Tumor Angiogenesis Promoted by *Ex vivo* Differentiated Endothelial Progenitor Cells Is Effectively Inhibited by an Angiogenesis Inhibitor, TK1-2

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Abstract

Neovascularization plays a critical role in the growth and metastatic spread of tumors and involves recruitment of circulating endothelial progenitor cells (EPC) from bone marrow as well as sprouting of preexisting endothelial cells. In this study, we examined if EPCs could promote tumor angiogenesis and would be an effective cellular target for an angiogenesis inhibitor, the recombinant kringle domain of tissue-type plasminogen activator (TK1-2). When TK1-2 was applied in the *ex vivo* culture of EPCs isolated from human cord blood, TK1-2 inhibited adhesive differentiation of mononuclear EPCs into endothelial-like cells. In addition, it inhibited the migration of *ex vivo* cultivated EPCs and also inhibited their adhesion to fibronectin matrix or endothelial cell monolayer. When A549 cancer cells were coimplanted along with *ex vivo* cultivated EPCs s.c. in nude mice, the tumor growth was increased. However, the tumor growth and the vascular density of tumor tissues increased by coimplanted EPCs were decreased upon TK1-2 treatment. Accordingly, TK1-2 treatment reduced the remaining number of EPCs in tumor tissues and their incorporation into the host vascular channels. In addition, overall expression levels of vascular endothelial growth factor (VEGF) and von Willebrand factor in tumor tissues were decreased upon TK1-2 treatment. Interestingly, strong VEGF expression by implanted EPCs was decreased by TK1-2. Finally, we confirmed *in vitro* that TK1-2 inhibited VEGF secretion of EPCs. TK1-2 also inhibited endothelial cell proliferation and migration induced by the conditioned medium of EPCs. Therefore, we concluded that EPCs, as well as mature endothelial cells, could be an important target of TK1-2. [Cancer Res 2007;67(10):4851–9]

Introduction

Neovascularization is required for the growth and spread of tumors, which recruit neighboring vessels and vascular endothelial cells to support their own blood supply (1, 2). Due to these characteristics of tumors, inhibition of tumor-induced neovascularization has been an effective anticancer approach. Recently, the existence of circulating endothelial progenitor cells (EPC) in the adult has been suggested (3–8) and increasing body of evidence has indicated that neovascularization involves the recruitment of EPCs as well as endothelial cells to tumor vasculature (6, 9, 10). Circulating EPCs are mobilized from the bone marrow into the bloodstream and contribute to new blood vessel formation during tissue ischemia, vascular trauma, and tumor growth (6, 11, 12). Clinical studies using EPCs have been started for neovascularization of ischemic organs (13, 14). Transplantation of *ex vivo* cultivated EPCs has been also reported to contribute to therapeutic neovascularization (15, 16) and home to the angiogenic tumor vasculature (17). Recently, EPCs or EPC-like cells have been proposed to improve angiogenesis by secretion of angiogenic cytokines, which might activate adjacent endothelial cells (18, 19). In this context, the modulation of recruitment and differentiation of these cells may be an efficient target for tumor angiogenesis.

Although the evidence that EPCs promote tissue repair is strong, the molecular and cellular mechanisms underlying EPC recruitment and differentiation are not yet understood. Recruitment and incorporation of EPCs require a coordinated sequence of multistep events, including adhesion, migration, chemoattraction, and differentiation to endothelial cells (20). In embryonic EPCs, such multistep has been visually captured and defined (21). More recently, bone marrow–derived $CD34^+$ cells of human species also showed $\alpha_4\beta_1$-mediated attachment to endothelial cells at tumor periphery and then migration into tumor tissues (22). These studies suggest several inhibition targets of EPCs. In fact, some inhibitors have been tested for their effects on EPC mobilization or *in vitro* differentiation. For an example, endostatin has been found to inhibit mobilization of circulating EPCs from bone marrow in mice bearing lymphoma xenografts (23) and vascular endothelial growth factor (VEGF)–induced mobilization of EPCs (24). Interestingly, angiostatin more sensitively inhibits human EPCs than mature endothelial cells *in vitro* (25). Previously, we have identified TK1-2, the recombinant two-kringle domain of tissue-type plasminogen activator, as an angiogenesis inhibitor with kringle architecture despite of low sequence identity with angiostatin (26). It also suppresses tumor growth *in vivo* in xenograft animal models of lung and colon cancers (27, 28). Until now, the exact molecular and cellular mechanism of this fragment remains unclear. Although antiangiogenic molecules specifically target endothelial cells, their effects on EPCs are largely unknown. Therefore, we have examined the effects of TK1-2 on EPCs *in vitro* and *in vivo* by using *ex vivo* cultivated EPCs more likely to commit to endothelial-like cells, and an A549 xenografted animal model coinjected with cultured EPCs, to investigate their effects on the process of vascular formation in tumor tissue rather than the mobilization process.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

***Ex vivo* cultivation of EPCs from cord blood.** The institutional review board at the Catholic University of Korea College of Medicine approved all protocols. Cord blood (80–100 mL) was obtained from donors with informed consent. Mononuclear cells (MNC) were isolated from the cord blood using the Ficoll-Hypaque density gradient centrifugation method as described before (29). Isolated MNCs (1×10^7 cells per well) were plated into a six-well plate coated with 0.1 mg/mL human fibronectin (Sigma) and incubated for 3 days. After that, nonadherent cells were removed and selected adherent cells were maintained in M199 medium supplemented with 20% fetal bovine serum (FBS; Life Technologies), 30 μg/mL endothelial cell growth supplements (Sigma), 90 μg/mL heparin (Sigma), and 1% antibiotics for 3 to 4 days.

Cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described before (26). Human lung cancer A549 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics.

Treatment of TK1-2 under *ex vivo* culture condition. Yeast-derived TK1-2 was prepared by expression in *Pichia pastoris* as previously described (27). Isolated MNCs were incubated with TK1-2 at the indicated concentration for 30 min and then plated on eight-chamber wells coated with fibronectin (2,500 cells/mm^2). Then, the cells were incubated in M199 supplemented with 10% FBS and 90 μg/mL heparin. After 3 days of culture, nonadherent cells were removed by washing with PBS, and new medium and TK1-2 were again added to each well. The culture was maintained through 7 days and examined for cell number and shape. Four randomly selected fields per well were evaluated.

To confirm EPC phenotypes, binding of FITC-labeled *Ulex europaeus* agglutinin 1 (UEA-1) and ac-LDL uptake of adherent cells were also determined at day 7 as described before (29).

