

Antiangiogenic and Antitumor Activities of Aflibercept, a Soluble VEGF Receptor-1 and -2, in a Mouse Model of Hepatocellular Carcinoma



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Abstract

BACKGROUND & AIM: Aflibercept known as ziv-aflibercept in the United States is a soluble decoy receptor of both vascular endothelial growth factor (VEGF) receptor-1 and -2 known to inhibit the binding of VEGF and placental growth factor (PlGF) to VEGF receptor-1 and -2. Here, we analyzed the mechanisms of the antitumor effects of aflibercept in mouse hepatoma models. **METHODS:** In *in vitro* studies, we determined the effects of aflibercept on human umbilical vein cell (HUVEC) proliferation and bone marrow (BM) cell differentiation to endothelial progenitor cells (EPCs). In *in vivo* experiments, aflibercept was injected intraperitoneally in hepatoma cell tumor-bearing mice, and its inhibitory effects on tumor growth and BM cell migration to tumor tissues were evaluated. **RESULTS:** Aflibercept suppressed phosphorylation of VEGF receptor-1 and -2 in HUVEC and dose-dependently inhibited VEGF-induced HUVEC proliferation. It suppressed the differentiation of BM cells to EPCs and migration of BM cells to tumor tissues. It also suppressed tumor growth and prolonged survival time of tumor-bearing mice without side effects. In tumor tissues, aflibercept upregulated the expression of hypoxia inducible factor-1 α , VEGF, PlGF, fibroblast growth factor-2, platelet derived growth factor-BB, and transforming growth factor- α and reduced microvascular density. It also reduced sinusoidal density in noncancerous liver tissues. **CONCLUSIONS:** Our results demonstrated potent antitumor activity for aflibercept in a mouse model of hepatocellular carcinoma. These effects were mediated through inhibition of neovascularization, caused by inhibition of endothelial cell proliferation, EPC differentiation, and BM cell migration to tumor tissues.

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Introduction

Neovascularization is an important process in solid tumor growth [1]. The presence of a highly upregulated angiogenesis process results in the formation of abnormal and leaky vessel structures, which induce abnormal blood flow [2,3]. Vascular endothelial growth factor (VEGF) family members [e.g., VEGFs-A to -E and placental growth factor (PlGF)-1 and -2] are potent angiogenic factors [4,5]. Recent studies have suggested that circulating bone marrow (BM)-derived endothelial progenitor cells (EPC) as well as other BM cells migrate into tumor tissues to support neovascularization and tumor development [6–8]. The migration of EPC and other BM cells is

mainly regulated by the local release of VEGF, PlGF, and stromal derived factor-1 in tumor tissues [7,9,10].

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Hepatocellular carcinoma (HCC) is a common solid tumor and a major cause of cancer-related death globally [11]. Although the portal blood supply to HCC is predominant at the early stage of hepatocarcinogenesis, HCC ultimately becomes a highly vascular tumor with the development of neoarteries in parallel with tumor growth [12,13]. Several reports have stressed the role of VEGF in such neovascularization process [14,15].

Targeting tumor vasculature as adjunct anticancer therapy was first advocated by Folkman in 1971 [16]. Since then, numerous antiangiogenic agents have been used clinically and/or preclinically to investigate the benefits of such approach in various types of tumors. Because advanced HCC is a hypervascular tumor, antiangiogenic therapy might be particularly suitable so as to prevent or halt tumor growth and even promote tumor regression or dormancy. In 2008, the therapeutic use of sorafenib, an inhibitor of VEGF receptor-2 (VEGFR)-2, platelet-derived growth factor (PDGF), and Raf/MEK/ERK signaling, was approved for patients with advanced HCC [17]. However, the therapeutic efficacy of sorafenib proved to be limited.

Aflibercept, a new antiangiogenic agent, is a soluble decoy VEGFR constructed by fusing the second Ig domain of VEGFR-1 and the third Ig domain of VEGFR-2 with the constant region (Fc) of human IgG1 [18]. Because PlGF binds specifically to VEGFR-1 and VEGF binds to VEGFR-1 and -2, aflibercept shows one-to-one high-affinity binding to all isoforms of VEGF and PlGF [19,20]. Clinical randomized phase 3 trial using aflibercept has been performed for several solid cancers except HCC [21,22]. Then, aflibercept has been approved by the US Food and Drug Administration for metastatic colorectal cancer in 2012 [23]. This approval is based on the results of a randomized, double-blind, placebo-controlled, multicenter trial enrolling patients with metastatic colorectal cancer, which showed that a statistically significant improvement of overall survival was observed in patients in the chemotherapy plus ziv-aflibercept group compared with the chemotherapy plus placebo group (hazard ratio, 0.82) [24].

In the present study, we investigated the antitumor effects and antiangiogenic mechanisms of aflibercept in a mouse hepatoma model. The results demonstrated that aflibercept suppressed tumor development by inhibiting neovascularization through the suppression of proliferation of vascular endothelial cells, migration of BM cells to tumor tissue, and differentiation of EPC.

Materials and Methods

Reagents, Cells, and Animals

Human umbilical vein endothelial cells (HUVECs), mouse hepatoma cell line (Hepa 1-6), and human hepatoma cell line (HuH-7) were obtained from CAMBREX Bio Science Walkersville Inc. (Walkersville, MD). Human hepatoma cell lines (KYN-2 and HAK1-B) were provided by the Department of Pathology, Kurume University School of Medicine [25,26]. Male 5-week-old nude mice (BALB/c nu/nu) and C57BL/6 mice were purchased from Kyudo KK (Fukuoka, Japan). Male 5-week-old C57BL/6-Tg (act-EGFP) C14-Y01-FM131Os mice were generous gifts from Professor Okabe (Genome Information Research Center, Osaka University, Osaka, Japan) [27]. All mice were acclimatized to the new laboratory environment and cared for in separate cages. Furthermore, all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National

Institute of Health. The experimental protocol was approved by the Laboratory Animal Care and Use Committee of Kurume University.

Proliferation Assay of HUVEC and Hepatoma Cells

HUVEC and hepatoma cells (HuH-7, KYN-2, HAK1-B, Hepa1-6) were seeded (1×10^3 /well) in 96-well plate containing a suitable medium [EGM-2 supplemented with 5% fetal bovine serum (FBS) for HUVEC and Dulbecco's modified Eagle's medium with 10% FBS for hepatoma cells]. After 24-hour incubation at 37°C, the media were replaced with media containing various concentrations of recombinant VEGF-165 (0, 0.1, 1, 10, 100 pM, 1 nM) (PeproTech, Inc., Rocky Hill, NJ) or PlGF-2 (0, 0.1, 1, 10, 100 pM, 1 nM) (PeproTech, Inc.) and 5% FBS for HUVEC cell proliferation assay. To inhibit HUVEC and hepatoma cell proliferation, the media were replaced with media containing various concentrations of aflibercept (0, 10, 100 pM, 1, 10, 100 nM) combined with recombinant VEGF-165 (0.2 nM) or PlGF-2 (1.0 nM), and 5% FBS. After 72-hour incubation, cell proliferation was evaluated by the tetrazolium-based assay (Cell Count Reagent SF; Nakalai Tesque Inc., Kyoto, Japan). The experiment has been done at least twice to confirm reproducibility.

