Blockade of the vascular endothelial growth factor-receptor 2 pathway inhibits the growth of human renal cell carcinoma, RBM1-IT4, in the kidney but not in the bone of nude mice

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Abstract. Primary and metastatic RCCs are consistently resistant to radiotherapy, chemotherapy, or immunotherapy. As recurrent or metastatic RCC after surgery is related with poor prognosis and cancer-related death, development of therapeutic modalities that can control RCC and improve patient survival is urgently needed. We determined whether blockade of the vascular endothelial growth factor-receptor 2 (VEGF-R2) signaling pathway inhibits the growth of human renal cell carcinoma cells in the kidney and bone of nude mice. Male nude mice implanted with $1x10^6$ RBM1-IT4 cells in the kidney or in the tibia were treated with oral administrations of TSU-68, anti-VEGF-R2 tyrosine kinase inhibitor beginning 5 days after implantation. The tumor incidence, tumor weight and bone destruction were determined at twelve weeks after commencing the therapy. VEGF production by RCCs was determined by ELISA and alterations in VEGF production related with genetic instability were also analysed. VEGF-R expression of mouse osteoclast precursors (mOCPs) and human umbilical vascular endothelial cell (HUVEC) was determined by RT-PCR and Western immunoblotting. *In vitro*, the effects of TSU-68 on the cellular proliferation of HUVEC, normal human renal proximal tubule epithelial cell (RPTEC) and mOCPs were determined. RBM1-IT4 cells had loss of heterozygosity and frame shift mutation on chromosome 3p, inactivating the von Hippel-Lindau (VHL) tumor suppressor gene and resulting in the production of relatively higher levels of VEGF than the RCCs without VHL mutation. TSU-68 significantly inhibited the growth of RBM1-IT4 in the kidney ($p<0.05$). In contrast, TSU-68 did not inhibit the growth of RBM1-IT4 in the tibia or bone lysis. Although HUVEC, RPTEC and mOCPs expressed VEGF-R2, TSU-68 directly inhibited the VEGF-stimulated cell growth of HUVEC and RPTEC but not the mOCPs *in vitro*. These data indicate that the VEGF-VEGF-R2 pathway is not required for survival of the osteoclasts and anti-VEGF-R2 therapy did not contribute to the suppression of metastatic RCC growth in the bone.

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Introduction

Due to the lack of cancer-related symptoms before quite advanced stages of renal cell carcinoma (RCC), it is frequently diagnosed incidentally and a high portion of patients have distant metastases at the time of diagnosis. Primary tumors are mostly well vascularized (hypervascular) and metastasize to lung, liver and bone via hematogenous spread. Bone metastasis occurs in 20-60% of patients with RCC (1-4) and reportedly accounts for about 10% of pathologic fractures and 5% of instances of spinal cord or nerve root compression (5). These complications result from excessive bone metabolism, principally bone resorption that characterizes osteolytic bone lesions causing severe bone pain. Furthermore, the bone metastasis of RCC remains a clinical problem because the lesions are resistant to conventional therapeutic modalities, such as chemotherapy, radiotherapy, immunotherapy or other systemic therapies, and surgery is currently not curative but palliative. Therefore, there is a great need for developing therapies that effectively inhibit bone resorption, thereby reducing the risk of skeletal complications (6).

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors, inducing tumor angiogenesis that is critical and essential for tumor growth and metastasis (7). Its specificity is demonstrated by the preferential expression of its two receptors, VEGF-R1 (flt-1) and VEGF-R2 (flk-1/KDR), on the surface of endothelial cells of the tumor-associated vessels. Therefore, VEGF-Rs are promising potential targets for the inhibition of tumor angiogenesis. Studies with the VEGF-R2 tyrosine kinase inhibitor, PK787, demonstrated anti-tumoral and anti-angiogenic activity in murine renal cell carcinoma models (8). The prolongation of the survival of patients with metastatic RCC by inhibition of the VEGF receptor pathway was demonstrated in a randomized Phase II trial with the anti-VEGF antibody, bevacizumab. This study involved 116 patients with metastatic renal cell carcinoma

and revealed that this drug significantly prolonged the time to progression of disease when compared with the placebo group (9).

Studies of the hereditary form of clear cell type RCC observed in von Hippel-Lindau syndrome identified the von Hippel-Lindau (VHL) tumor suppressor gene. The gene is mutated both in hereditary RCC (where one mutation is a germ-line mutation) and in most cases of sporadic clear cell type-RCC (where both alleles have acquired mutations or deletions) (10,11). One consequence of these mutations is the induction of overproduction of VEGF through a mechanism related to hypoxia-inducible factor-1α (12-16), which implicates that the VHL protein is tightly linked to angiogenesis by regulating VEGF. VEGF stimulates the growth of endothelial cells and appears to be a central factor in tumor angiogenesis and metastasis (17).

In the present study, we investigated whether inhibition of the VEGF/VEGF-R pathway by a potent inhibitor of flk-1/KDR kinase activity, TSU-68 (SU6668), can be used as a treatment for primary or metastatic clear cell type-RCC in an animal model.

