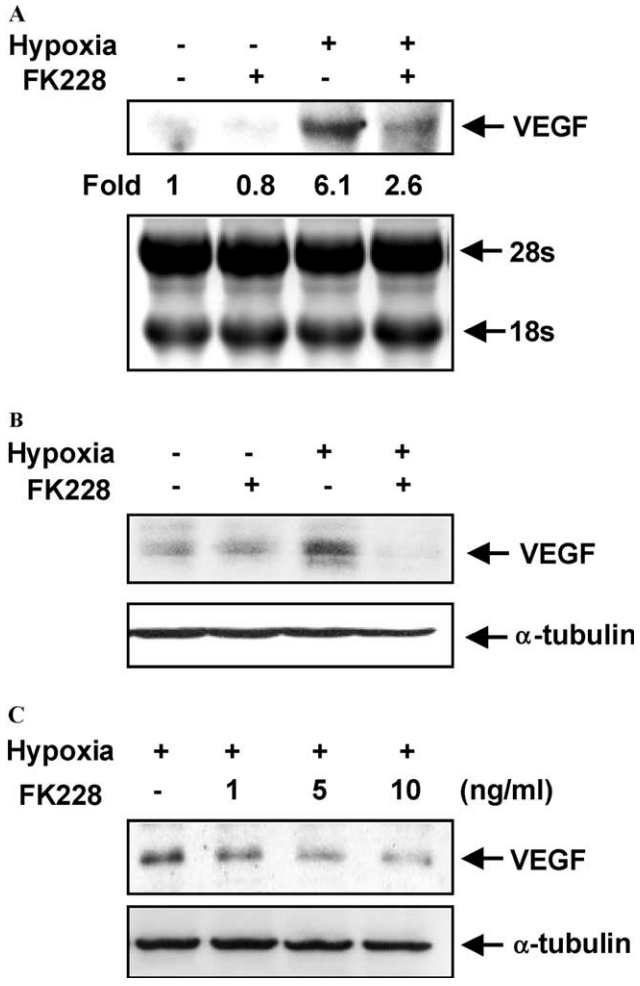

Fig. 2. Effect of FK228 on hypoxia-induced VEGF expression. (A) Northern blot analysis was performed for VEGF with total RNA (20 μg) isolated from LLC cells cultured under normoxia or hypoxia treated with FK228 (10 ng/ml) for 16 h. The 18 and 28 s rRNA was indicated as a quantity control. (B) Cell lysates from LLC cells treated with 10 ng/ml FK228 for 16 h under hypoxia or normoxia were electorphoresed onto SDS–PAGE gel, blotted into the membrane, and probed with anti-VEGF antibody. (C) Detection of VEGF from protein extract obtained LLC cells treated with different doses of FK228 (1, 5, and 10 ng/ml) under hypoxia for 16 h. Cells were seeded in the same density and changed with 10% serum containing media when exposed to hypoxia. Protein extracts (20 μg) were examined by 6% SDS–PAGE and analyzed by Western blotting with VEGF-specific antibody as described in 'Materials and methods.' Western blot analysis with anti-α-tubulin was performed as a control.

To investigate the dose-dependent effect of FK228 on the down-regulation of VEGF protein, we treated FK228 from 1 to 10 ng/ml. The increased VEGF protein level by hypoxia was significantly reduced by treatment of FK228 in a dose-dependent manner (Fig. 2C). These results suggest that the down-regulation of VEGF by FK228 may be due to the inhibition of HIF-1α activity.

Inhibition of tumor angiogenesis in a hypoxia-specific manner by FK228

In our previous report [17], we investigated the effect of FK228 on in vitro and in vivo angiogenesis. FK228 potently inhibits angiogenesis as well as hypoxia-induced endothelial cell proliferation and migration. To confirm whether the anti-angiogenic activity of FK228 is the hypoxia-specific regulated mechanism, we performed in vivo tumor generation experiments with LLC cells.

Seven days after inoculation of tumor cells, tumor-bearing mice were treated with FK228 (0.5 mg/kg body weight) as well as a vehicle. Tumor mass was rapidly grown and reached above 600 mm^3 in size at 10 days after inoculation. However, a significant decrease of tumor size was observed at 10 days of FK228 treatment (Fig. 3). These data supported anti-tumorigenic activity of FK228 in mice [16]. Although it has a stronger tumor inhibitory activity for longer than 10 days treatment of FK228, it was optimal for the observation of hypoxia-induced angiogenesis at 17 days after inoculation of tumor cells (treatment started 7 days after inoculation). Many large vessels were grossly observed in the dissected control tumor. Hypoxic regions were highly detected by the hypoxia marker, pimonidazole, at around center and sporadical regions of tumor mass, and HIF-1α was co-localized with hypoxic regions in the tumor sections (Figs. 4A and C).