Cell-cell adhesion. HUVECs were seeded in 48-well plates by adding 2.5×10^4 cells per well 24 h before this assay. Confluent HUVEC monolayers were stimulated for 12 h with endothelial growth medium-2 SingleQuots (EGM-2 SingleQuots) containing endothelial basal medium-2 (EBM-2), 2% FBS, and growth factors. *Ex vivo* cultivated EPCs (for 7 days) were labeled with CM-1,1′-dioctadecyl-3,3,3′,3′-tetramethyliodocarbocyanine (CM-DiI; Molecular Probe) at a concentration of 2.5 μg/mL in PBS for 5 min at 37°C and for 15 min at 4°C. DiI-labeled EPCs (1×10^5/well) preincubated with TK1-2 for 30 min were added to the HUVEC monolayers. After 3 h of incubation (37°C), the plates were washed twice with EBM-2 to remove nonadherent cells. The DiI-labeled EPCs adhering to HUVEC layer were quantified in triplicates.

Cell-matrix adhesion. Ninety-six–well plates were coated overnight (4°C) with 0.1 mg/mL fibronectin and blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. *Ex vivo* cultivated EPCs (for 7 days) preincubated with TK1-2 in M199 medium at the indicated concentrations for 30 min were seeded at 8×10^3 cells per well. After removal of nonadherent cells by two washing steps, adhesion was quantified in triplicate by counting adherent cells in four randomly selected fields per well.

Migration. Migration was measured by a modified Boyden chamber assay using a 48-well chemotaxis chamber (Neuroprobe, Inc.). After *ex vivo* cultivated EPCs (for 7 days) were detached by 1 mmol/L EDTA and suspended in M199 medium, 5×10^4 cells in 56 μL of serum-free medium were incubated with TK1-2 for 30 min and then added to each well in the upper chamber. The filter was placed over a 48-well bottom chamber containing 10 ng/mL of VEGF in serum-free M199 with 0.1% BSA. The assembled chamber was incubated for 6 h at 37°C with 5% CO_2 to allow cells to migrate through the filter. The membrane was removed from the chamber and stained with Diff-Quik solution (Sysmex, Kobe, Japan). Nonmigrated cells on the upper surface of the membrane were removed. The number of migrated cells was counted in random five fields (×200) of each well. Each experiment was done in triplicate.

The conditioned medium was prepared by incubating 4-day-old EPCs in 1% FBS-containing M199 medium for 3 days, and then filled in bottom chambers. For testing inhibitory activity, HUVECs were preincubated with TK1-2, VEGF-neutralizing antibody (5 μg/mL, anti-human VEGF polyclonal antibody, R&D Systems), or control isotype IgG (5 μg/mL; Zymed), in M199 medium in an incubator for 30 min before being added to the upper chamber.

Animal studies. Six-week-old male mice (BALB/c Slc-nu/nu; Japan SLC) were s.c. implanted at the right flank with A549 cells (1×10^7) alone or along with *ex vivo* cultivated EPCs (1×10^6). Before implantation, *ex vivo* cultivated EPCs (for 5–7 days) were obtained from the blood of several donors to have enough number of the cultured cells for the animal study. Then, they were pooled and labeled with PKH26 red (Sigma) according to the manufacturer's instructions. Three days after implantation, the mice of the treatment group were injected i.p. every day for 17 days with TK1-2 protein (30 mg/kg), whereas control groups of mice were injected with sterile PBS. The size of tumor in all groups was measured every 2 days using a caliper, and the volume of tumors was determined using the formula width^2 × length × 0.52. Institutional guidelines for animal welfare and experimental conduct were followed.

For the second experiment, nonlabeled EPCs were coimplanted and the mice (A549, A549+EPC) were treated with TK1-2 for a longer period to evaluate the effect of TK1-2 on EPC kinetics for tumor growth. All the other experiment details were carried out identically to the above experiment, except increasing the dose of TK1-2 from 30 to 50 mg/kg/d after 24 days of treatment.

Histologic and immunohistochemical analysis. This analysis was done as described before (27). Rabbit polyclonal antibody against human von Willebrand factor (vWF; DAKO) and mouse monoclonal antibody against human VEGF (clone 26503, R&D Systems) were used. The negative control study for immunohistochemistry was done simultaneously without each antibody.

Immunofluorescent staining. For cryosectioning, the tumors were fixed in 4% paraformaldehyde, incubated in 30% sucrose in PBS overnight at 4°C, embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek Europe), and frozen at −70°C. Tissue sections, cut with cryostat (Leica CM1800) at a thickness of 8 μm and dried on glass slides, were rinsed in PBS and then blocked with 10% normal goat serum/0.3% Triton-X in PBS for 1 h. Tissue sections were incubated with mouse anti-human VE-cadherin monoclonal antibody (clone BV6, Chemicon) or human VEGF specific polyclonal goat antibody (R&D Systems) as primary antibodies overnight at 4°C. Samples were washed and incubated with FITC-conjugated anti-mouse or conjugated anti-goat antibodies (Molecular Probes) for 1 h at room temperature. Sections were treated with biotinylated *Bandeira simplicifolia* lectin B4 (Vector Laboratories) and then with fluorescein streptavidin to identify murine endothelial cells according to the manufacturer's instructions. Finally, the sample was rinsed in PBS and mounted (DABCO; Sigma). Figures were taken using an inverted fluorescent microscope.

Digital image analysis. The digital images were analyzed as previously described (30). Digital images were acquired from Olympus AX70 fluorescence microscope using DP70 camera via DP Manger version 2, 1, 1, 163 software (Olympus) and stored as TIFF files. The PKH26-labeled cells were identified by red fluorescence, and *B. simplicifolia* lectin B4–binding cells were identified by green fluorescence. Fields were chosen randomly from various section levels to ensure objectivity of sampling. Images were analyzed in Adobe Photoshop (Adobe, Inc.) or ImageJ v.1.34s.[3] The fraction of PKH26 (red) positive pixels or *B. simplicifolia* lectin B4 (green) positive pixels was binarized to black and white and a common threshold was set such that correct vascular morphology was represented with a minimum background noise. The percentage of white pixels, representing each positive staining, was determined by histogram analysis [fluorescence intensities ranging from black (0) to white (255)]. *B. simplicifolia* lectin B4–stained images were further analyzed to investigate the changes in vessel architecture using ImageJ software. Images were skeletonized, which reduced all vessels to a single pixel width. Total length of vessels was determined.

Data and statistical analysis. All data are presented as mean ± SE. *In vitro* experiments were done in triplicate. Statistical significance was

[3] http://rsb.info.nih.gov/ij/

evaluated by means of Sigma plot t test. $P < 0.05$ was regarded as being statistically significant.

ELISA for VEGF levels. The MNCs (4×10^6 cells per well, six-well plate) were incubated under *ex vivo* culture condition for 4 days and then floating cells were removed by washing with PBS. Then, the remaining adherent cells were incubated in M199 containing 1% FBS. To assess whether TK1-2 itself inhibits VEGF secretion of EPCs, TK1-2 was added to the medium of a treatment group. After 3 days, only the culture medium was stored at −70°C, after being filtered with a 0.22-μm filter. Before the assay, the conditioned medium was concentrated five times using Centricon YM3 (Millipore). The concentrated medium (200 μL) was added into the VEGF antibody precoated 96-well plates and VEGF levels were measured by a VEGF ELISA kit (DVE00, R&D Systems) according to the manufacturer's instructions.

