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A Radicicol Derivative, KF58333, Inhibits Expression of Hypoxia-inducible Factor-1α and Vascular Endothelial Growth Factor, Angiogenesis and Growth of Human Breast Cancer Xenografts

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A novel oxime derivative of radicicol, KF58333, binds to the heat shock protein 90 (Hsp90) and destabilizes its associated signaling molecules. These effects play a critical role in the growth inhibition of tumor cells. To further investigate the effects of this agent, it was administered to two human breast cancer cell lines, KPL-1 and KPL-4, both *in vitro* and *in vivo*. KF58333 dose-dependently inhibited the growth and vascular endothelial growth factor (VEGF) secretion, concomitantly with a decrease in VEGF mRNA expression, in each cell line. This agent also suppressed the increase of VEGF secretion and expression induced by hypoxia (1% O_2). Intravenous injections of this agent into nude mice bearing either KPL-1 or KPL-4 xenografts significantly inhibited the tumor growth associated with a decrease in the Ki67 labeling index and microvascular area and an increase in apoptosis and the necrotic area. These findings indicate that the antitumor activity of this radicicol derivative may be partly mediated by decreasing VEGF secretion from tumor cells and inhibiting tumor angiogenesis. To explore the action mechanisms of the anti-angiogenic effect, the expression level of hypoxia-inducible factor (HIF)-1α was investigated. KF58333 provided a significant decrease in the HIF-1α protein expression under both normoxic and hypoxic conditions. In contrast, the mRNA expression of HIF-1α was not decreased by this agent. It is suggested that the post-transcriptional down-regulation of HIF-1α expression by this agent may result in a decrease of VEGF expression and tumor angiogenesis.

Key words: Radicicol — Hypoxia-inducible factor-1 — Vascular endothelial growth factor — Breast cancer — Angiogenesis

Heat shock protein (Hsp) 90, a molecular chaperone, interacts with and stabilizes a variety of signaling molecules, such as tyrosine kinases, serine/threonine kinases, mutated *p53* gene product and nuclear transcription factors.[1)] These signaling molecules play a role in the development and progression of human cancer. It has been reported that Hsp90 is constitutively expressed at a higher level in cancer cells than in their normal counterparts.[2)] Three antibiotics, geldanamycin, radicicol and novobiocin, have been reported to bind to Hsp90 and destabilize Hsp90-associated signaling molecules.[3–5)] Geldanamycin and radicicol have been reported to exhibit anticancer activities.[6)] Moreover, a geldanamycin derivative, 17-allylaminogeldanamycin, has been entered into a clinical trial in cancer patients.[7)] Our previous studies have indicated that novel oxime derivatives of radicicol, KF25706 and KF58333, bind to Hsp90, deplete Hsp90-associated signaling molecules and exhibit antitumor activity both *in vitro* and *in vivo*.[8–10)] It has been suggested that KF58333 and other Hsp90 inhibitors cause the down-regulation of cyclin-dependent kinases CDK4 and CDK6, up-regulation of cell cycle-dependent kinase inhibitor p27 (Kip1), G1 arrest, induction of apoptosis and cell differentiation and growth inhibition in human cancer cells.[9–13)]

Angiogenesis plays critical roles in tumor growth and metastasis.[14)] Many angiogenesis inhibitors have been developed, and some are under clinical trials against various malignancies.[15)] Radicicol has been reported to inhibit *in vivo* angiogenesis.[16)] To clarify if a radicicol derivative, KF58333, has anti-angiogenic activity, it was administered to two human breast cancer cell lines, KPL-1 and KPL-4.[17, 18)] The findings of the present study show that this agent significantly inhibits vascular endothelial growth factor (VEGF) expression *in vitro* and angiogenesis *in vivo*. VEGF expression is reported to be partly regulated by the hypoxia-inducible factor (HIF)-1 signaling pathway.[19)] A critical subunit of HIF-1, HIF-1α, has been reported to be overexpressed in human cancers.[20)] In addition, HIF-1α has been reported to interact with Hsp90 and to transactivate various target genes, such as VEGF.[21)]

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These findings prompted us to investigate the effects of this radicicol derivative on the regulation of HIF-1α expression in cancer cells.

MATERIALS AND METHODS

Drugs Radicicol was produced by fermentation, and its derivative, KF58333, was chemically synthesized according to methods to be published elsewhere (manuscript in preparation).

Cell lines Both KPL-1 and KPL-4 human breast cancer cell lines were established in our laboratory from the pleural effusion of two different patients with recurrent breast cancer. The characteristics of these cell lines was reported elsewhere.[17, 18] In brief, the KPL-1 cell line expresses a high level of estrogen receptors and a low level of HER2. The KPL-4 cell line expresses a low level of estrogen receptors and a high level of HER2. This cell line secretes interleukin-6, and its transplanted tumors induce cachexia. Subcutaneous injections of both cell lines produce rapid-growing tumors in female intact nude mice. Both cell lines express high levels of VEGF family members.[22]

