**In vivo** imaging of the molecular distribution of the VEGF receptor during angiogenesis in a mouse model of ischemia

Running Title: **In vivo** distribution of the VEGF receptor

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Abstract

Vascular endothelial growth factor (VEGF) plays a critical role in angiogenesis and has been applied to medical therapy. However, as vascular imaging at the molecular level is impossible, the detailed in vivo dynamics of VEGF and its receptor (VEGF-R) remain unknown. In this study, to understand the molecular distribution of VEGF and the VEGF-R, we prepared ischemic mice with a new surgical method and induced angiogenesis in the gastrocnemius muscle. Then, we made a VEGF-conjugated fluorescence nano-particle and performed staining of VEGF-R-expressing cells with the fluorescent probe, demonstrating the high-affinity of the probe for VEGF-R. To observe the physiological molecular distribution of VEGF-R, we performed in vivo single particle imaging of gastrocnemius in the ischemic leg with the fluorescent probe. The results suggested that only a 3-fold difference of VEGF-receptor distribution is involved in the formation of branched vasculature in angiogenesis, although previous ex vivo data showed a 13-fold difference in its distribution, indicating that a method inducing a several-fold local increase of VEGF-R concentration may be effective in generating site-specific angiogenesis in ischemic disease. This new in vivo imaging of ischemic mice could make useful contributions to understanding the mechanisms of angiogenesis and to developing a VEGF-R-related drug.
Introduction

Angiogenesis and arteriogenesis play a critical role in neovascularization in adults. Angiogenesis is defined as the sprouting of new capillaries from postcapillary venules, whereas arteriogenesis is defined as the process of artery maturation or the de novo growth of collateral conduits. Our laboratory studies the mechanisms of angiogenesis, and clarification of these mechanisms is crucial for the development of new treatments for arteriosclerotic disorders. Recently, medical applications for recombinant VEGF proteins or genes have been developed. However, no placebo-controlled trial has yielded overwhelmingly positive results. An understanding of the detailed molecular mechanisms of this angiogenesis factor in vivo is thought to be very important for the effective design of a VEGF-related drug delivery system. However, neither VEGF activity nor VEGF-R distribution has been quantitatively analyzed in vivo at a molecular level with respect to therapeutic angiogenesis. In previous animal studies, the efficacy of treatment for atherosclerotic disease was primarily evaluated using angiography, laser Doppler imaging, and the determination of histological capillary density. Angiography can be used to non-invasively visualize vessel size, vessel branching, and the vascular network throughout the body. However, as it is difficult to visualize vascular structures several hundred micrometers beneath the imaging surface, this method is not adequate for the observation of early stage angiogenesis at a molecular level. Laser Doppler imaging provides a non-invasive measurement of blood flow by determining the Doppler frequency shift of reflected light due to the motion of red blood cells. This technique enables quantitative analysis of improvements in blood flow following injury to the vasculature. However, the Doppler shift measurement is easily influenced by movement artifacts, room temperature, and blood pressure. Additionally, it is difficult to analyze microvascular structures using laser Doppler imaging because the resolution of the obtained image is limited by diffusion of the reflected light due to distance between the red blood cells and the detector. Histological measurements of capillary density can reveal quantitative increases in blood vessel density, and many previous studies have used this metric as a standard evaluation of angiogenesis. However, continuous observation of the same tissue is impossible with this measurement technique as protein structure is influenced by fixation of the tissues. For this reason, histological techniques are not recommended for physiological observations of the angiogenesis process. In the above methods, as resolution is limited to the micrometer level and imaging at the molecular level is currently impossible, the detailed in vivo dynamics of individual VEGF and VEGF-R molecules remains unknown. We have developed an in vivo single-particle imaging system using bright and photostable fluorescent nano-particles, or quantum dots (QDs), with a spatial precision of 7-9 nm. This was performed to clarify the molecular mechanisms of a anti-HER2 antibody-based drug delivery system and of cancer metastasis in tumor-bearing mice.
The use of an ischemic mouse model is highly effective for understanding the *in vivo* molecular dynamics of angiogenic factors and their effects on vascular remodeling. However, two surgical concerns impose limitations on previous mouse models\textsuperscript{10}. First, angiogenesis and arteriogenesis were not separately evaluated in previous models. As both processes contribute to an increase in the rate of blood flow\textsuperscript{1}, it is necessary that the effects of arteriogenesis be eliminated if angiogenesis is to be analyzed. Second, inflammation and edema at the surgical site are known to affect angiogenesis\textsuperscript{11}. To overcome these problems, it is critical to establish an improved ischemic mouse model that selectively induces angiogenesis at a specific muscle. Here, we demonstrate the development of an imaging method for determining the molecular distribution of VEGF-R labeled with QD-conjugated VEGF. This technique was designed to observe angiogenesis in a novel ischemic mouse model that induces angiogenesis in the gastrocnemius. Our results suggest that only a several-fold difference in VEGF-R distribution is required for the promotion of angiogenesis. This novel imaging method may aid in the development of drugs and treatments for atherosclerotic diseases.
Methods