Proliferation assay. Proliferation assay was done using the conditioned medium from EPC culture by a [^{3}H]thymidine incorporation assay as described before (26). HUVECs were stimulated by the conditioned medium (1×) or VEGF 10 ng/mL in the absence or presence of TK1-2 (0.5 or 1 μmol/L) or in the presence of VEGF-neutralizing antibody or isotype IgG antibody (5 μg/mL).

Western blot analysis. Western blot analysis for tumor tissues was done as described before (31).

Results

TK1-2 inhibits *in vitro* adhesive differentiation of EPCs. In the previous studies, we confirmed that total MNCs isolated from human cord blood give rise to spindle-shaped and endothelial-like cells when cultured on a fibronectin matrix in M199 containing 10% to 20% FBS for 7 days (29, 32). EPCs were characterized as adherent cells double positive for DiI-ac-LDL uptake and UEA-1 lectin binding. We also showed by immunocytochemistry that cultured EPCs express several important endothelial lineage markers, including CD31, VE-cadherin, vWF, and KDR/Flk-1. Using this established culture system, we examined whether TK1-2 affects the differentiation of EPCs, which exist in a subfraction of MNCs of human cord blood.

Incubation of isolated MNCs with TK1-2 on fibronectin matrix for 7 days decreased the number of adherent cells in a dose-dependent fashion (Fig. 1*A*). Most of the adherent cells of untreated group are spindle shaped, whereas the adherent cells treated with TK1-2 changed their cell morphology into round shape at high concentrations (Fig. 1*B*). In addition, the number of adherent cells double-positive for ac-LDL(+)-lectin(+) (yellow) was also decreased dose-dependently (Fig. 1*A*). The double-positive cells significantly reduced at 0.1 μmol/L, and IC_{50} was ~1 μmol/L. Because TK1-2 did not induce cell death against MNCs and cultured EPCs *in vitro* (Supplementary Fig. S1*A* and S1*B*), these results indicate that TK1-2 inhibits adhesion of early undifferentiated EPCs, a subset of MNCs, on matrix, thereby inhibiting their adhesive differentiation into endothelial-like cells.

Inhibition of *ex vivo* cultivated EPC functions by TK1-2. Because EPCs exist in MNCs at a very low ratio and their pure isolation is technically unapproachable, we examined the inhibitory activity of TK1-2 further using *ex vivo* cultivated EPCs, which are partially selected on fibronectin-coated dishes. Before a migration assay, we confirmed that *ex vivo* cultivated EPCs expressed VEGFR-2 (KDR) by fluorescence-activated cell sorting and immunostaining (data not shown) and that EPC migration was induced dose dependently by VEGF. Inhibition assays were conducted using 10 ng/mL of VEGF allowing maximum migration of EPCs. As shown in Fig. 2*A*, TK1-2 effectively inhibited *ex vivo* cultivated EPC migration induced by VEGF in a dose-dependent manner at a concentration range of 0.05 to 0.5 μmol/L.

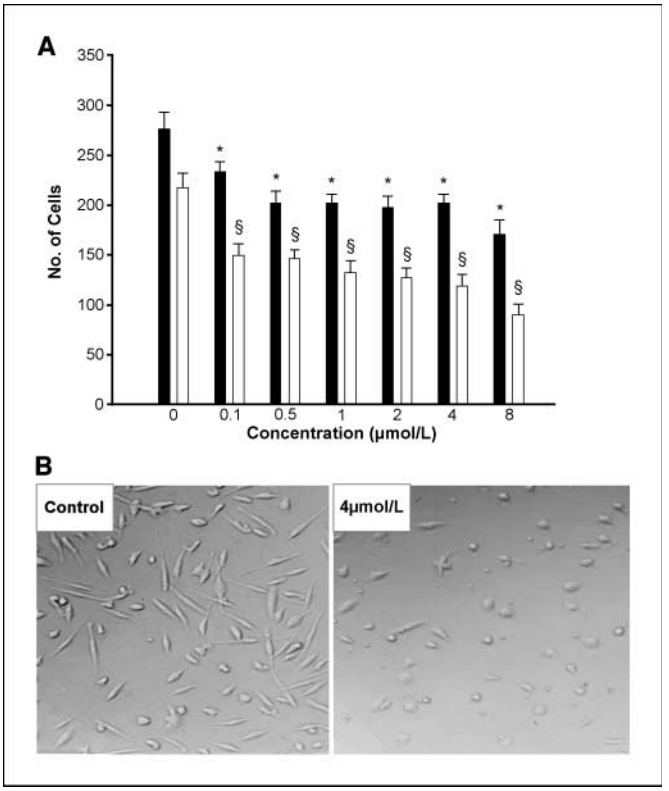

Figure 1. Effect of TK1-2 on *in vitro* EPC differentiation on fibronectin matrix. *A,* isolated MNCs were cultured on a fibronectin-coated plate under treatment with TK1-2 for 7 d at the indicated concentrations. TK1-2 dose-dependently decreased the number of adherent cells (*black columns*) and seemed to change cell morphology (*B*). *, $P < 0.05$, compared with no treatment of TK1-2. Adherent ac-LDL/lectin double-positive cells were also counted (*white columns*). §, $P < 0.05$, compared with no treatment of TK1-2. *B,* representative digital microscopic images of adherent cells. Magnification, ×200.

Next, we assessed whether TK1-2 inhibits the adhesion of *ex vivo* cultivated EPCs to activated endothelial cells, or to extracellular matrix, fibronectin. Fibronectin has been reported to be expressed at a high level at the endothelium of tumor periphery in a breast cancer xenografted model (22). When the cultured EPCs were incubated on fibronectin-coated plates for 30 min after pretreatment of TK1-2, a significant decrease in the number of adherent cells was observed upon TK1-2 treatment (Fig. 2*B*). The number of adherent cells decreased dose dependently, and a maximal effect was achieved at 2 μmol/L (at >2 μmol/L, no significant difference was noted in the number of adherent EPCs). We also investigated whether TK1-2 could affect adhesion of EPCs to endothelial cell monolayer. The HUVECs activated with serum and growth factors showed the increased adhesion of EPCs to a greater extent than quiescent HUVECs, whereas the addition of TK1-2 significantly inhibited EPC adhesion to HUVECs dose dependently (Fig. 2*C*). Therefore, all the results show that TK1-2 effectively inhibits the important functions for vessel formation, migration, and adhesion of *ex vivo* cultivated EPCs.