***In vitro* experiments** To reduce endogenous estrogenic activity, phenol red-free RPMI-1640 medium (Gibco BRL, Bethesda, MD) supplemented with 2% dextran-coated charcoal-stripped fetal bovine serum (FBS, Hyclone, Logan, UT) (estrogen-deprived medium) was used for *in vitro* experiments.[23] Approximately 1×10^5 KPL-1 or KPL-4 cells per well were inoculated into 12-well plates (Costar Corning, Inc., Corning, NY) and cultured in Dulbecco's modified Eagle's medium supplemented with 5% FBS for 2 days. Then, the cells were washed twice with phosphate-buffered saline (PBS) and cultured for 2 days in the estrogen-deprived medium with 10−1000 n*M* KF58333 or vehicle under normoxia or a hypoxic condition (1% O_2) using a CO_2/Multi-Gas Incubator APM-30D (Astec, Fukuoka). The medium of the last 2-day incubation was collected for measuring VEGF concentration. After cell dispersion with 0.05% trypsin (Difco, Detroit, MI) and 0.02% EDTA in PBS for 10 min, the cell numbers were measured with a Coulter counter (Coulter Electronics, Ltd., Harpenden, UK). Triplicate wells were treated in each experiment. To investigate the effects of KF58333 on the VEGF and HIF-1α mRNA expression levels in KPL-1 or KPL-4 cells, semi-confluent cells in 6-well plates (Costar Corning, Inc.) were incubated with the estrogen-deprived medium plus 1 μM KF58333 or vehicle under normoxia or the hypoxic condition for 24 h. Collected cells were stored at −80°C until use.

***In vivo* experiments** KPL-1 or KPL-4 xenografts were minced with scissors in Hank's balanced solution, and approximately 27 mm^3 pieces were inoculated using a trocar into the flank of 9-week-old female BALB/c *nu*/*nu* mice (Nippon Clea Co., Tokyo). Nude mice were randomly divided into two groups (control and KF58333-treated groups, $n=16$ each) 24 days after the inoculation (day 0). The sizes of the KPL-1 and KPL-4 transplanted tumors on day 0 were 157.2±38.6 and 156.8±46.3 mm^3 (mean±SD), respectively. KF58333 (50 mg/kg, daily) or saline was intravenously injected for 5 days from day 0 to day 4. For the evaluation of antitumor activity, the tumor volume was calculated using the following formula:

$$\text{Tumor volume } (mm^3)=(\text{Length}\times\text{Width}^2)/2$$

Drug efficacy was expressed as the ratio of the mean V/V_0 value to that of the control group (T/C ratio), where V is the tumor volume at the day of evaluation and V_0 is the tumor volume at the day of the initial treatment (day 0) with the drug. On days 4 and 7, four mice in each group were sacrificed, and the other mice were sacrificed on day 18. The resected tumor samples were weighed and cut into halves. A half of the sample was fixed with 5% formalin in PBS, and the other half was dipped into liquid nitrogen and stored at −80°C until use.

Ki67 labeling, angiogenesis, apoptosis and necrosis in xenografts Paraffin sections of tumor samples were dewaxed with xylene, hydrated with PBS, treated with hydrogen and then processed using the immunoperoxidase procedure. Monoclonal anti-Ki67 mouse antibody (Immunotech, Marseille, France) and polyclonal anti-laminin rabbit antibody (CHEMICON, Temecula, CA) were used as the first antibodies. The reaction was visualized by streptavidin-biotin (Nichirei, Tokyo) techniques. The specimens were initially evaluated with a light microscope at a magnification of ×10 to permit localization of malignant areas and assessment of the heterogeneity of immunostaining within the tumor components. Then, color microphotographs of 5 representative areas were taken at a magnification of ×400. Ki67 labeling was assessed by determining the percentage of tumor cells stained.

To evaluate tumor angiogenesis, tubular areas, which were surrounded by endothelial cells positively stained for laminin and which contained red blood cells, were recognized as vascular structures. The vascular area was measured by two-dimensional morphometry using a point counting system.[24] The number of dots inside the structures per field (approximately 0.43 mm^2) was counted using a transparent plastic grid to evaluate the vascular area of each tumor sample.[25]

DNA breaks were detected by nick end labeling using an ApopTag *in situ* apoptosis detection kit (Oncor, Inc., Gaithersburg, MD) as described elsewhere.[26] The areas of most intense staining, exclusive of necrotic areas, were identified in each sample, and the percentage of cells showing positive staining was calculated.

Since the histologic examination of treated tumors showed massive necrosis inside the tumors, the percentage of the necrotic area inside the xenografts was compared

among the control and treated groups. To quantify the necrotic area, the central section of each tumor was stained using the conventional hematoxylin-eosin method and subjected to computer-assisted image analysis using an IBAS system (Zeiss-Kontron, Eching, Germany) as described elsewhere.[27] Briefly, the image of each whole tumor was projected into an Ikegami ITC-370M camera (Ikegami, Tokyo) and fed into the image analysis system. After shading correction, contrast enhancement, median filtering and interactive editing, a binary image was created based on gray-value-based thresholding to discriminate the necrotic area from the area showing viable tumor cells.