Animals

C57BL/6J male mice (Charles River Laboratories, Kanagawa, Japan) weighing 20-27 g and 8-9 weeks of age were used for all experiments. All surgical processes were performed under anesthesia with ketamine (100-120 mg/kg) and xylazine (8-10 mg/kg). Anesthesia was maintained for the course of the imaging session. Animals were used in accordance with guidelines approved by the committee on animal experiments of Tohoku University.

Hemi hind limb ischemic mouse model

The hair of each mouse was removed from the abdomen and both hind limbs with an electric shaver and depilatory cream. The skin from both thighs was then incised to expose the arteries, veins, and nerves. To induce selective ischemia in the gastrocnemius, which is located in the deep layer of the thigh muscles, three vessels of the femoral area were ligated in the right hind limb. First, the proximal end of the superficial femoral artery and vein were ligated with surgical silk, size 6-0. Second, the origins of the popliteal artery and vein were ligated. Third, the distal portions of the saphenous artery and vein were ligated to avoid the backflow of blood. Femoral nerves were carefully preserved. Each of the vessels that were surrounded by the three ligated points was excised. The left leg was not treated except for an incision in the skin of the thigh. Finally, the overlying skin was closed (Figure 1).

Histological capillary density

To confirm that the surgical operation induced angiogenesis in the gastrocnemius, we performed immunohistological staining of the muscle with anti-CD31 antibody, a marker for vascular endothelial cells. Mice were sacrificed at predetermined times (7, 14, 21, and 28 days after operation). The gastrocnemius was removed and fixed overnight in 10% formalin in phosphate buffered saline (PBS). After fixation, the tissue was embedded in paraffin, and the tissue sections were prepared and mounted on slides. The tissue samples were deparaffinized and antigen retrieval was performed with proteinase K treatment. After this treatment, the tissue samples were incubated with a rat anti-CD31 monoclonal primary antibody (Angio-Proteomie, Boston, MA, USA) at 5 μg/ml for 12 h at 4 °C. After being washed with PBS, the samples were incubated with a biotinylated anti-rat IgG secondary antibody (Vector Laboratories, Inc, CA, USA) (100-fold dilution) for 30
min at 25 °C. Following incubation, the samples were incubated with horseradish peroxidase-conjugated streptavidin (Nidchirei, Japan) for 30 min at 25°C. Samples were then treated with DAB chromogen reagent (Dojindo, Japan) and counter-stained with hematoxylin. The samples were observed using light microscopy (BX51, Olympus, Japan) equipped with a camera (DP2-BSW, Olympus, Japan).

Laser Doppler Perfusion Imaging

Blood perfusion of the hind limb was measured using a Laser Doppler Perfusion Imaging system (MoorLDI2-IR, Moor Instruments Ltd, Devon, England). This imaging technique provides a non-invasive measurement of blood flow by determining the Doppler frequency shift of light reflected off of moving red blood cells. Mice under the same anesthetic dose described above were scanned from the lower abdomen to the end of the toes. After scanning, colored images were obtained with original software (RESEARCH 3.09, Moor Instruments Ltd., Devon, England). Each pixel in the acquired images reflected an original blood flow value, referred to as a Perfusion Unit (PU). The mean of the PUs of the lower thighs in a control limb and a treated hind limb was determined. The PUs of ischemic legs were obtained at different time points (prior to operation, soon after operation, and 7, 14, 21, and 28 days after operation). The relative ratios of the mean PUs between the ischemic and control legs in the same mouse were calculated.