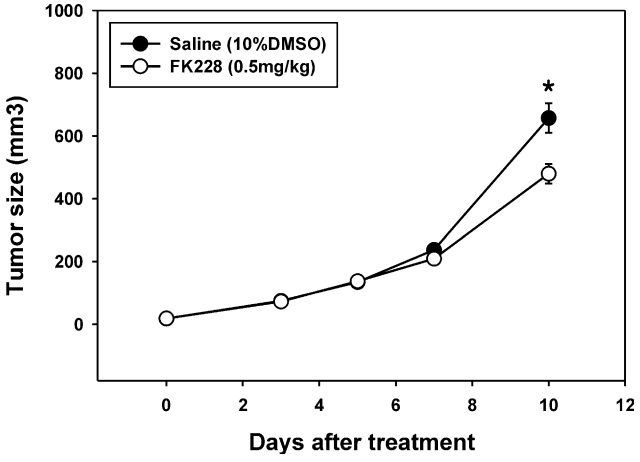

Fig. 3. Inhibition of LLC tumor growth by FK228. C57BL/6J mice were inoculated with LLC about 1×10^6 cells/0.1 ml of media at the flank of mouse. Seven days after implant, mice were treated with FK228 0.5 mg/kg body weight or the same amount of saline (containing 10% DMSO) daily intraperitoneally. The tumor size was measured using microcaliper at days indicated. Significant decrease of tumor size was observed at 10 days of treatment. Each values represents the mean ± SE, and $*p < 0.05$ compared to control tumor.

There were normoxic regions near the large vessels, but hypoxia was predominant at some distance to large vessels and even at near or around microvessels in control tumor (Fig. 4, left panel). It was prominent that there were a lot of microvessels even in hypoxic region (Fig. 4, left panel, arrowheads). Those microvessels may not be able to supply proper and/or enough oxygen to the highly proliferative tumor cells.

In FK228-treated tumor, there were also hypoxic regions at some distances from large vessels and microvessels (Fig. 4, right panel, arrows). However, it is interesting that in FK228-treated tumor, most of large and micro-vessels were regressed especially in hypoxic regions detected by pimonidazole and HIF-1α (Figs. 4B and D). PECAM staining also showed that endothelial cells were not found in hypoxic region of FK228-treated tumor (Fig. 4F). These results indicate that the induction of new blood vessels in the hypoxic region of tumor mass was largely inhibited by FK228 treatment. Therefore, we suggest that FK228 might efficiently inhibit the hypoxia-induced angiogenesis in vivo.

Discussion

The outcome of hypoxia in cancer can differ according to the condition of chromatin structure of genes under different environments. Even deacetylation and acetylation of histone are recently accepted as a key regulatory mechanism of gene transcription, there are not enough evidences to suggest that genes involved in angiogenesis can be selectively modulated by histone acetylation under hypoxic condition.

In this study, we adopted a potent HDAC inhibitor, FK228, which has an anti-tumor activity [16]. We investigated whether angiogenesis of cancer under hypoxia can be regulated by FK228. The down-regulation of HIF-1 by FK228 (Fig. 1) indicates that HDAC is involved in oxygen-dependent gene expression and hypoxia-induced angiogenesis. We further reveal that FK228 may be involved in the modulation of genes regulated by HIF-1. The inhibitory effect of FK228 on HIF-1 activity prohibited the induction of VEGF in response to hypoxia both in transcriptional and translational levels (Fig. 2). This significant inhibition of VEGF by FK228 under hypoxia may play a direct and critical role in the inhibition of angiogenesis. And the fact that FK228 has an in vitro anti-angiogenic activity under hypoxia [17] may be substantially related to the inhibition of hypoxia-induced VEGF expression and HIF-1 activity.