Materials and methods

Cell lines and culture conditions. The cell lines used in this study are as follows; human umbilical vascular endothelial cell (HUVEC; from ATCC), mouse osteoclast precursors (mOCPs; Hokudo Co., Ltd., Hokkaido, Japan) (18), normal human renal proximal tubule epithelial cell (RPTEC; Sanko Junyaku Co., Ltd., Tokyo Japan) and various RCC cell lines. The RBM1 cell line was established from a metastatic bone lesion in a patient with RCC (gift from Dr Weber). Cell implantation into the tibia of nude mice induced the same osteolytic formation as human clear cell type-RCC bone metastasis. The cells in the resultant lytic lesion were recycled to yield the RBM1-IT4 cell line (19). The 786-O cell line (from ATCC) was originally derived from primary clear cell type-RCC containing a VHL mutation which is the deletion of Guanine on exon 1 of the VHL gene. 786-O sublines have been produced by stable tansfection with vectors carrying various mutant VHL genes (-Host, original cell line; -RD3, control vector; -RW2, point mutation on exon 3 leading to 167 amino residue Arg to Trp (from Dr Hiroi). The SN-12 cell line (gift from Dr Naito) is clear cell type-RCC without VHL mutation (20). Cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) for RCCs; in EBM-2 (Cambrex Bio Science Walkersville Inc., Walkersville, MD) supplemented with 2% fetal bovine serum, 0.1% human epidermal growth factor, 0.4% human basic fibroblast growth factor, 0.1% vascular endothelial growth factor, 0.1% R3-insulin-like growth factor, 0.1% ascorbic acid, 0.1% heparin, 0.04% hydrocortisone, and 0.1% gentamicin/amphotericin B for HUVEC; in modified Eagle's medium (MEM) supplemented with 100 ng/ml macrophage-colony stimulating factor (M-CSF) (Wako Chemicals Inc., Osaka, Japan) for mOCPs; and in REBM supplemented with 0.5% fetal bovine serum, 0.1% human epidermal growth factor, 0.1% insulin, 0.1% epinephrin, 0.1%, triiodothyronine, 0.1% transferine, 0.1% hydrocortisone, and 0.1% gentamicin/amphotericin (Cambrex Bio Science Walkersville Inc., Walkersville, MD) for RPTEC. Adherent monolayer cultures were maintained on plastic and incubated at 37˚C in a mixture of 5% CO_2 and 95% air. The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Determination of LOH by fluorescence in situ hybridization (FISH) assay. Metaphase chromosome spreads were prepared from RBM1-IT4 cells. The cells were grown for 72-96 h, harvested after arresting with colcemid (0.05 μg/ml) for 15 min during log-phase growth, treated in hypotonic KCl (0.54%) for 15 min at 37˚C, and fixed in a cold (-20˚C) methanol-acetic acid (3:1). Fresh slides were equilibrated in 2X SSC solution at 37˚C and dehydrated in increasing ethanol solutions of 70, 80, and 95%. FISH assay was performed using cosmid c3 (30 kb) probe (gift from Dr Zbar) (21), which includes the 3' portion of the VHL gene (a part of the reading frame and 3'-UTR). Whole printing probe, specific for chromosome 3 (Oncor, Gaithersburg, MD) was used for detecting chromosome 3. DNA was labeled with digoxigenin-11-dUTP by nick translation (Boehringer-Mannheim) and ethanol-precipitated in the presence of 50X herring sperm DNA and 50X Cot-1 human DNA. The DNA pellet was resuspended in the Hybrisol solution (50% deionized formamide/10% dextran sulfate/2X SSC) to a final concentration of 25 ng/μl. Slides were denatured in 70% formamide/2X SSC at 72˚C for 2 min, dehydrated sequentially in cold (-20˚C) ethanol solutions of 70, 85, and 100% for 2 min and air-dried. Probes were denatured at 78˚C for 10 min and then incubated for 30 min at 37˚C for preannealing. A total of 250 μg of the DNA probe was applied to the slide. Whole printing probe, specific for chromosome 3 (Oncor) was denatured separately and mixed with the cosmid probe immmediately prior to hybridization. Overnight hybridization was performed in a humidified chamber at 37˚C. Posthybridization washes were at 45˚C in 50% formamide/2X SSC (5-min x 3), /1X SSC (5 min x 2) and /0.1X SSC (5 min x 2). Detection was performed using avidin-FITC and antidigoxigenin Rhodamine (40 min at 37˚C) followed by washing in 4X SSC/0.1% Tween-20 solution at 45˚C and counterstaining with 4'6'-diaminophenylindole (DAPI)-antifade (0.25 μg/ml). FISH using probes from the VHL gene critical region in 3p25.3 was performed in a blind manner without knowing the clinical data for VHL family members. Hybridization signals were detected using an Olympus BX-50 fluorescence microscope equipped with single band-pass filters for Fluorescein, Rhodamine, and DAPI and with a cooled CCD camera (KAF 1400; Photometrics, Tucson, AZ, USA) and Mac Probe version 3.4-analysis system image software (Applied Imaging Corporation, Santa Carla, CA, USA; Sekitechnotoron, Tokyo, Japan).