Cell lines

A pancreatic islet endothelial mouse cell line, MS1, was obtained from the American Type Culture Collection (ATCC, VA, USA). MS1-VEGF cells, which express VEGF-R on the cell membrane via the transfection of the VEGF gene, were also acquired from ATCC. These cells were cultured in DMEM (GIBCO, Invitrogen, CA, USA) supplemented with 5% fetal bovine serum.

Immunostaining of cultured cells with an anti-VEGF-R antibody

MS1 and MS1-VEGF cells were cultured on slide glass chambers. After three days, the slides were placed in 2.5% formalin in DMEM for 10 min. After fixation, the cells were incubated with an anti-mouse VEGF-R monoclonal antibody (Pierce, Rockford, IL, USA) or a whole mouse IgG (Rockland Inc., Gilbertsville, Pennsylvania, USA) primary antibody at 10 μg/ml for 12 h at 25 °C. After the samples were washed with PBS, the cells were incubated with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (KPL Europe, Guildford, UK) (100-fold dilution) for 1 h at 25 °C. Following incubation, the samples were treated
with DAB chromogen reagent and counter-stained with hematoxylin.

**Preparation of angiogenesis factor-conjugated quantum dots**

Mouse vascular endothelial growth factor 164 (VEGF) (R&D System, MN, USA) and platelet-derived growth factor BB (PDGF) (Biovison, CA, USA) were biotinylated using the EZ-Link® Micro Sulfo-NHS-LC-Biotinylation Kit (Pierce). In this reaction, a 1:50 molar ratio of angiogenesis factors and Sulfo-NHS-LC-Biotin® was used according to the manufacturer’s instructions. The biotinylated VEGF and PDGF were then mixed with avidin-conjugated Qdot®705 nano-particles (QD705) (Invitrogen, CA, USA) at a molar ratio of 8:1 or 16:1 and incubated for 1 hr at 25 °C. The number of QD705 nano-particles determined the emission wavelength. QD705-conjugated VEGF and PDGF were termed VEGF-QD and PDGF-QD, respectively.

**Single particle imaging system**

The optical system used to observe the fluorescence of the angiogenesis factor-conjugated QDs consisted primarily of an epi-fluorescent microscope (IX-71, Olympus), a Nipkow disk-type confocal unit (CSU10, Yokogawa, Tokyo, Japan), and an EMCCD camera (Ixon DV887, Andor, Tokyo, Japan). An objective lens (60 ×, PlanApo, 1.40 NA, Olympus) was used for imaging. VEGF-QDs were illuminated using a green laser (532 nm, Spectra-Physics). The laser-excited fluorescence of the QDs was filtered with a 695-740 nm band-pass filter. Images were obtained at a rate of 5 frames/s. For *in vivo* imaging, to remove the oscillation due to heartbeats and respiration, a gastrocnemius window was developed and attached to the above microscopy system.

**Single particle imaging of VEGF-QDs in cultured cells**

To investigate the affinity of VEGF-QDs for VEGF-R, MS1 and MS1-VEGF cells were incubated with 1, 10, or 50 nM VEGF-QDs for 1 h at 25 °C. After three washes with DMEM, these cells were observed in a glass-bottom dish using the single particle imaging system. The fluorescence intensities of QD signals from the cells were analyzed as gray values using ImageJ software (http://rsb.info.nih.gov/ij/). The gray values of 100 frames (200 msec/frame) from a single cell were averaged, and the gray value of the background was subtracted from that of the cell. The mean gray value per pixel in the background-subtracted image of the cell was multiplied by the total number of pixels making up the image of the cell. The total fluorescence intensity
of the QDs per cell was thus determined. In the 10 nM VEGF-QD treatment, QD fluorescence signals were clearly observed on MS1-VEGF cells. In contrast, when MS1-VEGF cells were incubated with 1 nM VEGF-QDs, the QD fluorescence signals were low. When the cells were treated with 50 nM VEGF-QDs, QD signals were exceedingly high or even saturated (data not shown). In MS1 cells incubated with various concentrations of VEGF-QDs, the VEGF-QD fluorescence signals were very low. These results indicate that VEGF-QDs specifically recognize VEGF-R and that the 10 nM concentration was appropriate for imaging the binding of VEGF-QDs to VEGF-R. Moreover, to provide stronger evidence of the affinity of VEGF-QDs for VEGF-R, MS1 and MS1-VEGF, cells were incubated with 10 nM unconjugated QDs, PDGF-QDs, or VEGF-QDs for 1 h at 25 °C, and the resulting fluorescence intensities were examined.