VEGF secretion The VEGF concentrations in the culture medium were measured with an enzyme-linked immunosorbent assay kit (Otsuka Assay Laboratory, Tokushima).[25] Briefly, a mouse anti-human VEGF monoclonal antibody was used as the first antibody and a rabbit anti-human VEGF polyclonal antibody labeled with alkaline phosphatase as the second antibody. After removal of the unbound second antibody, horseradish peroxidase solution was added. As the standard, 20–10 000 pg/ml of human recombinant VEGF was used. The minimal detectable concentration of VEGF was 0.2 pg/ml. Since the VEGF concentration in the fresh medium was undetectable and increased linearly for at least 2 days, VEGF secretion into the medium was defined as follows:

VEGF secretion per cell per 48 h
=Concentration of VEGF×Volume of medium
/Mean cell number

mRNA expression levels of VEGF and HIF-1α Total cellular RNA was extracted with a TRIzol RNA extraction kit (GIBCO BRL Life Technologies, Gaithersburg, MD). One microgram of total RNA and 1 μM Oligo(dT) 18 primer in 12.5 μl of diethyl pyrocarbonate-treated water were heated to 70°C for 2 min followed by cooling on ice for 1 min. cDNA synthesis was initiated with 200 units of recombinant Molony murine leukemia virus reverse transcriptase (CLONTECH Laboratories, Inc., Palo Alto, CA), and the reaction was allowed to proceed at 42°C for 1 h. The reaction was terminated by heating at 94°C for 5 min. cDNA was dissolved to a final volume of 100 μl by adding 80 μl of diethyl pyrocarbonate-treated water and then frozen at −20°C until use. Oligonucleotide primers for the reverse transcription-polymerase chain reaction (RT-PCR) were designed using a published sequence of each target gene and synthesized by the solid-phase triester method. The primers and conditions used and the expected sizes from the reported cDNA sequence are shown in Table I. To amplify both the internal control gene (*β-actin*) and one of the target genes in a single reaction, multiplex PCR was carried out. The ratios of the primer-sets between the target gene and the control gene are also shown in Table I. These ratios and the numbers of PCR cycles were determined so as to amplify both products logarithmically. Each PCR reaction contained 1/100 cDNA, the indicated concentrations of primers of each target gene and the control gene, 200 μM deoxynucleotide triphosphates, 10 m*M* Tris-HCl (pH 8.8), 2.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.08% Nonident P-40, and 1 unit of recombinant *Thermus aquaticus* DNA polymerase (MBI Fermentas, Vilnius, Lithuania) in a final volume of 20 μl. After an initial denaturation at 94°C for 4 min, various cycles of denaturation (at 94°C for 15 s), annealing (at various temperatures as shown in Table I for 15 s), and extension (at 72°C for 30 s) were performed on a DNA Thermal Cycler 2400 (PC-960G Microplate Gradient Thermal Cycler, Mortlake, Australia). The final extension was performed for 5 min. After visualization of the PCR products on 1.2% agarose gel stained with ethidium bromide, gel images were obtained using the FAS-II UV-image analyzer (TOYOBO Co., Ltd., Tokyo), and the densities of the products were quantified using Quantity One version 2.5 (PDI, Inc., Huntington Station, NY). The gel images are shown in the inverted presentation. The relative expression levels were calculated as the density of the product of the respective target genes divided by that of the control gene.[28] Reproducibility was confirmed in at least two separate experiments.

Western blotting Anti-HIF-1α monoclonal antibody (clone 54), anti-Raf-1 (C-12) rabbit polyclonal antibody and anti-Erk2 monoclonal antibody (clone 1B3B9) were obtained from Transduction Laboratories (Lexington, KY), Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively.

KPL-4 or KPL-1 cells, plated in 6-well plates, were used for each sample. The indicated concentrations of

Table I. Primer Sequences, Conditions and Product Sizes for the Multiplex RT-PCR

Target genes	Primer sequences		Annealing temperature (°C)	Primer ratio	No. of PCR cycles	Expected size (bp)
	Forward	Reverse				
VEGF-A	5′-gcagaatcatcacgaagtgg-3′	5′-gcatggtgatgttggactcc-3′	58	1.0/2.0	38	212
HIF-1α	5′-tcatccatgtgaccatgagg-3′	5′-ttcatatccaggctgtgtcg-3′	56	0.5/2.0	37	345
β-actin	5′-tgacggggtcacccacactgtgcccatcta-3′	5′-ctagaagcatttgcggtggacgatggaggg-3′	NA[a]	NA	NA	661

a) Not assessable.

KF58333 were added to each well, and the cells were cultured for 48 h under normoxia or the hypoxic condition. Then, the cells were washed once with PBS without calcium (ICN Biochemicals, Aurora, OH), and were lysed by the addition of 50 or 100 μl per well of ice-cold lysis buffer (50 m*M* Hepes-NaOH [pH 7.4], 250 m*M* NaCl, 1 m*M* EDTA, 1% Nonidet P-40, 1 m*M* dithiothreitol, 1 m*M* phenylmethanesulfonyl fluoride [PMSF], 5 μg/ml leupeptin, 2 m*M* Na_3VO_4, 1 m*M* NaF, 10 m*M* β-glycerophosphate). The cells were lysed for 30 min on ice and clarified by centrifugation at 19 000*g* for 8 min. The cell lysate of the HeLa cervical cancer cell line treated with 150 μM $CoCl_2$ was used as a positive control for HIF-1α protein. Whole cell lysate was heated in Laemmli gel loading buffer for 5 min at 95°C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein was transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Tokyo) and immunoblotted with appropriate primary antibodies. For detection, the blots were incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham Life Sciences, Buckinghamshire, England), and developed using the enhanced chemiluminescence detection system (Amersham), according to the instructions of the manufacturer.[8)]