Determination of mutations of the VHL gene by sequence analyses. We analyzed RBM1-IT4 cells for mutations of the VHL gene by direct sequencing. Genomic DNAs were extracted from the cells by standard procedures. Briefly, the cells were lysed in a 25 mM EDTA solution, pH 8.0, containing 75 mM NaCl, 1.0% SDS, and 100 μg/ml proteinase K, and incubated at 50˚C for 18 h. After adding 0.33 volume of a 6 M NaCl solution, DNA was extracted with one volume of chloroform and precipitated with one volume of iso-propanol.

It was finally dissolved in 10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0. Genomic DNA (50-100 ng) was amplified by PCR in a standard PCR buffer containing 20 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 3% DMSO, 1.0 unit of AmpliTaq Gold polymerase (Perkin-Elmer), and 0.2 μM primers using 45 cycles of 95˚C for 45 sec, 59˚C for 45 sec, and 72˚C for 45 sec. PCR products were sequenced using a cycle sequencing kit with dye terminators (Perkin-Elmer) and an ABI 310 automated sequencer. Primers used for both PCR and sequencing were as follows: a) 1F, TGG TCT GGA TCG CGG AGG GAA T; b) 1R, GAC CGT GCT ATC GTC CCT GC; c) 2F, GTG GCT CTT TAA CAA CCT TTG C; d) 2R, CCT GTA CTT ACC ACA ACA ACC TTA TC; e) 3F, TTC CTT GTA CTG AGA CCC TAG T; and f) 3R, AGC TGA GAT GAA ACA GTG TAA GT. Amplification for sequencing was performed with 3 cycles of 95˚C for 10 sec, 55˚C for 5 sec, and 60˚C for 4 min; followed by 22 cycles of 96˚C for 10 sec, 50˚C for 5 sec, and 60˚C for 4 min.

Reagents. TSU-68 (SU6668) is a potent inhibitor of Flk-1/KDR kinase activity and also inhibits platelet derived growth factor-receptor (PDGF-R) and fibroblast growth factor-receptor 1 (FGF-R1) kinase activity (22,23).

Effects of TSU-68 on cell proliferation in vitro. *In vitro* anti-proliferation activity of TSU-68 on HUVEC, RPTEC and mOCPs was determined. Ten-thousand cells were plated and, after culturing for 24 h in fetal bovine serum-supplemented MEM, were incubated in the medium for low serum medium containing up to 10 μM TSU-68 with or without 30 ng/ml recombinant human VEGF165 (R&D Systems Inc., Minneapolis, MN) for 3-5 days, and viable cells were counted by using COULTER Z1 (Beckman-Coulter, Inc., Tokyo, Japan) and compared as the ratio of the number of viable cells in each groups treated with TSU-68 and/or VEGF to the number in the control group treated with dimethyl sulfoxide.

Evaluation of VEGF production by enzyme-linked immunosorbent assay. Cell culture supernatant was collected from RCCs (786-O-Host, -AC6, -SRD3, -RW2 and RBM1-IT4), HUVEC, RPTEC and 253J B-V human bladder cancer cells which highly express angiogenic factors, including VEGF (25). Levels of human VEGF proteins were determined using the commercial Quantikine ELISA kit (R&D Systems, Inc.). This kit has minimum cross-reaction with other species.

Western immunoblotting. Inhibition of phosphorylation of VEGF-R2 on HUVEC and mOCPs by TSU-68 was analyzed by Western blotting. Cells were treated with 30 ng/ml VEGF for 1, 5 or 10 min, 1 μM of TSU-68 with or without exogenous stimulation of VEGF. After removing media, cells were washed and lysed in NP40 lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotinin) at 4˚C. The supernatants were cleared by centrifugation. Protein concentrations were measured by the Bradford method. Equal amounts of crude extract were boiled in Laemmli SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with (1:1,000) rabbit anti-phosphorylated VEGF-R2 primary antibody (Chemicon, Temecla, CA), (1:200) rabbit anti-VEGF-R2 primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4˚C overnight. After the blots were incubated for another 1 h at room temperature with (1:7,500) horseradish peroxidase-labeled anti-rabbit secondary antibody (Amersham Life Science Inc., Arlington Heights, IL), signals were detected by using an enhanced chemoluminescence detection system (Amersham Life Science Inc., Arlington Heights, IL).

Detection of expression of VEGF-R1 and VEGF-R2 by RT-PCR analysis. RT-PCR analysis was performed by using standard technique. Briefly, total cellular RNA (1 μg) extracted from various cell lines was transcribed into cDNA using 0.5 μg oligo$(dT)_{12-18}$ primer and 50 U Superscript™ II reverse transcriptase by SuperScript™ First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA). The reverse transcription reaction was performed at 42˚C for 50 min. PCR was performed with 25 cycles of denaturation (95˚C for 1 min), annealing (57˚C for 1 min), and extension (72˚C for 2 min) for VEGF-R1 and with 25 cycles of denaturation (95˚C for 1 min), annealing (60˚C for 1 min), and extension (72˚C for 2 min) for VEGF-R2 and 7 min of extension after completion of all cycles. Amplified fragments were analyzed on the 2% gel, and bands of expected sizes were confirmed by sequencing. The primer sequences used were as follows: VEGF-R1 sense, 5'-AGC CCA CCT CTC TAT CCG CTG G-3'; VEGF-R1 antisense, 5'-GGC GCT TCC GAA TCT CTA ACG-3'; VEGF-R2 sense, 5'-AGC TTG GCT CAC AGG CAA CAT CGG-3'; and VEGF-R2 antisense 5'-TGG CCC GCT TAA CGG TCC GTA GG-3'.