**In vivo fluorescence imaging with the IVIS® Spectrum system**

To confirm that VEGF-QDs accumulated in the ischemic leg, we performed *in vivo* fluorescence imaging using the IVIS® spectrum imaging system (Caliper Life Sciences, MA, USA) at 4, 9, and 14 days after preparation of ischemic model mice. Unconjugated QDs or VEGF-QDs were injected intracardially into the mice. The final concentration of the QD probes in the blood was 10 nM, as determined by the single particle imaging data from cultured cells. The detection sensitivity of the fluorescent signal using the IVIS® spectrum is poorer than the single particle imaging system but allows for non-invasive visualization of the whole body of the mouse. Therefore, fluorescence images were taken 10 min after injection of fluorescent probes, before the fluorescence signals decreased due to their washing out with the blood. Fluorescence intensities of the QDs were analyzed using accessory software (Living Image® 4.0, Caliper LifeSciences, MA, USA). For data analyses, the relative ratio of fluorescence between the ischemic and control leg of the same mouse was calculated.

**Single particle imaging of VEGF-QDs in the gastrocnemius of ischemic model mice**

To examine the distribution of VEGF-QDs in the gastrocnemius of the ischemic leg, we performed *in vivo* single particle imaging of the vasculature 4, 9, and 14 days after operation. The skin of the ischemic leg was opened to expose the thigh muscles. The skin of the hind limbs was then fixed to a plastic plate using suture thread and superglue. Use of the superglue made both connections of the skin to the plate more stable without damaging the vasculature, eliminating the background oscillations from the heartbeat and respiration during observation. To expose the gastrocnemius, the skin and hemimembranous muscle, which is located on the superficial layer of the gastrocnemius, were then removed. The mouse, which was mounted as described on
the plastic plate, was then fixed to a handmade aluminum stage designed to stabilize the plate with screws. Unconjugated QDs or VEGF-QDs were injected intracardially into the mice. The sensitivity of the single particle imaging system to the fluorescence signal is extremely high, and immediately following injection, a part of probes are free in the blood and have not bound to VEGF-R. This initially prevents observation of the interaction between the VEGF-QDs and VEGF-R on the vascular wall. Therefore, in vivo single particle imaging of the fluorescent probes was carried out 1 h after injection. By this time, the concentration of the free probe in the blood was decreased. To quantitatively measure the affinity of the VEGF-QDs for the vasculature, an analysis was performed as follows. One hundred frames, each 512 pixels square and representing an exposure of 200 msec, were overlaid using image processing software (G-count, G-angstrom, Sendai, Japan). A portion of the overlaid (192 pixels square) image was examined to determine the number of QD particles in proper-sized vasculature. For each image analyzed, the fluorescence intensity (as gray values) of QD signals from the tissues was determined using Image J software. The mean gray value, derived from the tissues’ autofluorescence per pixel, was subtracted from the fluorescence value of the vascular wall area. The resulting gray value was then multiplied by the total number of pixels of the vascular wall. This value reflected the total fluorescence intensity of all QDs bound to the vascular wall (total-QD-value).

To precisely determine the number of QD particles on the vascular wall, it was necessary to define the fluorescence intensity of a single QD. Because QDs that fluoresce at the same wavelength are uniform in size, QD705 fluorescence intensity is proportional to the particle number. Moreover, the QD fluorescence is composed of fluorescent and non-fluorescent states referred to as on- and off-states. This property results in blinking of a QD. When the fluorescence and other properties of QD particles were analyzed immediately after their purchase, we determined that the mean duration of the off-state over 20 sec was approximately 4 sec and that the calculated s.e.m. was very low. In cases where QDs aggregate, the mean duration of the off-state per unit time is shortened because the on- and off-states of each particle in the aggregate occur randomly. Therefore, based on an off-state duration of 4 s, we selected a single particle QD from each image and measured the fluorescence intensity of the single QD particle (single-QD-value) in the same manner as the total-QD-value. The total-QD-value was divided by a single-QD-value, and the number of QD particles per 10 μm of vascular wall was calculated.