Statistical analysis All values are expressed as the mean±SE. ANOVA analysis with a StatView computer software (ATMS Co., Tokyo) was used to compare the differences in the cell number, secretion of VEGF, Ki67 labeling index, microvascular density, apoptotic index and the area of necrosis between two groups. A two-sided *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Antitumor activity *in vitro* and *in vivo* KF58333 (10–1000 n*M*) dose-dependently inhibited the growth of either KPL-1 or KPL-4 cells *in vitro* under both normoxia and hypoxia (Fig. 1). The 50%-inhibitory concentration under normoxia was approximately 100 n*M* for either cell line in this assay. KPL-4 cells appeared to be more sensitive than

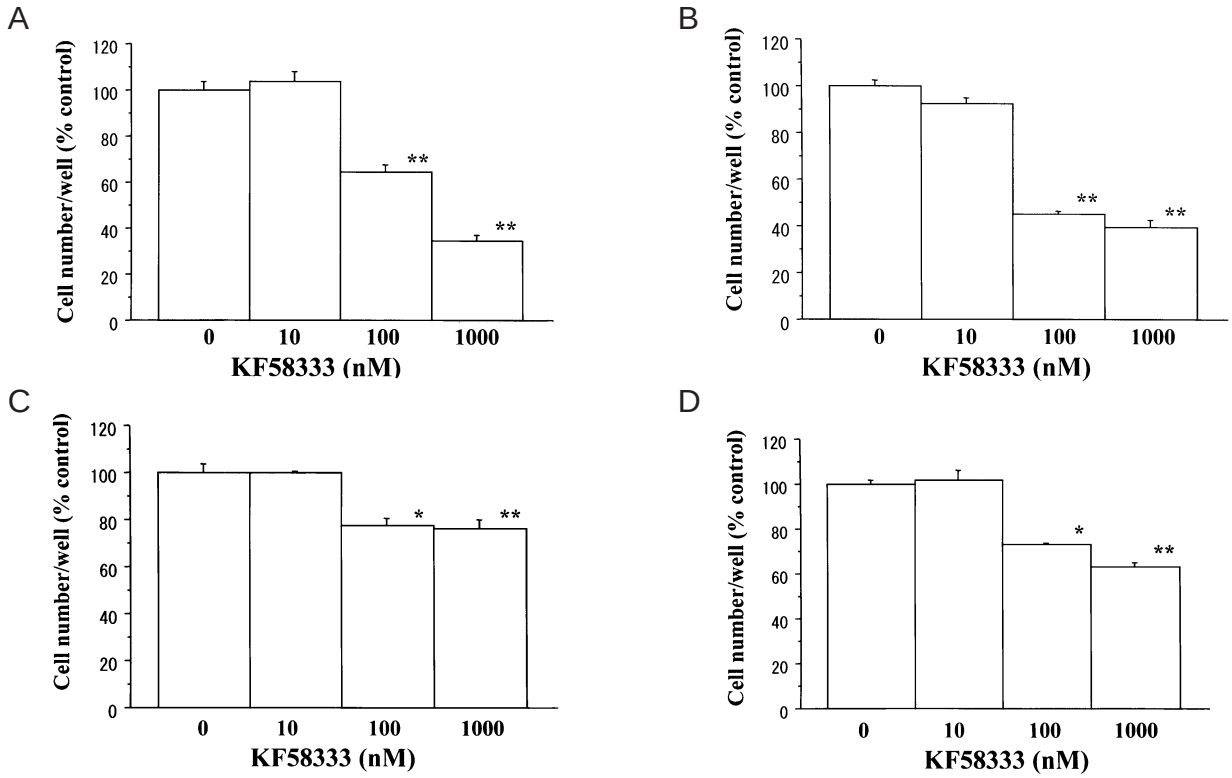

Fig. 1. Growth inhibitory effects of KF58333 on KPL-1 and KPL-4 breast cancer cells *in vitro* under normoxia and hypoxia. KPL-1 (A) or KPL-4 (B) cells were treated with 10–1000 n*M* KF58333 for 2 days under normoxia. Both cells were also treated with 10–1000 n*M* KF58333 for 2 days under hypoxia (1% O_2) using a CO_2/Multi-Gas Incubator (C (KPL-1) and D (KPL-4)). The number of cells per well was measured with a Coulter counter. The values are the means±SE. *, $P<0.05$; **, $P<0.01$ in comparison with controls.