Animals. Male athymic BALB/cA Jc1-nu nude mice were obtained from Clea Japan Inc., Osaka, Japan. The mice were maintained in a laminar-airflow cabinet in pathogen-free conditions and used at 6-8 weeks of age.

Implantation of tumor cells. Cultured RBM1-IT4 cells (60-70% confluent) were prepared for injection as previously described (24). Nude mice were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL). A percutaneous intraosseal injection was made by drilling a 27-gauge needle into the tibia immediately proximal to the tuberositas tibia. After penetration of the cortical bone, the needle was inserted into the shaft of the tibia, and 20 μl of the cell suspension ($1x10^6$ cells) was deposited in the bone cortex using a calibrated, push-button-controlled dispensing device (Hamilton Syringe Co., Reno, NV). To prevent leakage of cells into the surrounding muscle, a cotton swab was held for 1 min over the site of injection. For kidney implantation, a flank incision was made, and tumor cells were injected into the renal subcapsule of kidney upper pole. The formation of a bulla indicated a satisfactory injection. The kidney was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal clips (20). The animals tolerated the surgical procedure well, and no anesthesia-related deaths occurred.

Treatment of human RCC growing in the kidney or in the tibia of athymic nude mice. Five days after implantation of

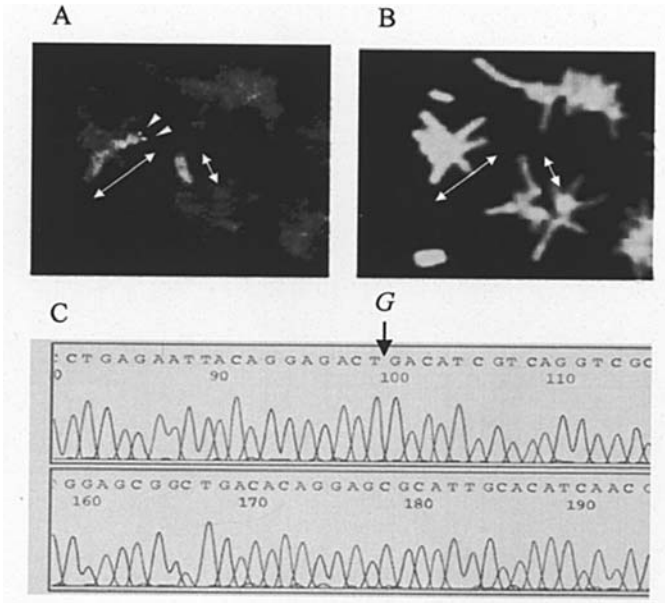

Figure 1. Loss of heterozygosity (LOH) of the VHL gene on chromosome 3 for RBM1-IT4 cells analyzed by FISH (A and B) and direct sequence method (C). (A) RBM1-IT4 cells had deletion of ipsilateral short arm of chromosome 3 (two-headed arrow) involving the VHL gene (double small arrow). (B) Whole chromosomes were detected by counterstaining with DAPI. Two-headed arrow indicates chromosome 3. (C) The RBM1-IT4 cells revealed deletion of guanine with frame shift on exon 3 of the VHL gene examined by the direct sequence method.

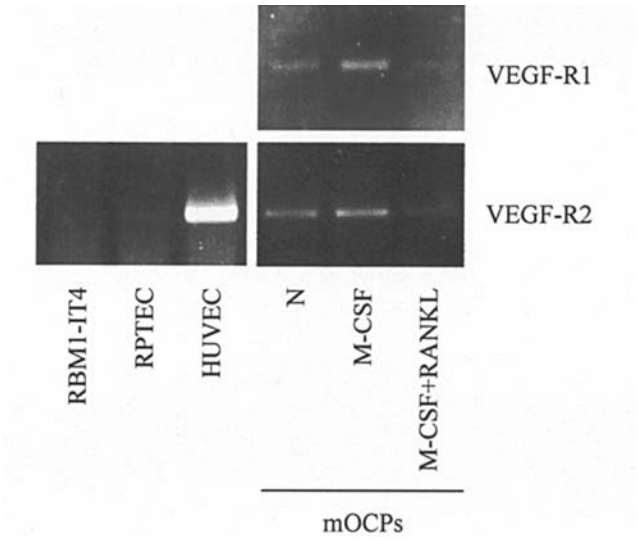

Figure 2. VEGF-R2 mRNA expression of RBM1-IT4 cells, HUVEC, and RPTEC, and also VEGF-R1 and -R2 mRNA expression of mOCPs measured by semi-quantitative RT-PCR. HUVEC and mOCPs expressed VEGF-R2 mRNA relatively more than RPTEC but not RBM1-IT4 cells. mOCPs cultured with normal medium or the medium containing M-CSF expressed VEGF-R2 mRNA more than mOCPs cultured with both M-CSF and RANKL.

tumor cells, mice were randomized into two groups (n=10-12): a) daily oral administration of carboxymethylcellulose-based vehicle solution; and b) daily oral administration of 400 mg/kg TSU-68. The mice were treated for 12 weeks. Tumor weight and status of the injected bone (lysis) were determined by gross observation and radiography as described below.