Colony-Forming Units (CFU) Assay of EPCs

Bone marrow cells were collected from both femurs of C57BL/6 mice, as reported by Nakamura et al. [28]. EPCs were measured according to the methods described by Sobrino et al. [29]. Mononuclear cells (5×10^6 /well) were seeded on fibronectin-coated six-well dishes in EndoCult Liquid Medium (StemCell Technologies, Vancouver, BC, Canada) for 2 days. Then, non-adherent cells (1×10^6 /well) were plated on fibronectin-coated 24-well dishes in a medium containing aflibercept (0, 10 nM). Colonies formed 3 days later were counted in 10 wells of each group. To confirm endothelial cell lineage, colonies were incubated with rabbit anti-mouse CD31 antibody (dilution 1:100; Abcam Japan, Tokyo, Japan) at 4°C overnight followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1:100; Dako, Kyoto, Japan). The experiment has been done at least twice to confirm reproducibility.

Western Blotting of HUVEC and Hepa1-6 Cells

For the investigation of phosphorylation of VEGFR-1 and -2, HUVEC and Hepa1-6 cells were cultured in serum-free, VEGF-free, and PlGF-free medium for 6 hours. These cells were preincubated with aflibercept (0, 3 nM) for 15 minutes. Then recombinant VEGF-165 or PlGF-2 (0, 1 nM) was added for 5 minutes. Total protein (10 µg) from each cell lysate was run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. The membranes were incubated overnight with rabbit anti-human phosphorylated-VEGFR-1 antibody (Ty1213) (*p*-VEGFR-1) (dilution 1:500; R&D Systems, Inc., Minneapolis, MN), rabbit anti-human *p*-VEGFR-2 antibody (Ty1175) (dilution 1:500; Cell Signaling Technology Inc., Danvers, MA), rabbit anti-human VEGFR-1 antibody (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-mouse VEGFR-2 antibody (dilution 1:200; Santa Cruz Biotechnology) at 4°C. After incubation with donkey anti-rabbit HRP-conjugated antibody (dilution 1:10,000; GE Healthcare Bio-Sciences GK, Tokyo, Japan) for 1 hour, the immunoreactive bands were stained using the enhanced chemiluminescence Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ).

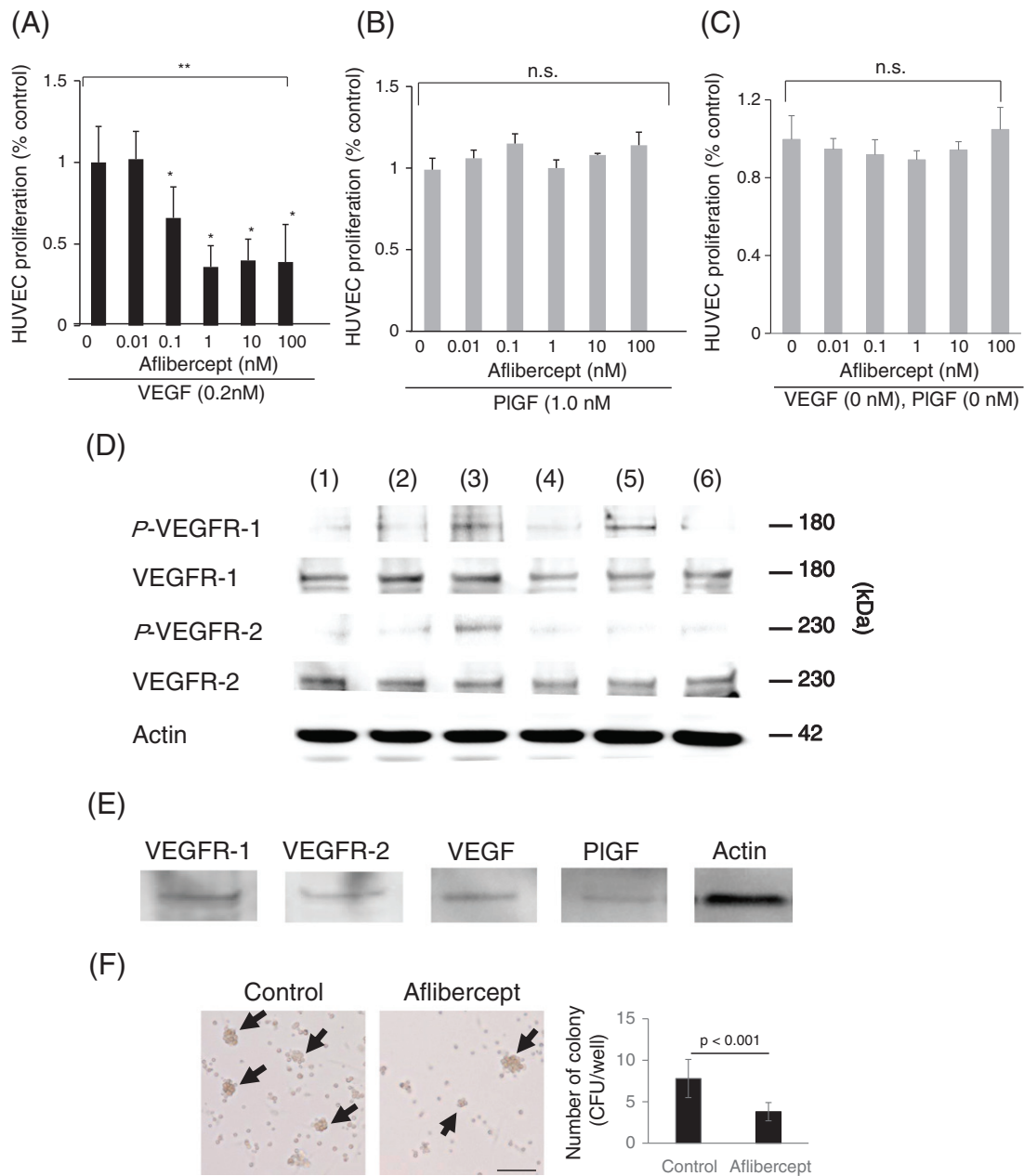


Figure 1. Inhibitory effects of aflibercept on cell proliferation and phosphorylation of VEGFR-1 and -2. HUVECs were cultured in media containing aflibercept (0-100 nM); FBS; and (A) VEGF 0.2 nM, (B) PIGF 1.0 nM, or (C) VEGF, PIGF 0 nM. Seventy-two hours after incubation, cell proliferation was evaluated by a tetrazolium-based assay. (C) HUVECs were cultured in media containing aflibercept and VEGF. (1) Control, (2) aflibercept, (3) VEGF, (4) VEGF/aflibercept, (5) PIGF, (6) PIGF/aflibercept. (D) BM-derived mononuclear cells were collected from both femurs of C57BL/6 mice. The expressions of VEGFR-1, VEGFR-2, VEGF, and PIGF were measured by Western blotting. (F) Images of colony formation assay of EPC in each control and aflibercept-treated group. Statistical comparisons with the control, **P* < .05, by Mann-Whitney U test, ***P* < .05, by Kruskal-Wallis test. *n.s.*, not significant.

Table 1. Tumor Growth in Three Different Mouse Xenograft Models and Effects of Treatment with Aflibercept.

	Baseline PBS Group	Baseline Aflibercept Group	After 3 Weeks PBS Group	After 3 Weeks Aflibercept Group
HAK1-B	73.0 ± 21.0	75.2 ± 11.5	213 ± 71.9	15.4 ± 6.6*
HuH-7	88.2 ± 6.0	92.1 ± 5.6	1,003 ± 573	75.1 ± 23.7*
Hepa1-6	80.3 ± 14.6	71.7 ± 13.5	1,025 ± 1,013	60.0 ± 42.9*

Data are mean ± SD of tumor volume (mm³) of six mice per group.
* *P* < .05, compared with the PBS group at 3 weeks of treatment.