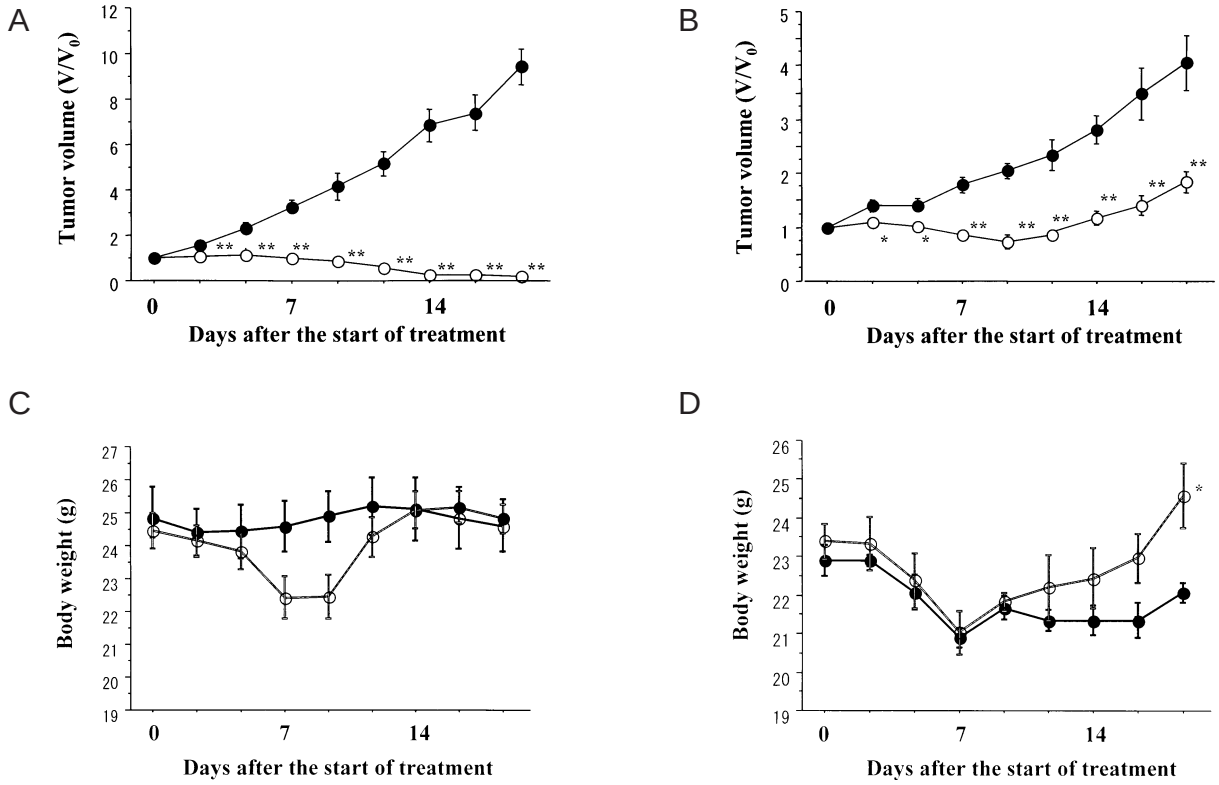

Fig. 2. Growth-inhibitory effects of KF58333 on KPL-1 and KPL-4 xenografts. Pieces of KPL-1 or KPL-4 breast cancer xenografts were inoculated s.c. into female athymic nude mice on day −24. KF58333 (50 mg/kg, daily) or saline (control) was intravenously injected into mice bearing KPL-1 (A) or KPL-4 (B) xenografts for 5 days from day 0 to day 4. The body weights of mice bearing KPL-1 (C) and KPL-4 (D) xenografts were monitored in the same experiment. The values are the means ±SE. Control, ●; KF58333-treated, ○. *, $P<0.05$; **, $P<0.01$ in comparison with controls.

Table II. Effects of KF58333 on the Ki67-labeling Index, Microvascular Area, Apoptotic Index and Necrotic Area in KPL-1 and KPL-4 Human Breast Cancer Xenografts

	Control (n=4 each)	KF58333-treated (n=4 each)
KPL-1 xenografts		
Ki67-labeling index[a)]	89.5±1.9	42.5±8.2**
Microvascular area[b)]	15.8±1.3	1.8±2.9**
Apoptotic index[c)]	2.0±0.6	5.3±0.8**
Necrotic area[d)]	10.9±3.6	83.3±12.4**
KPL-4 xenografts		
Ki67-labeling index	83.5±6.0	74.0±4.3*
Microvascular area	20.5±9.7	2.0±2.8**
Apoptotic index	3.2±0.6	5.6±0.3**
Necrotic area	14.7±3.4	57.2±6.9**

a) Percentages of Ki67-positive tumor cells.
b) Number of dots inside vascular structures per field.
c) Percentages of apoptotic tumor cells.
d) Percentages of necrotic area calculated by computer-assisted image analysis.
*, $P<0.05$; **, $P<0.01$ in comparison with controls.

KPL-1 cells under both normoxia and hypoxia. In addition, intravenous injections of KF58333 (50 mg/kg, daily) significantly inhibited the growth of KPL-1 xenografts in nude mice (Fig. 2A). The minimal T/C ratio was 0.02 on day 18. This agent also significantly inhibited the growth of KPL-4 xenografts (Fig. 2B). The minimal T/C ratio was 0.36 on day 9. To investigate the toxicity of KF58333, mouse body weight was monitored. This agent did not significantly reduce the body weight of mice bearing either KPL-1 or KPL-4 xenografts (Fig. 2, C and D). The body weight of mice bearing KPL-4 xenografts on day 18 was significantly higher than that of the control mice. This is probably because the reduction in tumor volumes of KPL-4 xenografts decreased the anti-cachectic effect of interleukin-6 secreted by KPL-4 cells.[18)]

Effects on the Ki67-labeling index, angiogenesis, apoptosis and necrosis in breast cancer xenografts Tumor samples obtained on day 7 were subjected to this experiment. KF58333 significantly decreased the Ki67-labeling index and microvascular area, and increased apoptotic

tumor cells and the necrotic area inside tumors in either KPL-1 or KPL-4 xenografts (Table II). In particular, this agent drastically decreased the Ki67-labeling index (T/C=0.47) and increased the necrotic area (T/C=7.64) in KPL-1 xenografts, probably because it drastically inhibited the growth of KPL-1 xenografts.