Radiography and harvest of tumors. After 12 weeks of treatment, mice from each treatment group were anesthetized with Nembutal and placed in a prone position. Soft X-ray photographs of the leg were taken to evaluate the bone destruction. The estimated area of bone destruction (osteolysis) was calculated by two axes (X,Y) using the formula of πXY.

Statistical analysis. The statistical differences in the amount of *in vitro* cell proliferation and VEGF expression were analyzed using the Mann-Whitney test. The incidence of tumors, estimated tumor volume and area of bone destruction were statistically analyzed using the χ^2 test. A value of $p<0.05$ was considered significant.

Results

Loss of heterozygosity (LOH) of human RCC. We performed FISH analysis to characterize the cytogenetic abnormality of the VHL gene on chromosome 3 for RBM1-IT4 clear cell-type RCC cells using cosmid c3 probe detecting the 3' portion of the VHL gene and whole printing probe for chromosome 3, and the DNA sequence of the VHL gene for the RBM1-IT4 cells was analyzed by using the direct sequence method. The RBM1-IT4 cells showed deletion of the ipsilateral short arm of chromosome 3 involving the VHL gene (Fig. 1A and B) and DNA sequence analysis revealed that guanin was deleted on exon 3 of the VHL gene with frame shift (Fig. 1C). The data suggested that RBM1-IT4 cells had LOH leading to functional alteration of the VHL gene based on a two-hit theory.

Alteration of VEGF-R2 mRNA expression on mOCPs at process of maturation to osteoclast. For assessment of micro-environment, VEGF-R2 mRNA expression in HUVEC, RPTEC and mOCPs was measured by semi-quantitative RT-PCR (Fig. 2). VEGF-R1 was expressed in mOCPs and VEGF-R2 was expressed in HUVEC, mOCPs and RPTEC (slightly). VEGF-R2 mRNA expression of mOCPs stimulated by M-CSF or M-CSF with RANKL was determined. The morphology of mOCPs cells changed from a round to spindle shape after culturing with excess amount of M-CSF (100 ng/ml) for a week. mOCPs cultured with both M-CSF and RANKL developed multinuclei thin-formed cells as mature osteoclasts. The expression of VEGF-R2 was higher in mOCPs cultured with normal medium or the medium containing M-CSF than the mOCPs cultured with M-CSF and RANKL.

VEGF protein expression of RCCs correlated with status of VHL mutation. VEGF protein production of RCCs with various mutations of the VHL gene, 253J B-V, and HUVEC was measured by ELISA. The cell lines had well-characterized VHL status as 786-O-Host, deletion on exon 1; -SRD3, control vector transfectant; -RW2, point mutation on exon 3; RBMI-IT4, deletion on exon 3; and SN-12, absent of VHL mutation. As control cell line, 253J B-V human bladder cancer cells which highly express VEGF were described previously (25). RCCs with VHL mutation expressed VEGF significantly more than RCCs without VHL mutation ($p<0.05$). Especially

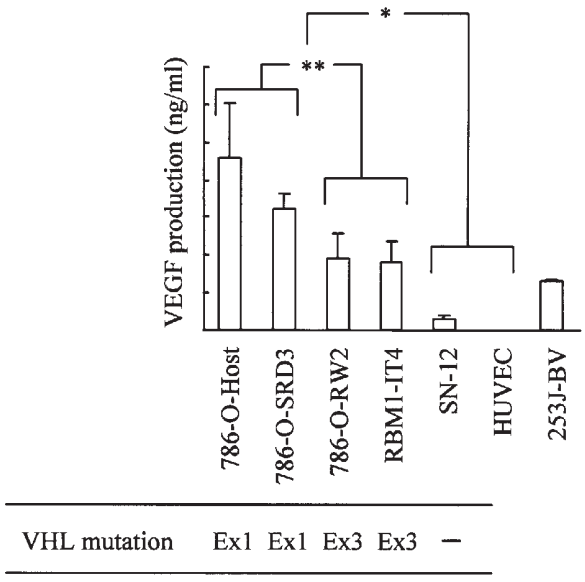

Figure 3. VEGF protein expression of RCCs, HUVEC, and 253J B-V cells measured by ELISA. The RCCs had well-characterized VHL status as 786-O-Host, deletion on exon 1; -SRD3, control vector transfectant; -RW2, point mutation on exon 3; RBMI-IT4, deletion on exon 3; and SN-12, absent of VHL mutation. RCCs with VHL mutation expressed VEGF significantly more than SN-12 or HUVEC ($p<0.05$). Both 786-O-Host and -SRD3 mutated exon 1 of the VHL gene expressed significantly higher levels of VEGF compared to 786-O-RW2 or RBM1-IT4 mutated exon 3 ($p<0.05$). The RBM1-IT4 cells mutated on exon 3 of the VHL gene expressed similar levels of VEGF compared to 253J B-V human bladder cancer cells which highly express VEGF.