Protocols of Tumor Growth Studies of Subcutaneous Tumor Model

Nude mice were anesthetized and then injected with 5×10^6 HuH-7, HAK1-B, KYN-2, or Hepa1-6 cells subcutaneously into the dorsal portions. The tumor-bearing mice of HuH-7, HAK1-B, and Hepa1-6 cells were randomly divided into phosphate-buffered saline (PBS)-administered groups and 25.0 mg/kg body weight (BW) aflibercept-treated groups. On the other hand, the tumor-bearing mice of KYN-2 cells were randomly divided into four treatment groups: one PBS-administered group and three aflibercept-treated

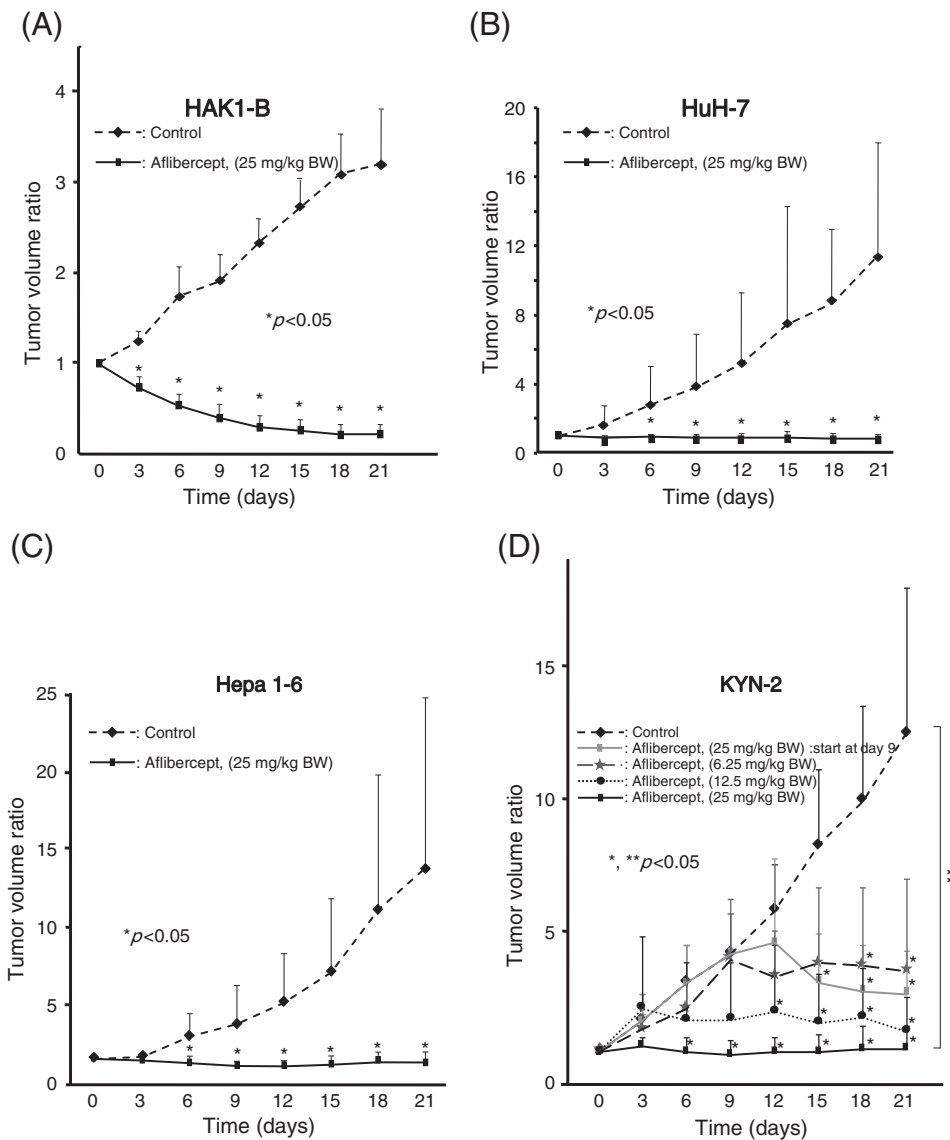


Figure 2. Aflibercept suppresses tumor growth in hepatoma-bearing mice. Data are mean \pm SD and expressed relative to the values immediately after the initiation of treatment ($n = 6$ per group). (A) HAK1-B cells, (B) HuH-7 cells, (C) Hepa1-6 cells. Initial treatment started when the average tumor size was 50 to 100 mm³. * $P < .05$, compared with PBS-treated mice (by Mann-Whitney U test). (D) KYN-2 cells. Tumor-bearing mice were treated with aflibercept (0-25 mg/kg BW) every 3 days. Gray line represents tumor volume in mice that initiated aflibercept treatment at day 10. Statistical comparisons with PBS-treated mice, * $P < .05$, by Mann-Whitney U test, ** $P < .05$, by Kruskal-Wallis test.

groups (6.25, 12.5, 25.0 mg/kg BW). Treatment was initiated when the average tumor size reached 50 to 100 mm³, and included intraperitoneal injection of aflibercept or PBS every 3 days. To

Table 2. Changes in Tumor Growth in KYN-2 Xenograft Mice Treated with PBS and Three Different Doses of Aflibercept.

	Baseline	After 3-Week Treatment
PBS	72.8 \pm 14.0	789 \pm 354
6.25 mg/kg BW aflibercept	70.3 \pm 18.9	299 \pm 318*
12.5 mg/kg BW aflibercept	79.3 \pm 15.8	121 \pm 57.2*
25 mg/kg BW aflibercept	73.2 \pm 13.4	77.7 \pm 53.5*

Data are mean \pm SD (mm³) of six mice of each group.
* $P < .05$, compared with the PBS group at 3 weeks.

evaluate the therapeutic efficacy of aflibercept in large tumor (i.e., equivalent to advanced HCC), treatment with aflibercept was initiated at 25.0 mg/kg BW in another group of tumor-bearing mice at day 9. Tumors size was measured by calipers in two dimensions every 3 days for 3 weeks, and tumor volume was calculated using the following equation: length \times width² \times 0.52. Each treatment group consisted of six mice. Tumor volume ratio was calculated by average tumor volume in each measured date/average tumor volume in the initial treatment date.

Protocols of Growth and Survival Studies of Liver Tumor Model

Nude mice were injected with 2×10^6 KYN-2 cells into the liver. The mice were randomly divided into PBS-administered group ($n = 6$) and aflibercept-treated group (25 mg/kg BW, $n = 6$). Seven days