***In vivo* tumor growth and angiogenesis are enhanced by *ex vivo* cultivated EPCs and inhibited by TK1-2.** On the basis of the *in vitro* experiment results, we set out the *in vivo* experiment whether TK1-2 inhibits the contribution of *ex vivo* cultivated EPCs to tumor neovascularization and growth. *Ex vivo* cultivated EPCs were labeled with a red fluorescent cell tracking dye (PKH26) and coinjected with A549 cancer cells to the flank of nude mice. In this

model, TK1-2 was systemically administered 3 days after implantation. Figure 3*A* shows each animal group and a period of TK1-2 treatment, and Fig. 3*B* indicates relative tumor growth for each group. TK1-2 treatment group showed inhibition of tumor growth compared with the control and EPC-coinjected control groups, corresponding to the antitumor effects of TK1-2 reported previously (27). The tumor growth seemed to be slightly promoted by EPC coimplantation. However, due to the limitation of experimental period, significant promotion of tumor growth by EPCs at later time points could not be observed. On the day 19 of implantation, we observed that average tumor volume in mice of TK1-2 treatment was $\sim 330 \pm 58$ mm^3 compared with 657 ± 95 mm^3 in control group ($P < 0.05$) and 735 ± 122 mm^3 in the EPC-coinjected group ($P < 0.05$). Thus, TK1-2 treatment decreased tumor growth by $\sim 55\%$ compared with EPC-coinjected group.

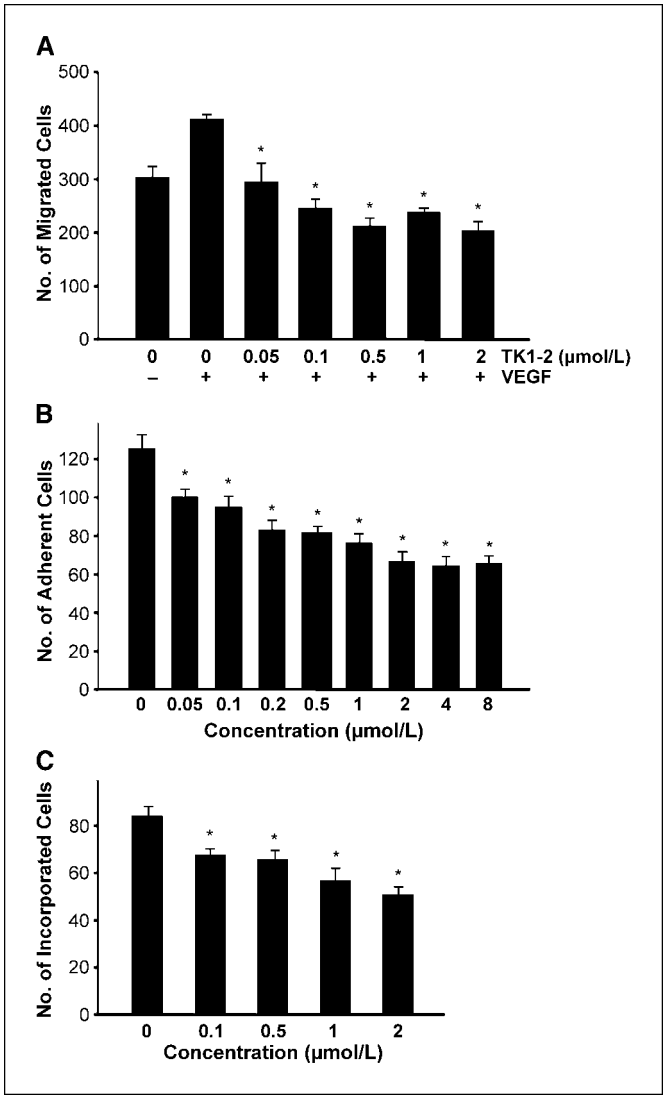

Figure 2. Effects of TK1-2 on migration and adhesion of *ex vivo* cultivated EPCs. *A,* migration. Migration of *ex vivo* cultivated EPCs was induced by 10 ng/mL VEGF in the presence or absence of TK1-2 after pretreatment for 30 min. *B,* cell-matrix adhesion. After 30 min of incubation with TK1-2 at the indicated concentrations in serum-free M199 medium, *ex vivo* cultivated EPCs were plated into fibronectin-coated plate and incubated for 30 min at 37°C. After removing nonadherent cells, the remaining adherent cells were counted. *C,* cell-cell adhesion. HUVECs were incubated for 12 h with EGM-2 medium. *Ex vivo* cultivated EPCs were labeled with a DiI fluorescent marker (*red*). The labeled cells (1×10^5) pretreated with TK1-2 for 30 min were added to each well containing HUVECs and incubated for 3 h at 37°C in the presence of TK1-2. After washing out nonadherent cells, the remaining adherent cells were counted. *, $P < 0.05$, compared with no treatment of TK1-2.

When we evaluated vessel density of tumor tissues of each group by staining host vessels with *B. simplicifolia* lectin B4, the coimplantation with cultured EPCs led to increase of vessel density, whereas TK1-2 treatment markedly reduced the increment of host vessel number (Table 1; Fig. 3*C*). Although the same number of cultured EPCs was coimplanted, the number of EPCs residing in tumor tissues on day 19 was also decreased upon TK1-2 treatment (Fig. 3*D*). A representative fluorescent microscopic feature of A549 + EPC tumor showed peripherally scattered EPCs in red (Supplementary Fig. S2). The central part of tumor tissue was almost acellular and fibrosed with sparce EPCs. PKH26-labeled EPCs were rarely incorporated into the vessels found in the core of the tumors; rather, they were usually incorporated into the peripheral vessels. Some labeled cells exist around vessels, not incorporating into the vasculatures. In the EPC-coinjected control group, the implanted EPCs were well aggregated and spread to form vascular channels, which were clearly incorporated with host vessels (Fig. 4*A*). However, in the case of EPC coimplantation followed by TK1-2 treatment, the remaining cell number of EPCs until day 19 was much less than the nontreated group, and the EPCs were poorly incorporated into the host vascular channels compared with the nontreated group. The TK1-2–treated group showed relatively scattered EPCs in the parenchymal tumor tissue, whereas the nontreated group showed highly aggregated EPCs. The latter was more likely to form vessels than the former (Figs. 3*D* and 4*A*). The PKH26-labeled cells in the tumor tissues were stained with 4′,6-diamidino-2-phenylindole and costained with human specific anti–VE-cadherin antibody, showing that the labeled cells were human type–cultivated EPCs (Fig. 4*A*). The PKH26-labeled cells were also detected as a part of the tumor vessels that were host derived and stained with mouse vessel–specific *B. simplicifolia* lectin B4.

After EPC coimplantation, the tumor tissue showed numerous EPCs incorporated into vessels and also showed increased positive reaction of VEGF (Fig. 4*B*). The VEGF expression was strongly detected usually around the incorporated EPCs (Fig. 4*C*). In the control group (without EPC coimplantation), there was slight VEGF staining in tumor tissues by immunofluorescent method but not enough to notify (data not shown). Such strong expression by the incorporated EPCs was reduced by TK1-2 treatment, in part, through the decreased number of residing EPCs or by still unknown mechanisms (Fig. 4*C*). Accordingly, vWF staining of human and murine endothelial cells also showed the decrement of positive reaction upon TK1-2 treatment (Fig. 4*B*), indicating again that TK1-2 inhibits neovascularization in tumors. In addition, TK1-2 treatment increased the terminal deoxyribonucleotide transferase–mediated nick-end labeling–positive cells in tumor tissues compared nontreated two control groups, corresponding to the previous results that TK1-2 or PK1-3 induced apoptosis in tumor tissues (refs. 27, 28, 31; Supplementary Fig. S3). However, we were not able to detect whether TK1-2 induced apoptosis in EPCs *in vivo*.