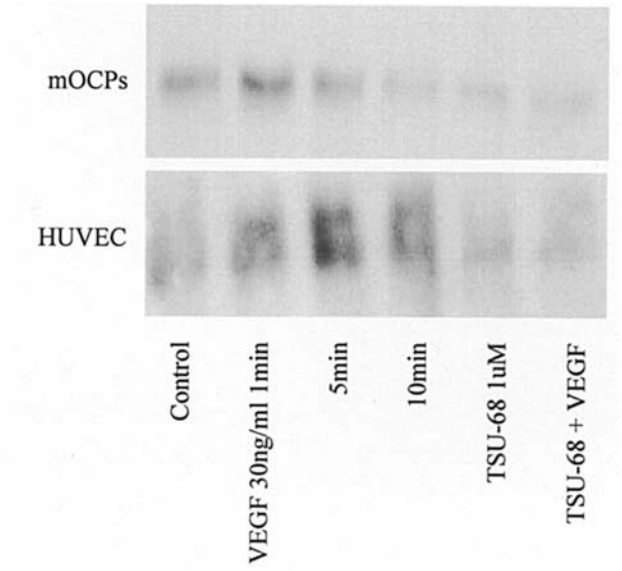

Figure 4. Inhibition of ligand-stimulated VEGF-R2 tyrosine phosphorylation in HUVEC and mOCPs detected by Western immunoblotting with anti-phosphorylated VEGF-R2 antibody. Under basal conditions in low serum medium, HUVEC and mOCPs cells demonstrated a low level of VEGF-R2 tyrosine autophosphorylation which was enhanced following exposure to 30 ng/ml of exogenous VEGF for 1-10 min. TSU-68 (1 μM) inhibited the VEGF-R2 tyrosine phosphorylation by HUVEC and mOCPs in response to exogenous VEGF stimulation.

with the farther VHL mutation existing close to the 5' terminus of the VHL gene, both 786-O-Host and -SRD3 expressed significantly higher levels of VEGF compared to 786-O-RW2 or RBM1-IT4 ($p<0.05$). The RBM1-IT4 mutated exon 3 of the VHL gene and 786-O-RW2 expressed similar levels of VEGF (Fig. 3).

Inhibition of VEGF-R2 tyrosine phosphorylation of VEGF-R2 with exogenous stimulation in HUVEC and mOCPs. We determined whether TSU-68 inhibits VEGF-stimulated tyrosine phosphorylation of the VEGF-R2 in HUVEC and mOCPs. Under basal conditions in low serum medium, HUVEC and mOCPs cells demonstrated a low level of VEGF-R2 tyrosine autophosphorylation, which was enhanced following exposure to VEGF for 1-10 min. TSU-68 (1 μM inhibited the VEGF-R2 tyrosine phosphorylation by HUVEC and mOCPs in response to exogenous VEGF stimulation (Fig. 4).

Treatment of RBM1-IT4 growing in the kidney or tibia of nude mice with TSU-68. We determined whether administration of TSU-68 inhibits the growth of RBM1-IT4 tumor cells implanted into the kidney or the bone of athymic nude mice. Therapy commenced 5 days after tumor implantation and continued for 12 weeks. Treated mice were closely monitored for any signs of progressive disease and were euthanized if they became moribund. The results of the therapy are summarized in Table I. The therapy with TSU-68 significantly decreased the tumor weight in the kidney compared with the control

Table I. Tumorigenicity of human RCC growing in the kidney and tibia of athymic nude mice.

	Therapy	Incidence	Tumorigenicity/osteolytic area Median (mg/cm^2)	Range (mg/cm^2)
Kidney tumor	Vehicle	10/12	870	450-1710
	TSU-68	9/12	470[a]	230-970
Tibia tumor	Vehicle	8/10	1.35	0.74-2.05
	TSU-68	8/10	1.51	0.17-1.95

[a]$p<0.05$ against vehicle kidney tumor (p, Mann-Whitney's U test). Mice were implanted with 1x10^6 RBM1-IT4 cells into the kidney or the tibia. Treatment commenced 5 days after tumor implantation. All mice were sacrificed at 12 weeks after implantation of tumor cells. Kidney tumors treated with TSU-68 were significantly reduced in tumor burden compared with kidney tumors treated with vehicle ($p<0.05$). The osteolytic area of the tibia tumor treated with TSU-68 was not significantly different from that of the vehicle control group.