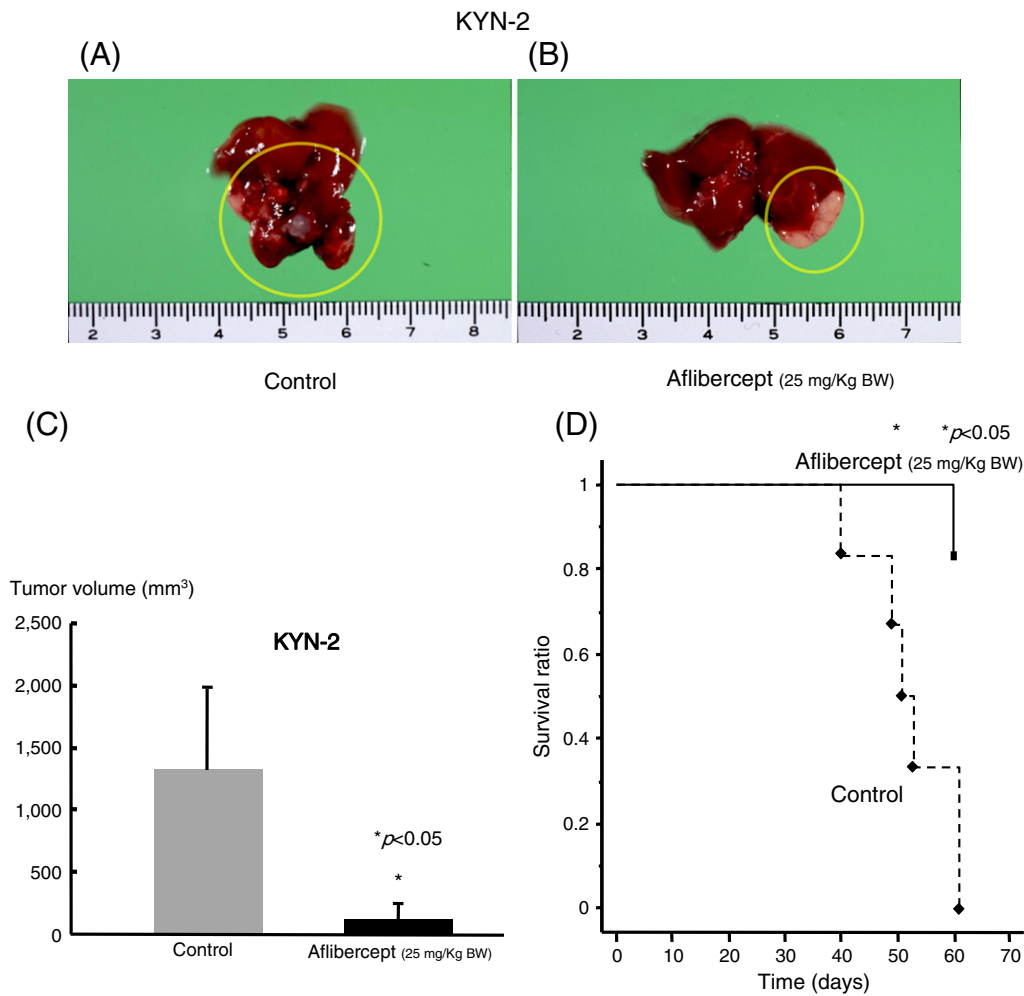


Figure 3. Aflibercept inhibits liver tumor growth in nude mice. (A, B) Representative photographs of the liver. Note the large tumor of KYN-2 cells in the control liver. Tumor growth is markedly suppressed by aflibercept. Yellow circle indicates tumor. (C) Data are mean \pm SD of tumor volume in mm³ ($n = 6$ per group). * $P < .05$, compared with PBS-treated mice by Mann-Whitney U test. (D) Kaplan-Meier estimates of survival of mice treated with aflibercept (25 mg/kg BW) and those treated with PBS. The survival time was counted from the day of tumor cell transplantation. * $P < .05$, compared with PBS-treated mice by log-rank test.

later, the mice were treated with aflibercept once every 3 days for 3 weeks. They were subsequently sacrificed at day 28, and tumor volume was evaluated. Serum levels of alpha fetoprotein (AFP) were measured at the time of sacrifice by radioimmunoassay.

For survival studies, KYN-2 cells were inoculated into another group of 12 nude mice, which was later divided at random into the control group (PBS-administered, $n = 6$) and aflibercept-treated group (25 mg/kg BW, $n = 6$). Mice were sacrificed when the clinical signs of weakness, anorexia, and/or $>20\%$ weight loss were noted.

Assessment of Microvascular Density, Plasma VEGF and PlGF, and Peripheral Free Aflibercept Levels of Subcutaneous Tumor Model

Sections of Hepa1-6 cell tumor tissues and nontumor liver tissues from mice treated with aflibercept (6.25, 12.5, 25 mg/kg BW) or PBS were incubated overnight with rabbit anti-mouse CD31 antibody at 4°C. Then, the sections were incubated with FITC-conjugated goat

anti-rabbit IgG. CD31-positive blood vessels in tumor tissues and sinusoids in noncancerous liver tissues were counted in 60 and 25 blindly selected random fields (z -series, 63 \times oil magnification), respectively.

Peripheral free aflibercept levels in Hepa1-6 cell tumor-bearing mice treated with aflibercept (6.25, 12.5, 25 mg/kg BW) were measured by ELISA [18,19] at the time of sacrifice. Plasma VEGF and PlGF levels were also measured by ELISA in mice treated with aflibercept (0, 25 mg/kg BW).

Western Blotting of Tumor Tissues from Subcutaneous Tumor Model

Hepa1-6 cell tumor tissues were obtained at sacrifice. Total protein (50 μ g) from Hepa 1-6 cell tumor tissues was run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane. The membrane was incubated overnight with rabbit anti-human p -VEGFR-1 antibody, rabbit

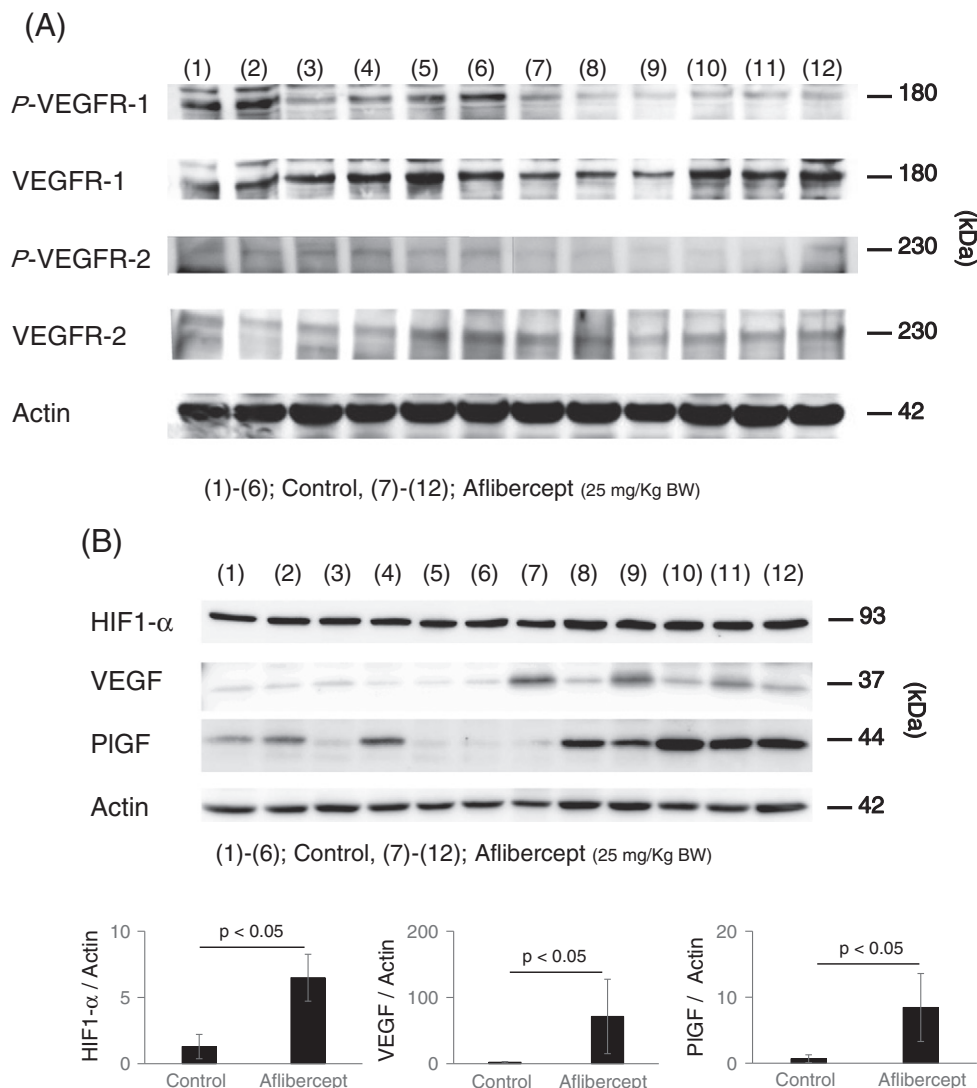


Figure 4. Effects of aflibercept on phosphorylation of VEGFR-1 and -2, and expression of HIF1- α , VEGF, and PIGF in tumor tissues. (A) Western blot analysis of *p*VEGFR-1, *p*VEGFR-2, VEGFR-1, and VEGFR-2 in Hepa1-6 cell tumor tissue. Lanes 1 to 6, tumor tissues of mice treated with PBS; lanes 7 to 12, tumor tissues of mice treated with aflibercept (25 mg/kg BW). (B) Western blot analysis of HIF1- α , VEGF, and PIGF expression in Hepa1-6 cell tumor tissue. Lanes 1 to 6, tumor tissues of mice treated with PBS; lanes 7 to 12, tumor tissues of mice treated with aflibercept (25 mg/kg BW). The quantified graphs were shown. (C) Quantitative real-time PCR analysis of EGF, FGF-2, PIGF, HGF, VEGF, Ang-1, Ang-2, PDGF-BB, and TGF- α expression. *EGF*, epidermal growth factor; *HGF*, hepatocyte growth factor.