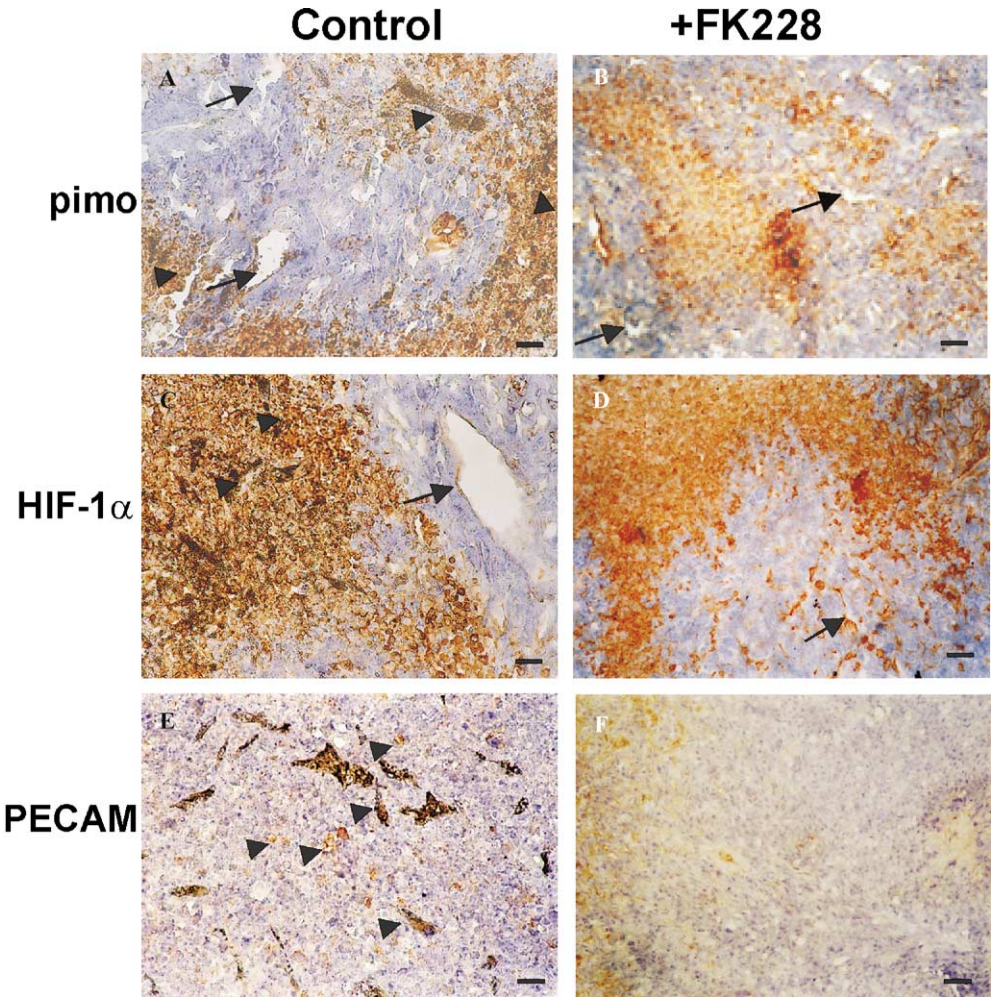

Fig. 4. Inhibition of hypoxia-induced angiogenesis in LLC tumor by FK228. FK228-treated tumor sections (15 μm) immunostained by hypoxia marker, pimonidazole (A,B), anti-HIF-1α (C,D), and PECAM-1 (E,F). Positive immunoreactive regions are almost co-localized with each other, judging by the spatial relationship with large or microvessels. Arrows indicate large vessels containing many erythrocytes and arrowheads indicate microvessels inside hypoxic region. Negative controls for immunohistochemistry were performed with PBS and normal IgG (data not shown). Bar = 100 μm.

We adopted a highly angiogenic LLC carcinoma as an in vivo animal angiogenesis model. FK228 significantly inhibited LLC tumor masses from when they started to grow fast; probably at the time angiogenesis is actively performed (Fig. 3), suggesting that FK228 may inhibit the starting angiogenic events that occurred inside the tumor. In control tumor, aggressive invasion of erythrocytes was found inside hypoxic regions determined by pimonidazole (Fig. 4). This result demonstrates that hypoxia substantially stimulates migration and invasion of endothelial cells. In contrast, FK228 significantly inhibited the tumor growth at 10 days after treatment and prohibited the growth of blood vessels especially in tumor hypoxic region. In FK228-treated tumor, there is a significant regression of microvessels even if they contain hypoxic regions, indicating that FK228 may efficiently inhibit angiogenesis in hypoxic region (Fig. 4).

Collectively, we demonstrated that an HDAC inhibitor, FK228, downregulates hypoxia-response genes and hypoxia-induced angiogenesis by the suppression of HIF-1α activity.

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