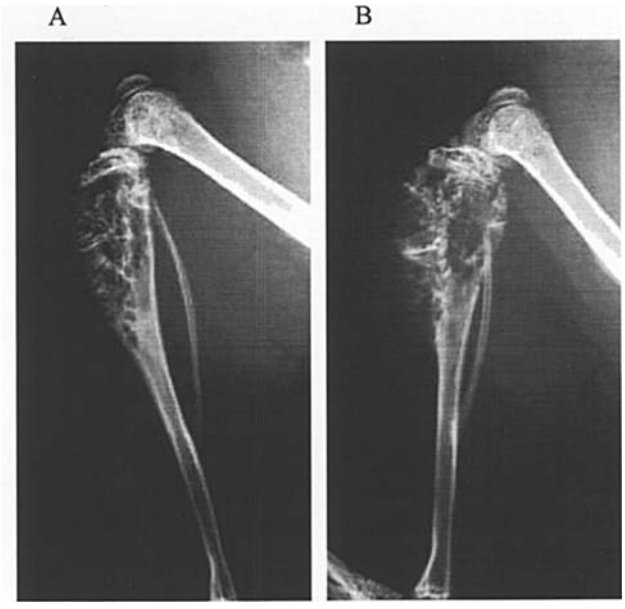

Figure 5. Representative soft X-ray photographs of the left legs of nude mice treated with control vehicle (A) or TSU-68 (B). Bone destruction (osteolysis) of the tibia of mice treated with daily administration of TSU-68 showed no difference compared to mice treated with control vehicle.

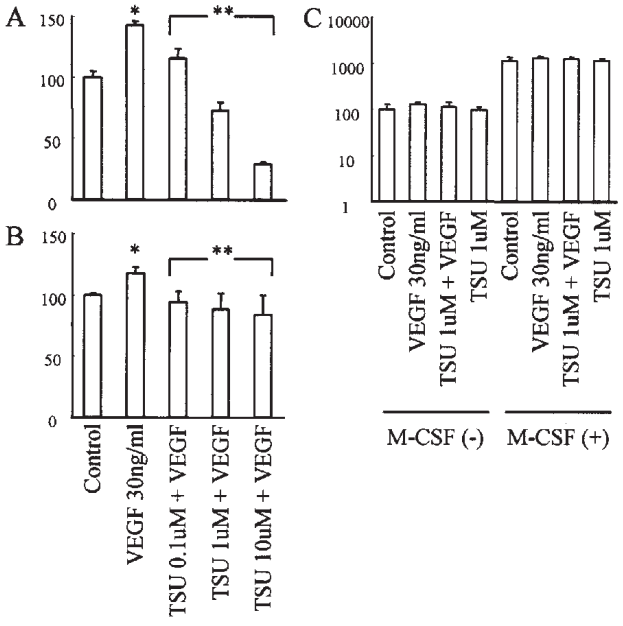

Figure 6. Percent inhibition of *in vitro* cell growth of HUVEC (A), RPTEC (B) and mOCPs (C) treated with TSU-68 in exposure to exogenous VEGF measured by auto-cell counter. Following exposure to 30 ng/ml of VEGF, the cell growth of HUVEC and RPTEC significantly increased compared to control ($^{*}p<0.05$). The therapy with TSU-68 ($\leq 10\ \mu M$) significantly suppressed VEGF-stimulated cell growth of either HUVEC or RPTEC ($^{**}p<0.05$). Especially, TSU-68 inhibited the cell growth of HUVEC in a dose-dependent manner. However, mOCPs did not respond to exposure to VEGF and the therapy with TSU-68 failed to inhibit the cell growth of mOCPs in the medium with or without M-CSF.

group ($p<0.05$). However, the therapy with TSU-68 did not significantly decrease the tumor growth in the bone compared with the control group ($p>0.05$) and, consequently, did not preserve bone structure (Fig. 5).

Effects of TSU-68 on cell proliferation of HUVEC, mOCPs and RPTEC in vitro. Next, we evaluated the inhibitory effects of TSU-68 on cellular proliferation of HUVEC, mOCPs and RPTEC *in vitro* by auto-cell counter (Fig. 6). Stimulation with exogenous VEGF significantly increased the cell proliferation of HUVEC and RPTEC ($p<0.05$, compared to control group). Inhibitory effects were dose-dependent with HUVEC but not with RPTEC ($p<0.05$). Cellular proliferation of mOCPs was not enhanced by exogenous VEGF but by exogenous M-CSF; however, treatment with TSU-68 did not affect the proliferation of mOCPs.

Discussion

The blockade of the VEGF/VEGF-R signaling pathway by VEGF-neutralizing antibodies, VEGF antisense, dominant-negative VEGF receptors or thyrosine kinase inhibitors has repeatedly shown, in all cases, significant inhibition of tumor growth and metastasis (26-33). These agents block VEGF-induced signaling in endothelial cells and inhibit the growth of human tumors in mice by an anti-angiogenic mechanism (29,31-33). TSU-68 (SU6668), a molecular-targeting drug, blocks the tyrosine kinase phosphorylation of VEGF-R2 and inhibits angiogenesis (23,34). *In vivo* studies using experimental models of cancers such as glioma, melanoma, lung, colon, ovarian, and epidermoid origin cancer have shown that TSU-68 has an inhibitory effect on tumor growth and metastasis through the suppression of tumor angiogenesis (23,34-37). Furthermore, a phase I surrogate end-point study of TSU-68 in patients with solid tumors showed decreased blood flow in tumors by functional CT, dynamic contrast enhanced magnetic resonance imaging and pharmacokinetics (38).