anti-human *p*-VEGFR-2 antibody, rabbit anti-human VEGFR-1 antibody, rabbit anti-mouse VEGFR-2 antibody, rabbit anti-hypoxia inducible factor- α (HIF1- α) antibody (Abcam Japan), rabbit anti-human VEGF antibody (Abcam Japan), rabbit anti-human PIGF-2 antibody (Abcam Japan), and goat anti-actin antibody (Santa Cruz Biotechnology) at 4°C. After incubation with donkey anti-rabbit HRP-conjugated antibody or donkey anti-goat HRP antibody (dilution 1:2,000; Nacalai Tesque Inc., Kyoto, Japan) for 1 hour, immunoreactive bands were stained by enhanced chemiluminescence Western blot analysis system.

Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNA of Hepa1-6 cell tumor tissues was isolated according to the Isogen method (Nippon Gene, Tokyo, Japan). One microgram of

total RNA was reverse transcribed by the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA) using random hexamer primers according to the manufacturer's instructions. The following TaqMan Gene Expression Assays were purchased: *hgf* (Assay ID Mm 01135193_m1), *plgf* (Assay ID Mm 00435613_m1), *fgf-2* (Assay ID Mm 00433287_m1), *vegfa* (Assay ID Mm 01281449_m1), *angiopoietin (ang)-1* (Assay ID Mm 00456503_m1), *ang-2* (Assay ID Mm 00545822_m1), *tgfa* (Assay ID Mm 000446232_m1), and *pdgfb* (Assay ID Mm 00440677_m1). The levels of PCR products were monitored with a StepOnePlus Real-Time PCR System (Applied Biosystems). The baseline and threshold values were adjusted according to the instructions provided by the manufacturer. The abundance of transcripts was expressed relative to the constitutive expression level

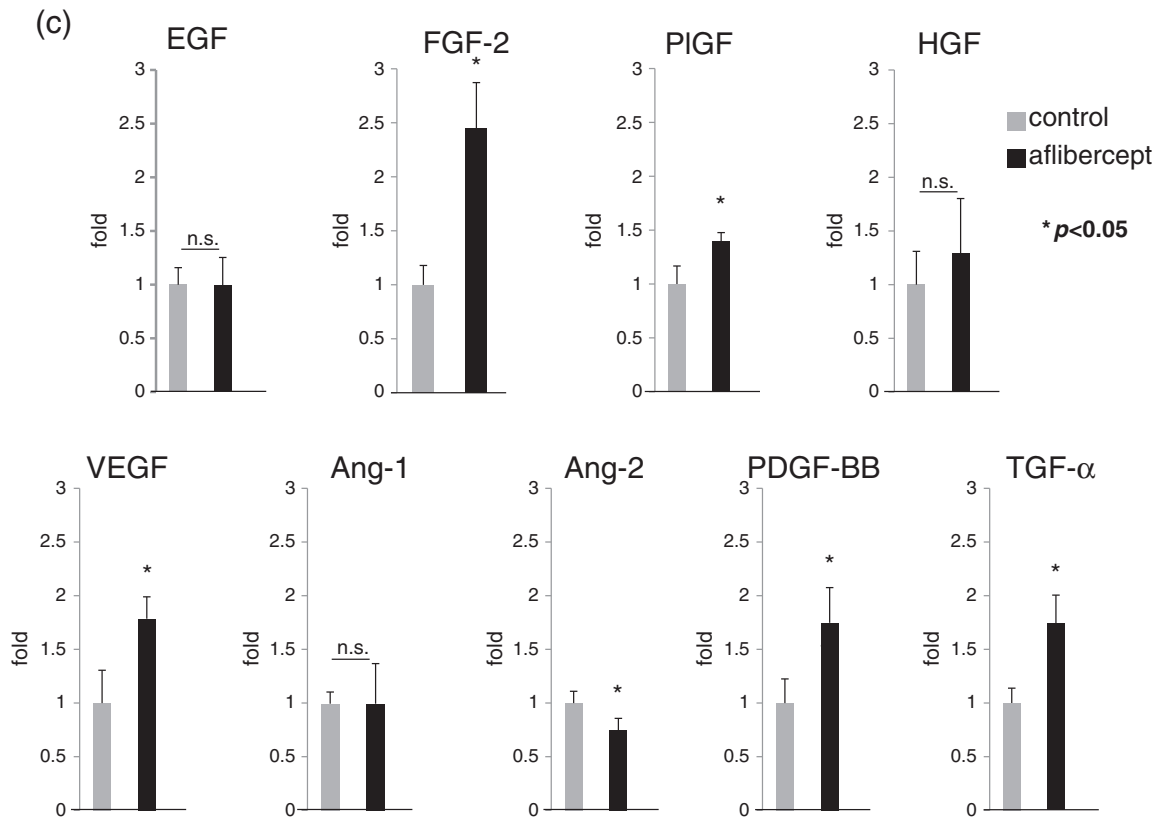


Figure 4. (continued).

of glyceraldehyde-3-phosphate dehydrogenase (gapdh: Assay ID Mm00433859_m1).

Migration of BM-Derived Cells to Tumor Tissues

BM-derived mononuclear cells (5×10^6) of C57BL/6-Tg (act-EGFP) C14-Y01-FM1310sb mice were injected into irradiated C57BL/6 mice through the tail vein. Six weeks later, 5×10^6 Hepa1-6 cells were implanted subcutaneously into the dorsal portions. The tumor-bearing mice were randomly divided into nontreated group, PBS-administered group, and aflibercept-treated (25.0 mg/kg BW) group. Before the application of any treatment, tumors of nontreated mice were fixed with 3.7% (vol/vol) formaldehyde. After 3-week treatment, tumors of PBS- and aflibercept-treated mice were harvested and fixed with 3.7% formaldehyde. Sections of tumor tissues were incubated with FITC-conjugated goat anti-green fluorescent protein (GFP) antibody (dilution 1: 100; Abcam Japan) for 1 hour at room temperature. GFP-positive cells in tumor tissues were counted in 10 blindly selected random fields (z -series, $63 \times$ oil magnification).

Measurement of Serum Alanine Aminotransferase (ALT) Level, BM Functions, and BW of Liver Tumor Model

Leukocyte count, platelet count, hemoglobin (Hb) level, and serum ALT level were measured at the time of sacrifice. BW was evaluated at the time of initial administration and at sacrifice.

Statistical Analysis

All data were expressed as mean \pm SD. Differences between groups were examined for statistical significance using the Mann-Whitney U

test, the Kruskal-Wallis rank test, and the log-rank test. A P value less than .05 denoted the presence of a statistically significant difference.