Effects on the expression level of VEGF under normoxia or hypoxia The expression level of VEGF mRNA was decreased by 1 μM KF58333 in either KPL-1 or KPL-4 cells under both normoxia and hypoxia (Fig. 3A). The T/C ratios of the relative expression levels of VEGF were 0.27 in KPL-1 cells and 0.30 in KPL-4 cells under normoxia and 0.52 in KPL-1 cells and 0.61 in KPL-4 cells under hypoxia, respectively. In addition, changes in VEGF secretion from these cell lines were investigated after treatment with 10–1000 nM KF58333 for 48 h. This agent dose-dependently decreased VEGF secretion from either KPL-1 or KPL-4 cells under both normoxia and hypoxia (Fig. 3, B–E).

Effects on the expression level of HIF-1α Since a

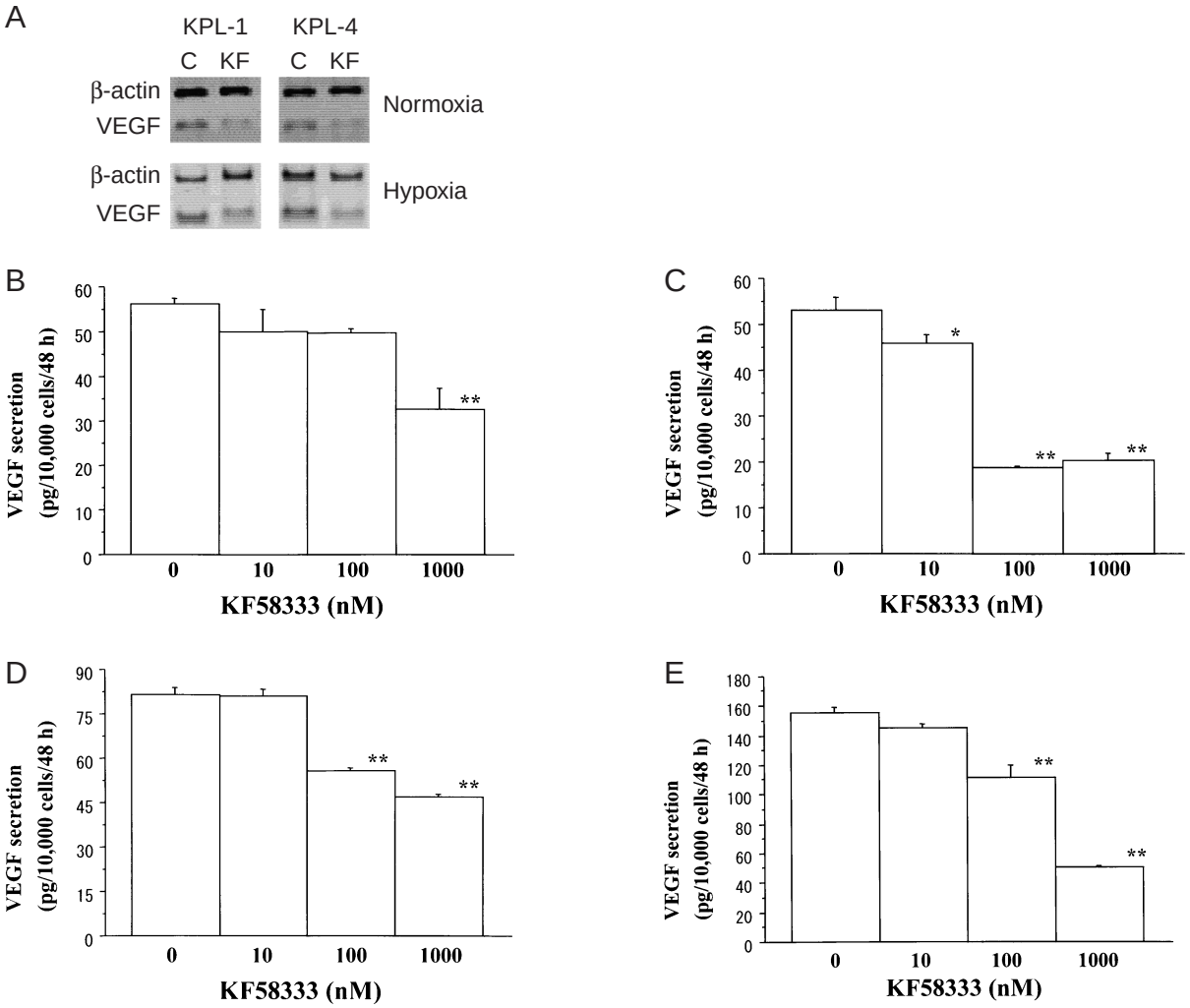

Fig. 3. Suppression of VEGF expression by KF58333 in KPL-1 and KPL-4 breast cancer cells. (A) VEGF mRNA expression levels. KPL-1 or KPL-4 cells were treated with 1 μM KF58333 or vehicle (control) for 24 h under normoxia or hypoxia. Total RNA extracted from the collected cells was subjected to the multiplex RT-PCR as described in "Materials and Methods." Representative gel images are shown in inverted presentation. C, control; KF, KF58333-treated. (B-E) VEGF secretion into medium. KPL-1 or KPL-4 cells were treated with 1 μM KF58333 or vehicle (control) for 48 h. VEGF concentrations in collected medium were measured with an enzyme-linked immunosorbent assay kit. VEGF secretion was calculated as described in "Materials and Methods." (B), KF58333-treated KPL-1 cells under normoxia; (C), KF58333-treated KPL-4 cells under normoxia; (D), KF58333-treated KPL-1 cells under hypoxia; (E), KF58333-treated KPL-4 cells under hypoxia. The values are the means±SE. *, $P<0.05$; **, $P<0.01$ in comparison with controls.