In a second set of experiments, nonlabeled EPCs were used for the coimplantation with A549 cancer cells and tumor-bearing mice were treated for a longer period; in an attempt to compare the efficacy of TK1-2 between two groups, A549-only and A549 + EPC groups. In this experiment, the growth rate of A549-only control group was slow compared with the previous experiment. Although

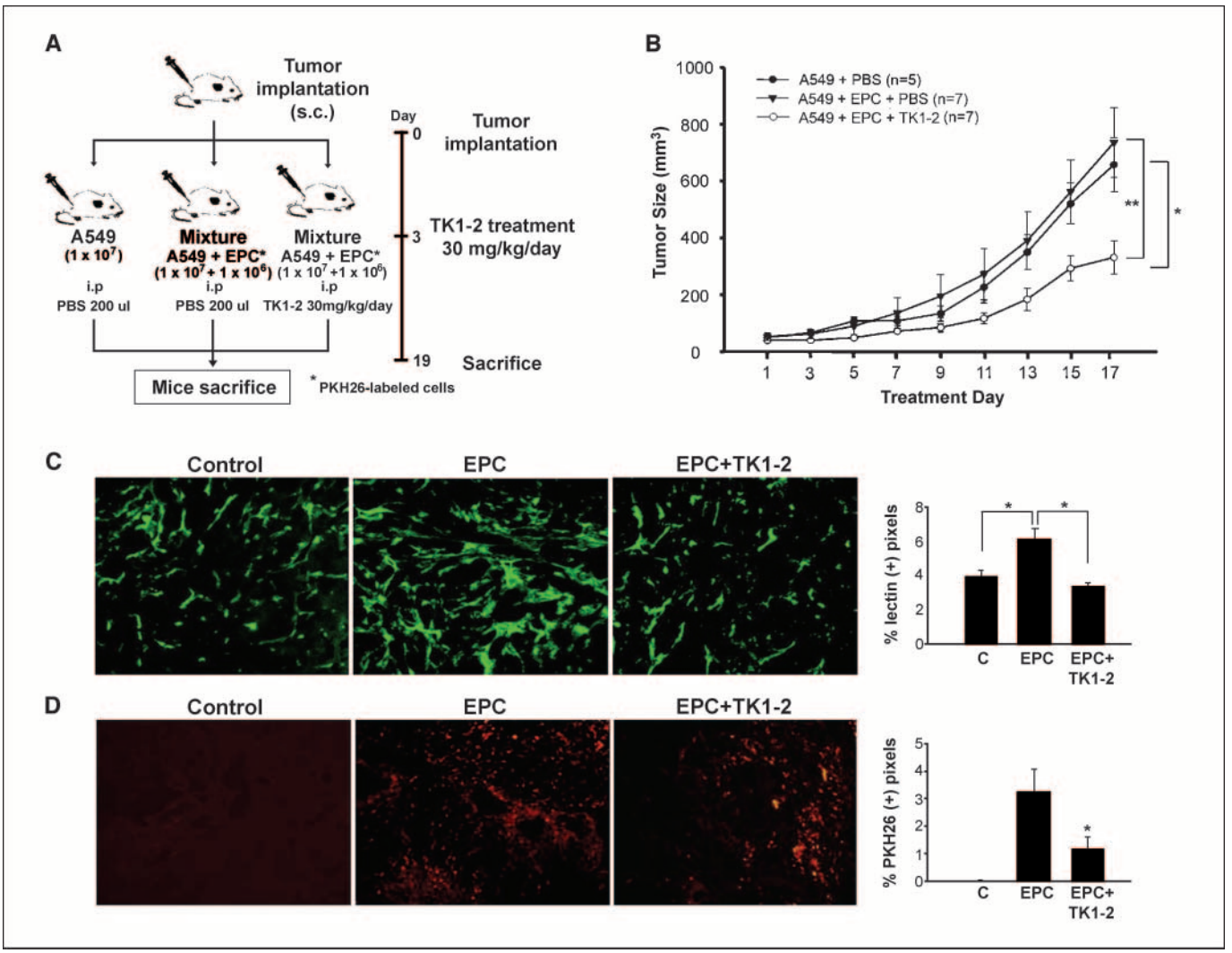

Figure 3. TK1-2 inhibits tumor growth and vessel formation in an A549 xenograft model coinjected with *ex vivo* cultivated EPCs. *A,* mice were injected s.c. with either 1×10^7 tumor cells or a mixture of 1×10^7 A549 cells and 1×10^6 PKH26-labeled EPCs. After 3 d, the mice were i.p. administered with PBS (control) or 30 mg/kg recombinant TK1-2 daily for 17 d. *B,* tumor volume of each group was measured. *, $P < 0.05$, A549 control versus TK1-2 treatment; **, $P < 0.05$, A549 + EPC control versus TK1-2 treatment. *C, B. simplicifolia* lectin B4 staining of tumor tissues of each group was done to stain the murine, host vessels. Only the green fluorescence was captured for *B. simplicifolia* lectin B4. *D,* implanted, red fluorescent EPCs of each tumor tissue were photographed and counted. No fluorescent cells were detected in control group. Fields were chosen randomly from various section levels to ensure objectivity of sampling. Images were analyzed in Adobe Photoshop or ImageJ v.1.34s. The fraction of PKH26 (*red*)–positive pixels or *B. simplicifolia* lectin B4 (*green*)–positive pixels was binarized to black and white, and a common threshold was set such that correct vascular morphology was represented with a minimum of background noise. *, $P < 0.05$.

the same number of the cultured EPCs was implanted, the enhancement of tumor growth by EPCs was more significant on day 31 compared with that by labeled EPCs (Figs. 3 and 5). Although PKH26 is being used to monitor cell division, the process of staining cells with PKH26 seemed to affect EPC activities. Therefore, in the case of A549 + EPC group, suppression level of tumor growth by TK1-2 was lower on day 25 (33% suppression with no significance) compared with the above data (55%) when

Table 1. Summary of computer-generated scoring variables

Measurement	Control (no EPC)	EPC	EPC + TK1-2
Lectin-positive pixel count*	123,689 ± 10,615	191,711 ± 19,473	105,637 ± 5,920
Total vessel length †	10,407 ± 857	15,291 ± 1,235	9,518 ± 525

*After binarization of the images, vessel density was estimated by scoring the total number of black pixels per field. Results show the mean black pixel count per image ± SD.

†Mean total vessel length per image in ± SD as determined after skeletonization of the images.