Results

Effect of Aflibercept for Endothelial Cell and Hepatoma Cell in Cell Proliferation Assay

VEGF at ≥ 100 pM stimulated HUVEC proliferation. However, PIGF did not induce cell proliferation when used at concentrations up to 1 nM. Under 0.2-nM VEGF stimulation, aflibercept dose-dependently suppressed VEGF-induced HUVEC proliferation ($IC_{50} = 34$ pM) up to 1 nM, then reached a plateau. Aflibercept had no effect on proliferation of HUVEC incubated with PIGF and control (without VEGF and PIGF) (Figure 1, A–C). Aflibercept did not suppress the proliferation of hepatoma cell lines (KYN-2, HuH-7, HAK1-B, and Hepa1-6) (data not shown).

Aflibercept Suppresses the Phosphorylation of VEGFR-1 and -2 on Endothelial Cells

VEGF stimulation upregulated the phosphorylation of VEGFR-1 and -2 on HUVEC and hepatoma cells. On the other hand, PIGF stimulation upregulated the phosphorylation of VEGFR-1 only. Aflibercept significantly inhibited the VEGF-stimulated phosphorylation of VEGFR-1 and -2 as well as the PIGF-stimulated phosphorylation of VEGFR-1 but had no effect on the expression of total VEGFR-1 or -2 in the same cells (Figure 1D).

Aflibercept Inhibits Differentiation of BM-Derived Cells to EPCs

BM-derived mononuclear cells expressed VEGFR-1 and -2. The same cells also produced VEGF-A and PIGF (Figure 1E). After 5-day

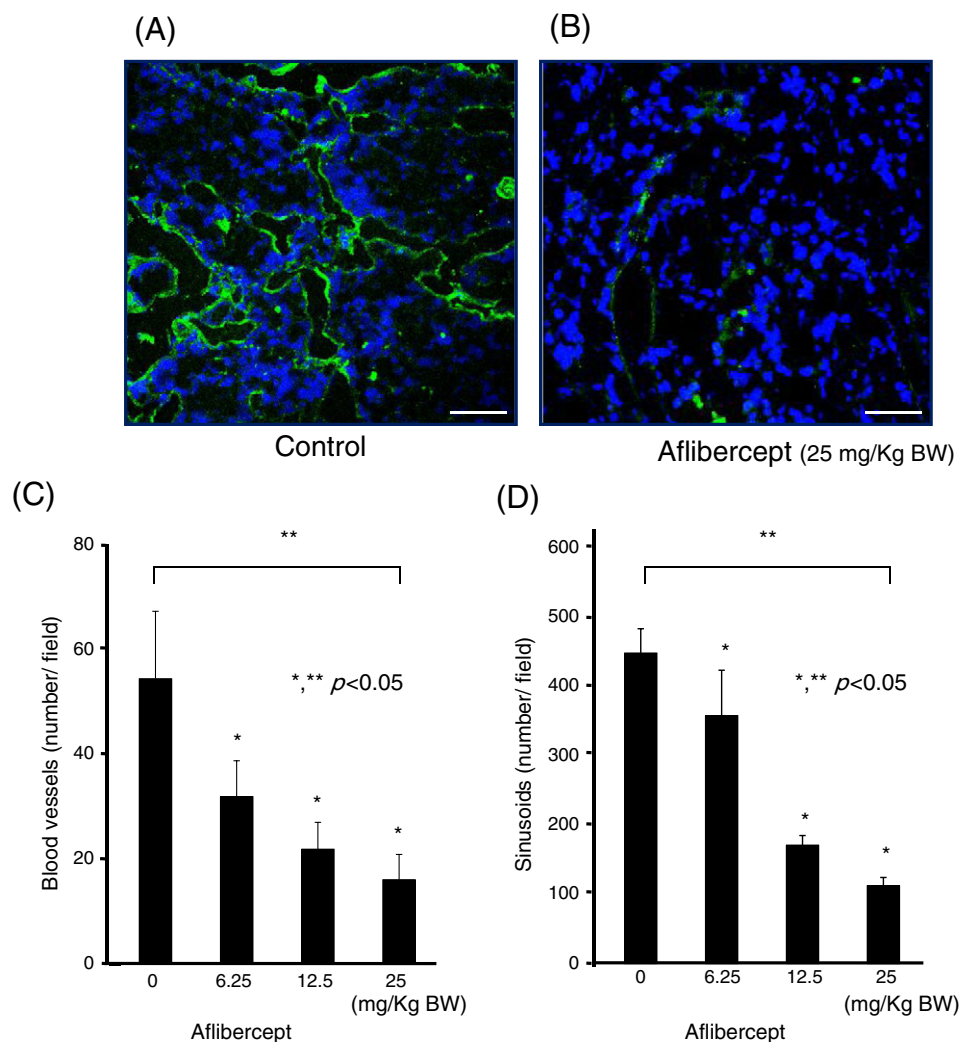


Figure 5. Aflibercept reduces vascularization in tumor tissue and noncancerous liver tissue. (A, B) Immunohistochemical analysis shows decreased number of CD31-positive vessels in tumor tissues of mice treated with aflibercept compared with PBS-treated tumor tissues. (C, D) Density of CD31-positive vessels in tumor and noncancerous liver tissues. Data are mean \pm SD (tumor tissues, 60 fields in 30 sections from each of 6 tumors; noncancerous liver tissue, 25 fields in 18 sections from each of 6 livers). Statistical comparisons with PBS-treated mice. * $P < .05$, by Mann-Whitney U test, ** $P < .05$, by Kruskal-Wallis test.

culture, BM-derived mononuclear cells differentiated and formed colonies of EPCs (7.8 ± 2.3 CFU/well). However, aflibercept significantly inhibited EPC colony formation (3.8 ± 1.1 CFU/well) (Figure 1F).

Anti-tumor Effect of Aflibercept for Hepatoma Cells in Subcutaneous Tumor Model

In HAK1-B-, HuH-7-, and Hepa1-6-xenograft models, there were no significant differences of tumor volume at baseline between PBS-administered group and 25 mg/kg BW aflibercept-treated group. Three weeks later, tumor growth was significantly suppressed in all mice groups treated with 25 mg/kg BW aflibercept compared with the respective PBS-administered groups (Table 1). Changes in tumor volume ratio are depicted in Figure 2, A–C.

In the KYN-2 xenograft model, there were no differences in tumor volume at baseline between the PBS-administered group and aflibercept-treated groups. Three weeks later, aflibercept significantly suppressed tumor growth dose-dependently (Table 2) (Figure 2D). In

another experiment, treatment commenced when the mean tumor volume was >350 mm³. The tumor volume at baseline was 360.5 ± 168.2 mm³. After 12 days of treatment, it decreased to 230.0 ± 141.3 mm³ in the 25 mg/kg BW aflibercept-treated group (Figure 2D). These results indicate that aflibercept significantly suppresses tumor growth of hepatoma cells in a dose-dependent manner and also suppresses the growth of advanced tumors.

Aflibercept Suppresses Tumor Growth of Hepatoma Cells and Serum AFP Levels, and Prolongs Survival of Tumor-Bearing Mice

Although liver tumors were noted in each mouse (Figure 3, A and B), in mice inoculated with KYN-2 cells, the tumor volume was significantly lower in 25 mg/kg BW aflibercept-treated mice than control mice (PBS, 1317 ± 661 ; aflibercept, 114 ± 115 mm³) (Figure 3C).

Serum AFP level was also significantly lower in the aflibercept-treated group than the control (PBS, $31,237 \pm 12,929$; aflibercept, 1422 ± 881 ng/ml).