decrease in VEGF expression by KF58333 might be mediated through the HIF-1 signaling pathway, changes in the HIF-1α protein expression after the treatment with 0.01 or 1 *μM* KF58333 for 48 h under the normoxic or hypoxic condition were studied by western blotting. The protein expression level of one of the client proteins of Hsp90, Raf-1, was significantly decreased and that of one of the non-client proteins of Hsp90, Erk2, was not changed by the treatment. KF58333 (1 *μM*) significantly decreased the HIF-1α protein expression in either KPL-1 or KPL-4 cells under both normoxia and hypoxia (Fig. 4A). In contrast, the treatment with 1 *μM* KF58333 for 24 h did not change the relative mRNA expression level of HIF-1α in either KPL-1 or KPL-4 cells (Fig. 4B).

DISCUSSION

Angiogenesis is one of the essential requirements for cancer growth and progression. Disruption of angiogenic processes is a promising strategy against cancer. Several anti-angiogenic agents are under phase trials for potential clinical use.[10, 11] On the other hand, a humanized anti-HER2 monoclonal antibody, trastuzumab, has been approved for use in patients with HER-2 overexpressing breast cancer. This antibody disturbs the HER2 signaling pathway and induces tumor regression.[29, 30] In addition, several tyrosine kinase inhibitors have been developed as promising anticancer agents.[31] Disruption of the growth-signaling pathway is also effective for controlling cancer growth.

Our previous studies have shown that a radicicol derivative, KF58333, more efficiently depletes several signaling molecules (client proteins), such as HER2, Raf-1, Akt, V-Src and mutant p53, and exhibit more anti-cancer activity both *in vitro* and *in vivo* than the parent agent, radicicol.[9, 10] Disruption of the interaction among Hsp90 and its client proteins by this agent is thought to cause these effects. Interestingly, the present study has demonstrated that KF58333 also has a significant anti-angiogenic effect on human breast cancer xenografts. These findings indicate that KF58333 has two different anticancer effects, that is, disrupting the growth signaling pathway and inhibiting tumor angiogenesis. Significant growth-inhibition *in vivo* by KF58333 (Fig. 2) may be synergistically induced by these two effects.

To explore the action mechanisms of the anti-angiogenic effect of KF58333, its direct effect on the expression levels of several angiogenic factors in cancer cells was investigated. Both the mRNA expression level and protein secretion of VEGF were significantly decreased by the treatment with this agent in two different human breast cancer cell lines under both normoxia and hypoxia (Fig. 3). In contrast, this agent had no influence on the mRNA expression levels of VEGF-B, -C, -D and platelet-derived endothelial growth factor (unpublished data). VEGF is known to be an important angiogenic factor for cancer growth and progression. Therefore, a decrease of VEGF secretion from cancer cells induced by KF58333 might be a main cause of the anti-angiogenic activity. However, it remains possible that this agent directly inhibits endothelial cell growth and function like radicicol.[16] Another possibility is that KF58333 may interfere with the angiogenic intracellular signaling pathway, such as inhibition of phosphorylation of the focal adhesion kinase (FAK) signaling molecule, like geldanamycin in endothelial cells existing in transplanted tumors.[32]

It has been shown that the VEGF expression is regulated through the HIF-1α signaling pathway. An increase

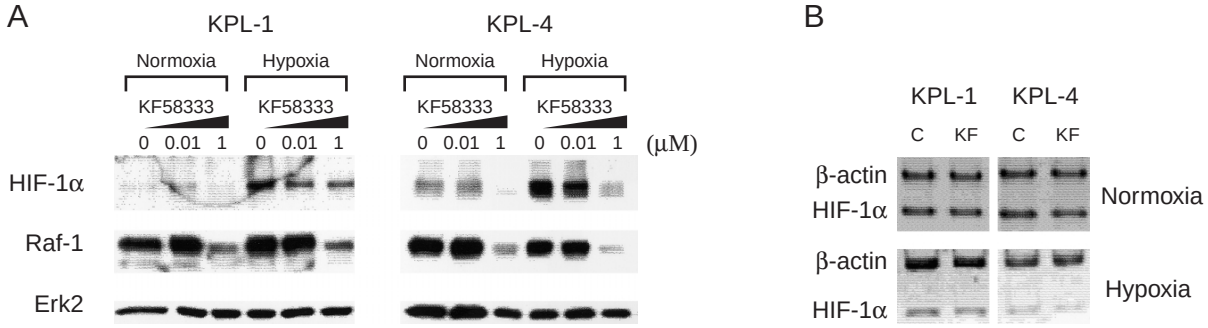

Fig. 4. Effects of KF58333 on the expression levels of HIF-1α in KPL-1 and KPL-4 breast cancer cells. (A) Protein expression levels. KPL-1 (left panel) or KPL-4 (right panel) cells were treated with 0–1 *μM* KF58333 under normoxia or hypoxia for 2 days. The cell lysate was subjected to western blotting for HIF-1α, Raf-1 and Erk2 as described in "Materials and Methods." (B) mRNA expression levels. KPL-1 or KPL-4 cells were treated with 1 *μM* KF58333 under normoxia or hypoxia for 24 h. These cells were subjected to the multiplex RT-PCR analysis. Representative gel images are shown in inverted presentation. C, control; KF, KF58333-treated.