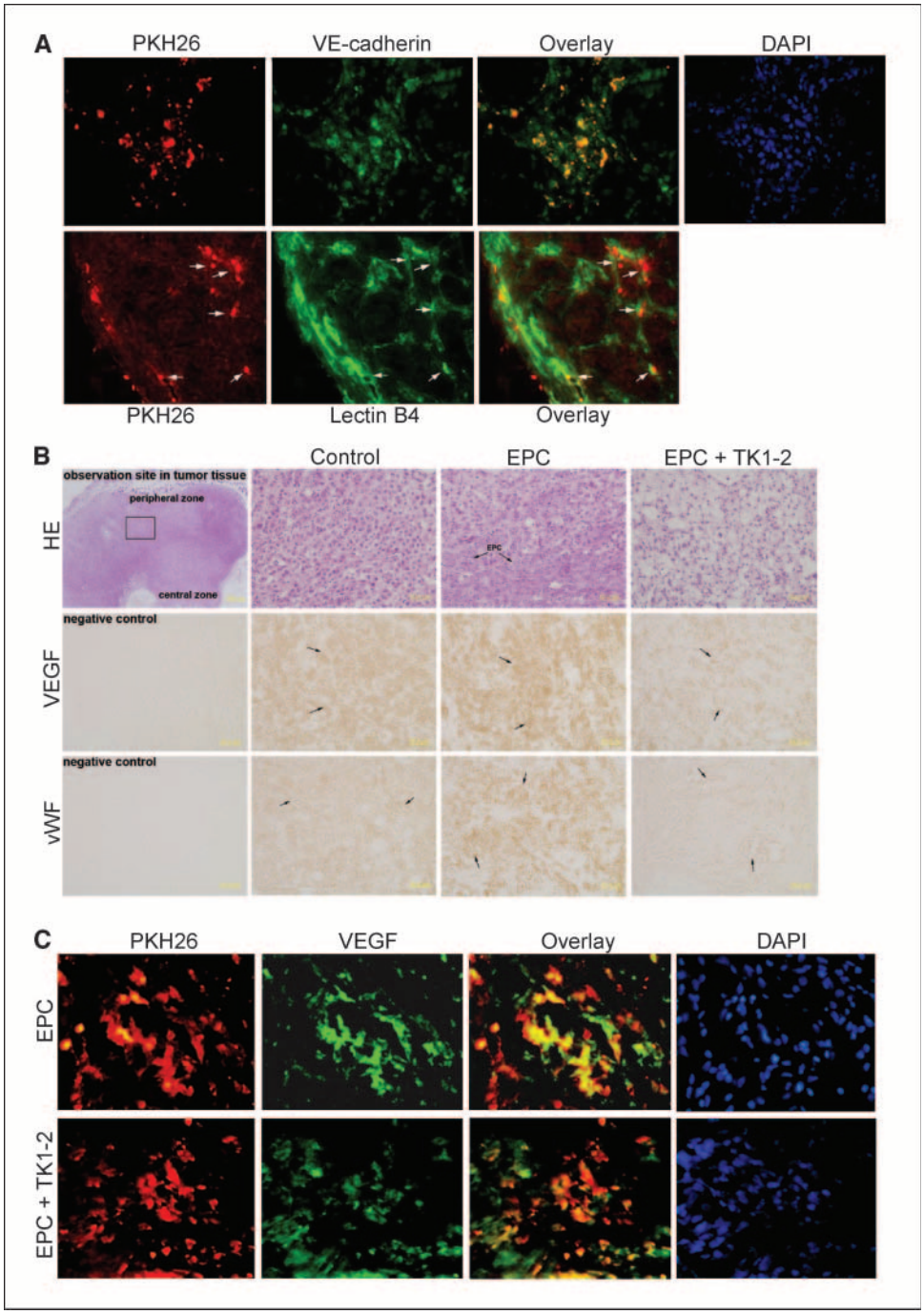

Figure 4. Immunohistochemical analysis of tumor tissues dissected from the mice. *A,* red fluorescent signals indicate the localization of coimplanted labeled EPCs. Several strongly positive PKH26-labeled cells covering the luminal surface were detected in animals injected with *ex vivo* cultivated EPCs. Sections were labeled with green fluorescent monoclonal anti-human VE-cadherin to visualize implanted EPCs. Red cells were consistent with green cells in overlay image. PKH26-labeled cells were also shown to incorporate into the *B. simplicifolia* lectin B4–stained host vessels. *B,* tumors embedded in paraffin were sectioned (4 μm thickness), and then stained with immunohistochemical staining using monoclonal antibodies against VEGF and polyclonal antibody against vWF. *Box,* the microscopic observation site chosen from the central area between peripheral and central zones of each tumor tissue. The tumor tissue implanted with EPCs and followed by TK1-2 treatment showed more loosely arranged parenchymal tumor cells and less immunoreactions of VEGF and vWF (*arrows*) compared with the control and EPC coimplanted tumors. vWF staining of human and murine endothelial cells also showed the decrement of positive reaction upon TK1-2 treatment. *C,* after EPC coimplantation, the tumor tissue containing numerous EPCs incorporated in vessels showed increased positive reaction of VEGF. VEGF expression was detected strongly around the incorporated EPCs. Such strong expression by the incorporated EPCs was inhibited by TK1-2 treatment.

tumor-bearing mice were administered at a dose of 30 mg/kg/d. However, when the dose was increased to 50 mg/kg/d from day 25 to day 30, the inhibitor effect of TK1-2 seemed significant on day 31 after 6 days of treatment (36%). TK1-2 suppressed more potently the growth of A549-only tumors (43%). The Western blot analysis of the tumor tissues obtained from this experiment also showed that EPC coimplantation increases VEGF expression, whereas TK1-2 potently suppressed VEGF expression in the tumor tissues of A549 and A549 + EPC groups, corresponding to the previous immunohistochemical data of the above-labeled EPC coimplantation experiment. In addition, the tissue extracts of tumors showed markedly contrast red colors due to the different levels of hemoglobin (Supplementary Fig. S4), indicating that EPC coimplantation increases tumor vessel density and TK1-2 treatment markedly inhibit this increment.

TK1-2 inhibits VEGF secretion from EPCs and conditioned medium–induced endothelial cell functions. From the immunohistochemical analysis of tumor tissues, we found that TK1-2 administration resulted in down-regulation of VEGF expression in A549 tumor tissues and such effect was especially accentuated in the coimplanted EPCs, which expressed VEGF at a higher level than the tumor cells *in vivo*. Thus, we examined if TK1-2 would directly inhibit VEGF expression in EPCs using *ex vivo* cultivation system. Indeed, the level of VEGF in the 5× conditioned medium

secreted by EPCs was ~230 pg/mL by an ELISA assay, and such VEGF secretion from EPCs was decreased upon TK1-2 treatment at 1 μmol/L (Fig. 6*A*).