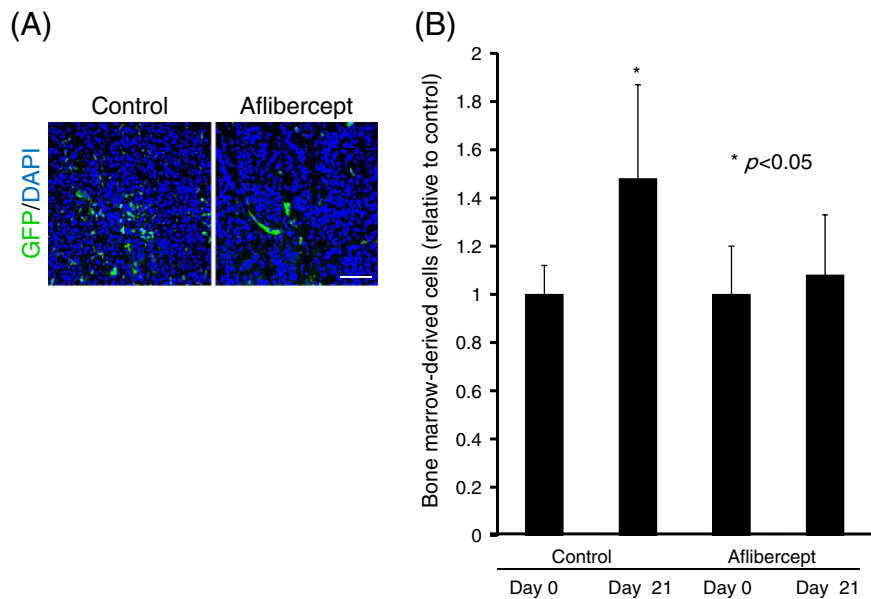


Figure 6. Inhibitory effect of aflibercept on migration of BM-derived cells to tumor tissues. (A) Photographs of representative tumor tissue showing GFP-positive BM-derived cells in each control and aflibercept-treated group on day 21. (B) Number of GFP-positive BM-derived cells per tumor field. Data are mean \pm SD (10 fields in 3 sections from each of 3 tumors per group).

The survival time of aflibercept-treated mice (range, 60–69 days; median survival time, 68 days) was significantly longer than that of PBS-administered group (40–61 days; 52 days) (Figure 3D). In this study, all tumor-bearing mice died of tumor progression.

Aflibercept Suppresses Phosphorylation of VEGFR-1 and -2 and Neovascularization, and Upregulates Expression of HIF1- α and Angiogenic Growth Factors

Treatment with 25 mg/kg BW aflibercept upregulated plasma levels of VEGF (PBS, 0.010 ± 0.008 ; aflibercept, 0.087 ± 0.045 nM) and PlGF (PBS, 0.0084 ± 0.0064 ; aflibercept, 0.266 ± 0.010 nM). In tumor tissues of Hepa1-6 cells, aflibercept significantly suppressed the phosphorylation of VEGFR-1 and -2 and upregulated the expression of HIF1- α , VEGF, and PlGF (Figure 4, A and B). Real-time PCR analysis showed that treatment with 25 mg/kg BW aflibercept significantly upregulated the expression of fibroblast growth factor-2 (FGF-2), PlGF, VEGF, PDGF-BB, and transforming growth factor (TGF)- β mRNAs. It also downregulated the expression of Ang-2 mRNA (Figure 4C).

Quantitative analysis of the number of tumor vessels showed that aflibercept dose-dependently suppressed neovascularization compared with the control (Figure 5, A–C). In nontumor liver tissues, aflibercept also reduced sinusoidal density dose-dependently (Figure 5D).

Peripheral free aflibercept level at sacrifice was 73.0 ± 34.8 , 184 ± 139 , and 454 ± 273.6 nM in 6.25, 12.5, and 25 mg/kg BW aflibercept-treated mice, respectively ($n = 3$).

Aflibercept Inhibits Migration of BM-Derived Mononuclear Cells to Tumor Tissues

GFP-positive BM-derived cells were identified in tumor tissues of Hepa1-6 cells (Figure 6A). The mean number of such cells was 100 ± 12 /high-power field at baseline, but aflibercept significantly suppressed the migration of these cells to tumor tissues (Figure 6B).

Effects of Aflibercept on Leukocyte Count, Hb Levels, Platelet Count, Serum ALT, and BW in Tumor-Bearing Mice

Treatment with aflibercept did not significantly alter leukocyte count, platelet count, and Hb levels compared with the control (Figure 7, A–C). Serum ALT levels and BW were not significantly different in mice free of tumors and in PBS-administered and 25 mg/kg BW aflibercept-treated tumor-bearing mice (Figure 7, D and E).

Discussion

The following were the main findings of the present study: 1) aflibercept significantly suppressed VEGF-induced endothelial cell proliferation, 2) aflibercept dose-dependently suppressed tumor growth, 3) the latter effect was mediated through inhibition of tumor-related neovascularization, and 4) aflibercept did not alter proliferation of hepatoma cells. These results suggest that the anticancer effects of aflibercept in our mouse model are mediated through inhibition of VEGFR signaling-induced tumor neovascularization.

Neovascularization in solid tumors is induced by several mechanisms, such as sprouting, intussusceptions, and/or co-option of local vasculature, and incorporation of BM-derived cells, such as EPCs [1,8]. The possible mechanisms of vascular growth inhibition of aflibercept are the following: 1) direct suppression of endothelial cell proliferation, 2) inhibition of differentiation of BM cells to EPC, and 3) inhibition of BM cell migration to tumor tissues. VEGFR-1 binds to VEGF with affinity of approximately 10 times that of VEGFR-2, but its signal-transducing properties are extremely weak [30]. Deletion of VEGFR-1 in the mouse embryo is lethal because of vascular overgrowth and disorganization induced by upregulation of VEGFR-2 signaling [31], whereas loss of the tyrosine kinase domain of VEGFR-1 alone produces a nearly healthy phenotype with normal vasculature [32]. In the present study, PlGF did not stimulate endothelial cell proliferation as reported in the previous study [33]. These findings imply that although VEGF and PlGF phosphorylate