in HIF-1α protein induced by hypoxia or other factors activates the HIF-1 signaling pathway and stimulates the transcription of the *VEGF* gene through a *cis*-element, hypoxia response element, existing in the promoter region of the *VEGF* gene.[19] It has also been suggested that Hsp90 plays a specific role in the function of HIF-1α.[21] In addition, it has been reported that HIF-1α is frequently overexpressed in common cancers including breast cancer.[20] Therefore, we investigated the influence of the radicicol derivative on the HIF-1α expression.

As expected, KF58333 decreased the HIF-1α protein expression level in both breast cancer cell lines under both normoxia and hypoxia (Fig. 4). To investigate the effect of this radicicol derivative on HIF-1α expression *in vivo*, the HIF-1α protein expression in the xenografts was also studied. No constitutive change in the expression levels of HIF-1α protein after the intravenous administration of KF58333 was observed (unpublished data). Since HIF-1α is easily degraded, the process of protein extraction from xenografts might reduce the amount of intact HIF-1α protein. In addition, contamination with stromal components might also influence the concentration of intact HIF-1α protein. According to the results *in vitro*, it is suggested that KF58333 depletes the HIF-1α protein and may decrease VEGF expression through the HIF-1 signaling pathway.

However, a role has been suggested for Hsp90 in the translocation of HIF-1α from cytoplasm to nucleus.[21] It is possible that KF58333 also disrupts the translocation of HIF-1α. Moreover, it has been suggested that the activated Raf-1-Mek1-Erk pathway stimulates HIF-1 transcriptional activity without affecting HIF-1α protein expression.[33, 34] A drastic down-regulation of Raf-1 by these radicicol derivatives (demonstrated in Fig. 4A) disrupts the mitogen-activated protein kinase (MAPK) pathway, decreases the HIF-1 transcriptional activity and down-regulates VEGF expression in association with a decrease in the HIF-1α protein expression. Furthermore, alternative dimerization partners of HIF-1β, which also transactivate target genes via the HIF-1 DNA recognition sites, have recently been identified and termed HIF-2α and HIF-3α.[35, 36] Interaction among these new HIF-1α family members and Hsp90 remains to be elucidated. No information on the effects of KF58333 on the protein expression levels of these new members is currently available. Further studies are clearly needed to elucidate the action mechanisms of this agent in the HIF-1 signaling pathway.

It should be noted that KF58333 significantly increased apoptotic tumor cells and necrotic areas inside tumors (Table II). A decrease in angiogenesis induces hypoxia inside tumors and results in an increase of apoptotic cell death.[37] The anti-angiogenic effect of KF58333 may induce apoptosis of tumor cells and may increase the necrotic areas. However, our previous studies have shown that this derivative directly induces apoptosis in K562, the human chronic myeloid leukemia cell line and in the KPL-4 cell line used in the present study.[9, 10] It is suggested that a synergistic effect with anti-angiogenesis and a direct induction of tumor cell apoptosis induced by this agent may cause massive apoptosis inside breast cancer xenografts.

Hsp90 is an abundant and unbiquitous protein in eukaryotes and plays important roles in the stability and function of various intracellular signaling molecules.[1] It is suggested that inhibition of the Hsp90 function, induced by certain agents such as radicicol derivatives, may cause serious toxicity. In the present study, KF58333 caused a slight loss of mouse body weight, although this recovered soon after the end of the treatment (Fig. 2). This suggests that the toxicity of this agent is reversible. Our previous studies have shown that this agent did not cause serious toxicity in the liver and kidney and that this did not influence the expression levels of Hsp90 client proteins in the spleen, as an example of normal tissue.[10] It has been shown that the Hsp 90 family chaperone proteins are expressed at higher levels in cancer cells than in their normal counterparts.[2] These findings suggest that the effects of KF58333 are relatively cancer-specific. However, careful monitoring must be performed when using this agent for the treatment of cancer patients.

In conclusion, the present study suggests, for the first time, that a radicicol derivative, KF58333, has a significant anti-angiogenic effect as well as inhibiting the Hsp90-associated signaling pathway. These two different action mechanisms may synergistically inhibit the growth of human breast cancer xenografts in association with an increase in apoptosis and necrosis. Additionally, it is suggested that these agents may down-regulate VEGF expression through the HIF-1α-dependent pathway in human breast cancer cells. A decrease in VEGF secretion from cancer cells may play a role, at least in part, in the anti-angiogenic activity of this agent. Further molecular analysis of the action mechanisms of KF58333 will be useful for clarifying the significant roles of Hsp90 in cancer cell biology and are needed for the future clinical use of radicicol derivatives.

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