Because EPCs seem to promote tumor growth by expression and secretion of VEGF and other factors, we tested if TK1-2 could inhibit endothelial functions induced by EPC-derived growth factors. In a thymidine incorporation assay, TK1-2 dose dependently suppressed endothelial cell growth induced by the conditioned medium of EPCs (Fig. 6*B*). Neutralizing antibody against VEGF, but not isotype IgG control, also inhibited such endothelial cell growth, indicating that one of the major EPC-derived growth factors is VEGF. The endothelial cell growth was profoundly increased by the conditioned medium (3.2-fold) compared with single treatment of VEGF (10 ng/mL). Considering the concentration of VEGF (~50 pg/mL) in the conditioned medium (1×), other growth factors derived from EPCs also seemed to promote endothelial cell growth potently and their stimulating activities might be also effectively inhibited by TK1-2. Next, the endothelial cell migration using the conditioned medium of EPCs was examined in a Boyden chamber migration assay. We found that TK1-2 also dose dependently inhibited HUVEC migration induced by the conditioned medium. In the same setting, VEGF-neutralizing antibody could inhibit HUVEC migration induced by the conditioned medium, but not isotype IgG control (Fig. 6*C*). Thus, these results suggest that TK1-2 does not only inhibit VEGF secretion from EPCs, but also effectively inhibits endothelial functions induced by growth factors (i.e., VEGF derived from EPCs).

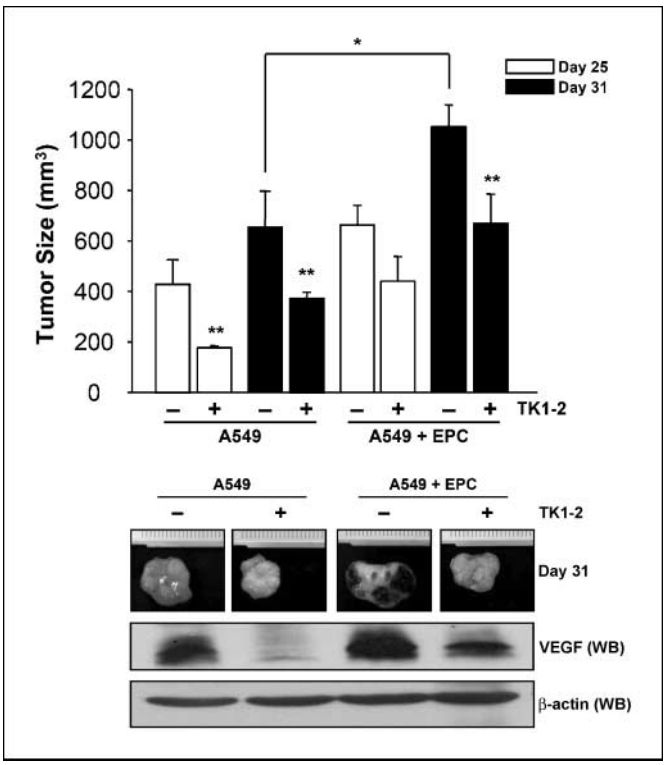

Figure 5. Enhancement of tumor growth and VEGF expression by coimplanted EPCs and its inhibition by TK1-2. Without labeling the cultivated EPCs with fluorescence dye, A549 cancer cells and EPCs were implanted s.c. in nude mice (n = 6 for each group). Three days after implantation, TK1-2 was administered i.p. at a dose of 30 mg/kg every day until day 24, and then at a dose of 50 mg/kg for another 6 d. The tumor volume was compared on day 25 and day 31. *, $P < 0.05$, A549 versus A549 + EPC; **, $P < 0.01$, control versus TK1-2 treatment. The representative pictures of the tumors removed from the mice are shown for each group. The Western blot (*WB*) analysis was done using the tumor tissue obtained from each group.

Discussion

Although there were some discrepancies already reported, the role of EPCs in vessel formation in adult ischemia and tumor has become widely accepted (9). $CD34^+$ cells, or $CD133^+$ cells have been used as a fraction more selective for EPCs in experimental studies. However, $CD34^-$ cells, $CD34^-/CD14^+$, or $CD14^-$ cells have been claimed for exhibiting incorporation into neovasculature in ischemic tissues (33, 34). In case of $CD14^+$ cells, without *ex vivo* expansion, no functional improvement of neovascularization was detected in ischemic tissues (34). Thus far, although the identity of EPCs has not been clear yet, the *ex vivo* cultivated EPCs, which already commit to differentiation at some levels, have been also proved to contribute to tumor vessel formation (17, 35). *Ex vivo* cultivated EPCs derived from MNCs of human peripheral blood or cord blood have been successfully shown to incorporate into neovasculature and contribute to functional recovery of ischemic tissue (15, 16). In this study, we have shown that *ex vivo* cultivated EPCs derived from human cord blood contributed to new vessel formation in tumor tissues of A549 xenografted mice, when coinjected with cancer cells. In addition, we also confirmed that EPC implantation promotes tumor growth through *de novo* angiogenesis. Finally, through this model, we found that TK1-2 inhibits the multisteps of vessel formation by EPCs and more importantly suppresses the VEGF expression of EPCs.

Angiostatin, with similar structure to TK1-2, was found to inhibit human EPC growth in culture by 75%, while having no effect on endothelial cell growth (25). However, in this study, an *in vivo* demonstration of angiostatin inhibiting vasculogenesis in a vasculogenic tumor model was lacking. Another naturally occurring endostatin has been found to block EPC mobilization increased by VEGF (24). Endostatin was also shown to increase EPC apoptosis. Again, *in vivo* effects of this agent on tumor vasculogenesis were not addressed. Therefore, this study showed for the first time that angiogenesis inhibitor inhibits tumor vasculogenesis by EPCs *in vivo*.

Because VEGF increases EPC mobilization from bone marrow into circulation, decrement of VEGF expression can be a critical control point (11, 36). In fact, when the effects of the VEGF-specific antibody, Bevacizumab, on the number of circulating EPCs in six patients with rectal adenocarcinoma were investigated, the amount of $CD133^+$ EPCs decreased in all patients, and all six patients experienced tumor regression (36, 37). Interestingly, TK1-2 treatment decreased VEGF expression in tumor tissues with coimplanted EPCs. In the previous studies, we have shown the similar pattern of VEGF suppression in tumor tissues upon TK1-2 treatment in several tumor models without EPC coimplantation. Similar results were also shown in angiostatin fragment treatment in a U87 glioma model (31). Endostatin and angiostatin have been also reported to inhibit tumor growth by modulation of VEGF expression in tumor cells *in vitro* and *in vivo*, with unknown mechanisms (37). This notion was also supported by the previous report that angiostatin down-regulates VEGF expression in the retina in rats with oxygen-induced retinopathy or with streptozotocin-induced diabetes, but not in normal rats (38). In this study, we showed that TK1-2 inhibits VEGF expression of EPCs *in vitro* and *in vivo*. Because the labeled EPCs strongly react with anti-VEGF antibody over the background level of VEGF expression by A549 cells, EPCs are expected to be a major source of VEGF