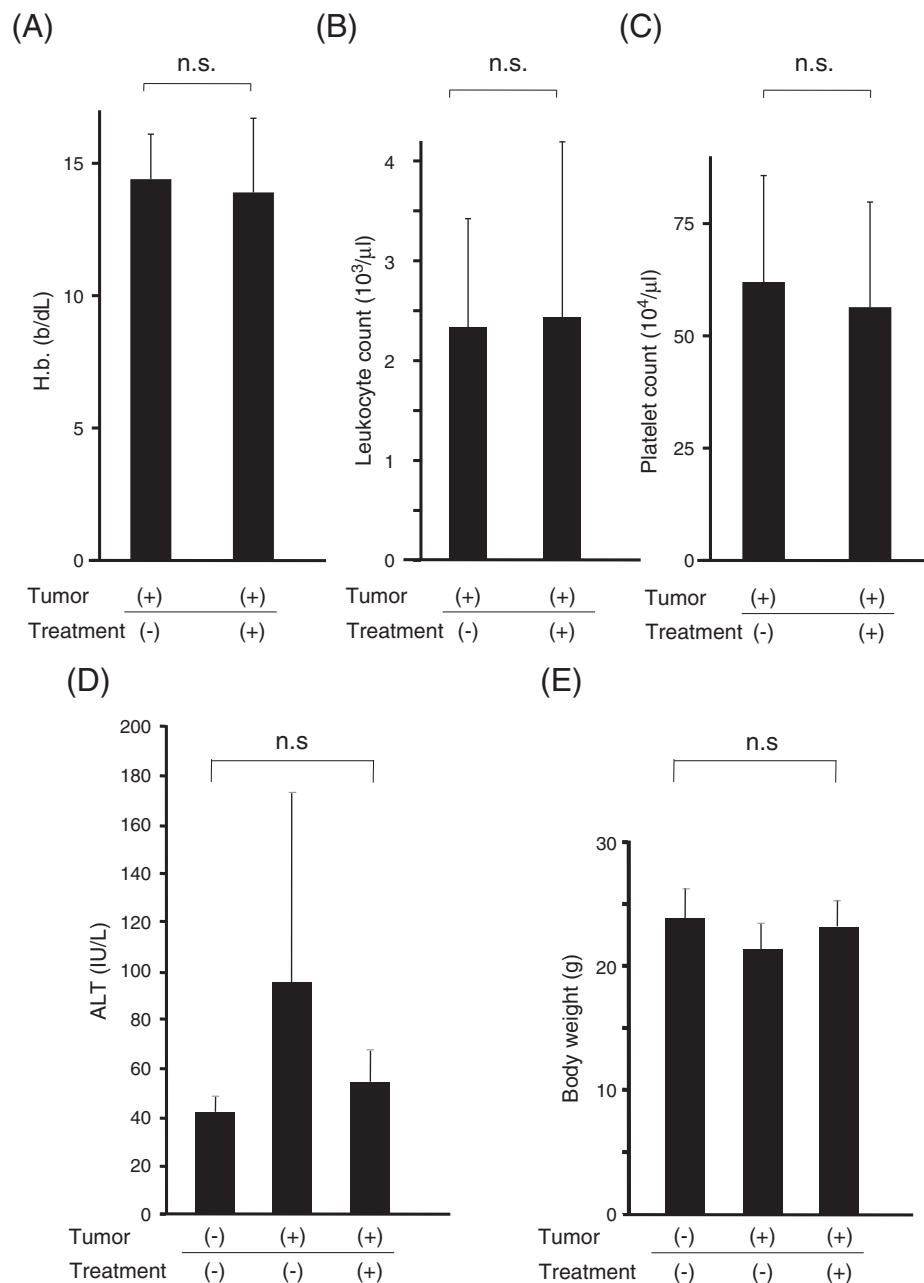


Figure 7. Lack of side effects of aflibercept on BM function, serum ALT level, and BW. (A) Hemoglobin, (B) leukocyte count, (C) platelet count, (D) serum ALT, (E) BW.

VEGFR-1, VEGFR-2 signaling by VEGF mainly promotes endothelial cell proliferation [30]. VEGF (0.2 nM)-induced proliferation of HUVEC was completely inhibited by 1 nM of aflibercept in our *in vitro* study. Because aflibercept binds to VEGF at a rate of 1:1 [19], the concentration of peripheral free aflibercept in aflibercept-treated mice (6.25 mg/kg BW) was sufficient to induce complete suppression of endothelial cell proliferation. If this is true, why were the suppressive effects of aflibercept on tumor neovascularization and tumor growth dose-dependent? Two possibilities exist. The first is the difference in concentrations of aflibercept and VEGF in peripheral blood compared with those in tumor tissues. The other is that aflibercept does not completely bind to VEGF in the presence of even sufficient concentrations of aflibercept.

Circulating EPC levels are significantly elevated in patients with advanced HCC compared with patients with early resectable HCC, patients with liver cirrhosis, and healthy controls [34]. We also reported previously that EPCs injected into the circulation migrated to tumor tissues of hepatoma cells and participated in neovascularization [35]. Whyte et al. reported that VEGF-A was strongly induced by Notch [36]. Coordinated Jagged-1-mediated Notch signaling and VEGF signaling direct cell differentiation to EPCs in the BM [37]. In our *in vitro* study, the addition of aflibercept to media containing VEGF inhibited the differentiation of BM-derived cells to EPCs. Aflibercept binds to VEGF, leading to inhibition of VEGF signaling in BM-derived cells, with subsequent suppression of BM cells differentiation to EPCs.

In the present study, BM-derived cells migrated to tumor tissues. Previous studies showed that the proportions of tumor-associated macrophages (TAM) and cancer-associated fibroblasts (CAF) were BM derived [38,39]. CAF, TAM, and other BM-derived cells participate in tumor neovascularization [8]. CAF and TAM secrete numerous growth factors, including VEGF, and participate in neovascularization, EPC recruitment, and tumor cell survival [40–42]. In the present study, BM-derived cells expressed VEGFR-1 and -2 and also produced PlGF and VEGF. PlGF produced in tumor tissues participates in the recruitment of VEGFR-1⁺ angiocompetent BM-derived cells, which mostly indirectly promote neovascularization by secreting angiogenic factors [43]. In comparison, VEGF mainly recruits VEGFR-2⁺ EPCs from the BM to sites of tumor neovascularization [5]. In our *in vivo* study, serum aflibercept level was high enough to bind to VEGF and PlGF in the circulation. Thus, it seems that aflibercept binds to VEGF and PlGF and then inhibits the mobilization and recruitment of BM-derived cells to tumor tissues.

Our results also showed that aflibercept upregulated HIF1- α expression in tumor tissues, in addition to the expression of VEGF, PlGF, FGF-2, PDGF-BB, and TGF- α . Aflibercept-induced suppression of neovascularization in tumor tissues should result in reduced local blood supply, leading to a worsened state of tumor tissue hypoxia. Hypoxic condition triggers upregulation of angiogenic factors through upregulation of HIF1- α . Upregulated FGF-2, PDGF-BB, and TGF- α might somewhat induce neovascularization. Because VEGF is a major mediator of tumor neovascularization [8] and serum aflibercept concentration was high enough to completely bind to VEGF and PlGF in tumor-bearing mice, strong inhibition of VEGF and PlGF signaling seems to suppress tumor neovascularization as a whole. Aflibercept downregulated Ang-2 expression. In the absence of VEGF, Ang-2 is known to promote endothelial cell apoptosis and vessel regression [44,45]. Based on the inhibitory effect of aflibercept on VEGF signaling, tumor tissue might downregulate Ang-2 expression to resist the antiangiogenic effect of aflibercept.

In the orthotopic liver tumor xenograft model, which mirrors the clinical course of hepatoma more accurately than the subcutaneous xenograft model, aflibercept did not only induce the suppression of tumor growth without severe side effects but also improved survival. In addition, aflibercept also suppressed the growth of large tumors. These findings suggest that aflibercept is potentially useful for patients with advanced HCC. However, in this study, we used xenograft HCC models and thus could not evaluate the effects of liver cirrhosis on treatment outcome. In fact, the results showed that aflibercept reduced sinusoidal density in the noncancerous liver tissue. In our mouse hepatoma model, noncancerous liver tissue was normal. Thus, aflibercept had no effect on liver function even though sinusoidal density and sinusoidal blood flow were diminished. Further investigation using HCC model with liver cirrhosis is required before any clinical application of aflibercept is possible.

In recent clinical studies on the use of aflibercept in patients with brain tumors, the most common side effects that resulted in discontinuation of aflibercept were fatigue, thromboembolic complications, wound healing complications, and central nervous system ischemia [46]. Coleman et al. reported that the side effects associated with aflibercept included grade 1 and 2 hypertension and grade 2 proteinuria in patients with ovarian cancer and fallopian tube cancer. We did not experience any severe adverse events in tumor-bearing nude mice [47]. These differences could represent species differences.

Aflibercept is not a multisignaling inhibitor, like sorafenib, but only inhibits VEGFR-1 and -2 signaling. Accordingly, it might have less severe side effects compared with other antiangiogenic multimolecular targeting agents.

In conclusion, we have demonstrated in the present study that the antitumor effects of aflibercept in mouse hepatoma model are based on inhibition of neovascularization through the suppression of endothelial cell proliferation, BM cell differentiation to EPCs, and BM-derived cell migration to tumor tissues.

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