The VHL gene was identified in 1993 as a causative gene for von Hippel-Lindau disease (39). VHL gene alterations were also observed at high frequencies of up to 80% in cases of sporadic RCC, especially in clear cell-type and it was recognized as a causative tumor suppressor gene for sporadic RCC (40-43). The functions of the VHL protein (pVHL) have been well analyzed (15,44-46). pVHL suppresses the transcription of angiogenic, growth, and mitogenic factors, including the VEGF gene via disruption of ubiquitination following degradation of hypoxia-inducible factor (HIF)-1α, a transcription factor (15,47,48). pVHL inhibits Sp1 and, consequently, VEGF transcription by interacting with Sp1 and decreasing its phosphorylation by protein kinase C (14,49). Thus, inactivation of the VHL gene may cause VEGF over-expression and angiogenesis, resulting in the development and progression of clear cell-type RCC. Actually, it has been shown *in vitro* that up-regulation of VEGF is a consequence of VHL alteration (13,50). Brieger *et al* demonstrated an inverse relation between the expression level of VHL mRNA and that of VEGF protein in sporadic RCC (51). The present study indicates that RBM1-IT4 cells have LOH leading to functional alteration of the VHL gene based on a two-hit theory, that RCCs with VHL mutation express relatively higher VEGF than RCCs without VHL mutation, and that the expression level of VEGF may correlate with the status of VHL mutation. Thus, we assumed that human clear cell-type RCCs could be a good therapeutic target for anti-VEGF-R therapy and investigated whether the oral administration of TSU-68 could

inhibit the growth and metastasis of human clear cell-type RCC in the kidneys of nude mice. Treatment of RBM1-IT4 growing in the kidneys of nude mice with TSU-86 showed significant inhibition of tumor growth and lymph node metastasis.

Metastasis of renal cell carcinoma to the bone developed osteolytic lesion through the stimulation of osteoclasts by locally secreted unknown factors (52). The bone mineral matrix contains numerous growth factors, such as insulin-like growth factor 1 and 2, transforming growth factor ß, acidic and basic forms of fibroblast growth factor, platelet-derived growth factor, and bone morphogenetic protein, which are released during normal bone remodeling, providing a fertile microenvironment for tumor cell colonization and proliferation (53,54). Furthermore, tumor cells release a variety of growth factors that promote bone resorption and increase the risk of skeletal complications. Agents targeting osteoclast have shown benefits in the treatment of cancer patients with bone metastases. For example, Bisphosphonates are potent inhibitors of osteoclast activity that have demonstrated efficacy in the treatment of bone metastases. Bisphosphonates bind avidly to the bone matrix and are released during bone resorption and, subsequently, internalized by osteoclasts where they interfere with biochemical pathways and induce apoptosis of osteoclasts (55). SU11248 receptor tyrosine kinase (RTK) inhibitor may be an effective and tolerated therapy to inhibit the growth of breast cancer bone metastases, with the additional advantage of inhibiting tumor-associated osteolysis (56). Nakagawa *et al* demonstrated that, *in vitro*, both osteoblast and osteoclast express significant levels of $VEGF_{165}$ and $VEGF_{121}$ isoforms, as well as VEGF-R1 and VEGF-R2 receptors. Bone resorption was affected by direct effects of VEGF on osteoclast survival and consequent increase of osteoclasts during neovascularization, probably through activation of VEGF-R1 and/or VEGF-R2 (57,58). In the present study, mOCPs expressed VEGF (data not shown), VEGF-R1 and VEGF-R2, and RBM1-IT4 cells also expressed VEGF (relatively higher level). Therefore, we speculated that TSU-68 may inhibit human clear cell-type RCC growing in the tibia of nude mice. However, TSU-68 did not inhibit the tumor growth (data not shown) in the tibia or preserve bone structure. As therapeutic effects of tyrosine kinase inhibitors against solid tumors bone metastasis models were produced when they were combined with chemotherapeutic agents in previous reports, we may consider combination of chemotherapeutic agents in our model.

As VEGF-R2-neutralizing polyclonal antibody did not inhibit VEGF-mediated migration of VEGF-R1 expressing human peripheral blood monocytes, whereas VEGF-R1 neutralizing monoclonal antibody significantly suppressed VEGF-induced migration of the monocytes (59) and Flt-1 tyrosine kinase domain-deficient mice lost the capacity for VEGF-dependent cell migration of peritoneal macrophages (60), the VEGF-R1 pathway might be the major stream in the function of osteoclasts and mOCPs. Together with our data, (Fig. 4), this suggests that VEGF-R1 may be a more important target and M-CSF may be another key factor for the novel therapy of osteoclastic bone resorption of clear cell-type RCC bone metastasis.

In conclusion, blockade of the VEGF-R2 pathway by TSU-68, protein tyrosine kinase receptor inhibitor targeting VEGF-R2, produced significant therapeutic effects on human renal cell carcinoma, RBM1-IT4, growing in the kidney of nude mice but there was no significant inhibition of tumor growth or preservation of bone structure against RBM1-IT4 implanted into the tibia of nude mice. Further study revealing the mechanism of interaction between tumor cells and the microenvironment and the modifying regimen, such as combination of chemotherapeutic agents or suppressor for functioning osteoclasts, should be considered.

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