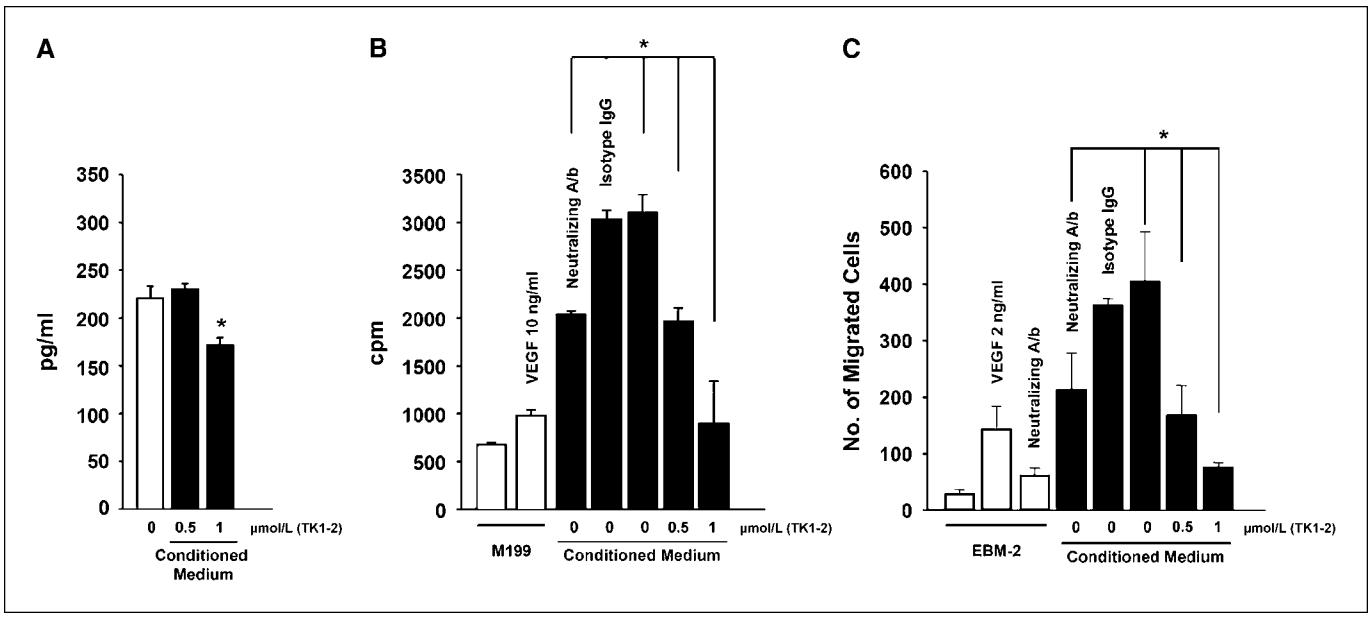

Figure 6. TK1-2 inhibits secretion of VEGF from EPCs as well as conditioned medium–induced endothelial cell functions. *A,* the conditioned medium of EPCs was measured for VEGF level by a VEGF ELISA kit. Upon TK1-2 treatment, VEGF secretion from EPCs in *in vitro* culture was reduced. *B* and *C,* the conditioned medium potently stimulated endothelial cell proliferation (*B*) and migration (*C*), which were inhibited by TK1-2– or VEGF-neutralizing antibody, but not by isotype IgG control, suggesting that TK1-2 inhibits the endothelial functions induced by angiogenic factors including VEGF secreted from EPCs. *, $P < 0.01$, compared with no treatment of TK1-2.

secretion *in vivo*. Thus, inhibition of VEGF expression of EPCs by TK1-2 may provide a partial mechanism for the suppression of tumor angiogenesis by TK1-2.

Through observation of the tumor tissue sections, we found that the TK1-2–treated mice revealed lesser number of labeled implanted EPCs than the nontreated mice, although the same number of EPCs was implanted. At this point, we cannot answer for those reasons. We can speculate some possibilities that TK1-2 may inhibit proliferation or induce apoptosis of the implanted EPCs *in vivo*, or that TK1-2 may stimulate inflammatory cells to phagocytose the EPCs.

It is interesting that TK1-2 inhibits the migratory activity of *ex vivo* cultivated EPCs, although we observed TK1-2 did not inhibit cancer cell migration.[4] As shown in cultured embryonic stem cells or isolated $CD34^+$ cells, the multistep events including adhesion, migration, chemoattraction, and differentiation to endothelial cells are required in recruitment and incorporation of EPCs into neovasculature (21, 22). Therefore, the inhibition of adhesion and migration of expanded EPCs by TK1-2 enhances its usefulness in antiangiogenic treatment. *In vivo*, TK1-2 also seemed to inhibit EPC-EPC interaction because TK1-2 treatment showed more separately locating implanted EPCs rather than aggregated form. Moreover, TK1-2 also inhibited adhesion onto fibronectin matrix of early undifferentiated EPC fraction from isolated MNCs in *in vitro* culture conditions, thereby providing the strong possibility of the inhibitory effect of TK1-2 on bone marrow–derived EPCs *in vivo*, not alone *ex vivo* cultivated EPCs. Therefore, our studies provide TK1-2 treatment as an efficient way of controlling EPC contribution to tumor vessel formation and tumor growth.

Estimates of EPC contribution to the tumor endothelium range from as much as 10% to 50% (8, 39), to 5% or less (40, 41). In contrast to ischemic condition, the role of circulating EPCs is controversial in tumor angiogenesis. However, the recent report indicated that acute recruitment of circulating EPCs occurs after vascular disrupting agents, such as combrestatin and OXi-4503 (42). These bone marrow–derived cells have been shown to home to the viable tumor rim and incorporate into or around the tumor vasculature, resulting in contribution to tumor regrowth. In addition, disruption of this circulating EPC spike by antiangiogenic drugs resulted in marked reductions in tumor rim size and blood flow. The results obtained from our models using *ex vivo* cultivated EPCs and TK1-2 also proved that antiangiogenic agents can effectively inhibit EPC incorporation into tumor vasculature at tumor periphery and EPC functions for tumor growth. Therefore, targeting EPCs may be an effective strategy for inhibition of tumor angiogenesis and growth, providing several possible ways, such as inhibitions of mobilization, differentiation, incorporation into endothelium, and other functions such as VEGF secretion.

At present, a homogenous population of EPCs is not available, and the characteristics of these cells are not clearly defined. Circulating EPCs and *in vitro* cultivated EPCs are both heterogeneous. In both cell types, some cells incorporate into vasculature and others exist around vasculature *in vivo*, thereby contributing to vessel formation and enhancing angiogenesis. In the future, if EPCs are more clearly understood, we can approach the role of EPCs in tumor angiogenesis and the effects of antiangiogenic agents in a more precise manner.

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Tumor Angiogenesis Promoted by *Ex vivo* Differentiated Endothelial Progenitor Cells Is Effectively Inhibited by an Angiogenesis Inhibitor, TK1-2

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