**Statistical analysis**

All data are presented as the mean ± s.e.m. An F-test was performed and equal variance was defined as p-values, (p) ≥ 0.05. Comparisons between groups were performed using the parametric Student’s t-test (p ≥ 0.05 at f-test) or Welch’s t-test (p < 0.05 at f-test). P < 0.05 was considered significant for both t-tests.
Results

Induction of angiogenesis in ischemic model mice.

To induce angiogenesis at a selected site and to eliminate the effects of inflammation and edema, we targeted the gastrocnemius. This muscle is primarily supplied by the popliteal and saphenous arteries, and no remarkable collateral arteries exist near the muscle. We therefore ligated the popliteal and saphenous arteries to induce angiogenesis in the gastrocnemius (Figure 1). Other muscles in the thigh are primarily supplied by the deep femoral artery, which was maintained as a collateral artery after the surgery. Therefore, we believe that our surgery is appropriate for analyses of angiogenesis mechanisms. The gastrocnemius is located deep among the thigh muscles, and the skin and semimembranosus muscle located on the upper layer of the gastrocnemius were removed just prior to in vivo imaging. Therefore, we avoided the effects of inflammation and edema-induced factors on normal angiogenesis. To determine whether the number of capillaries in the gastrocnemius of ischemic legs increased, we histologically determined the capillary densities over time (Figure 2A-C). Capillary densities in the control legs did not significantly change during observation (Figure 2D). In contrast, the capillary densities in ischemic legs increased gradually over 14 days, peaked at day 14 and were steady between day 14 and day 28. This finding demonstrates that angiogenesis was induced in the ischemic leg (Figure 2D). To investigate improvement of blood flow in the model mice using another method, we evaluated the change in blood flow using laser Doppler perfusion imaging (Figure 2E-J). The relative ratio of the mean perfusion unit (PU) between ischemic and control legs in the same mouse (ischemic leg value / control leg value) decreased to approximately 8% soon after operation (Figure 2E, F, K). This ratio then increased to approximately 34% 21 days after the surgery (Figure 2I, K). Both observations indicate that angiogenesis was effectively induced in the gastrocnemius between 7 days and 14 days following the procedure. The slight difference in the rate of increase of perfusion between the two imaging methods may be due to the fact that the laser Doppler perfusion imaging analyzed the thigh as a whole, and arteriogenesis induced by inflammation and edema of the semimembranosus muscle may have occurred in this larger volume.

Preparation and characterization of VEGF-QDs

To visualize the molecular distribution of VEGF using single-particle imaging, VEGF was conjugated with QDs (VEGF-QD). PDGF-conjugated QDs (PDGF-QD) were also prepared as control probes (Supplementary figure 1). To examine the binding of VEGF-QD and VEGF-R, staining was performed on MS1 and MS1-VEGF cells treated with unconjugated QDs, PDGF-QDs, and VEGF-QDs. High levels of
VEGF-R expression in MS1-VEGF cells were confirmed by immunostaining with an anti-VEGF-R antibody (Figure 3C, D), while low-level expression was observed in MS1 cells (Figure 3A, B). The analyses of QD-probe fluorescence indicated that the total fluorescence intensity of VEGF-QDs in MS1-VEGF cells was $21.0 \pm 2.4 \times 10^4$ (Figure 3H, I). This value was remarkably higher than the observed fluorescence values of stained MS1-VEGF cells treated with unconjugated QDs or PDGF-QDs (unconjugated QDs, $3.8 \pm 0.3 \times 10^4$; PDGF-QDs, $4.4 \pm 0.3 \times 10^4$) (Figure 3G, I) and those of MS1 cells treated with unconjugated QDs, PDGF-QDs or VEGF-QDs (unconjugated QDs, $5.7 \pm 0.4 \times 10^4$; PDGF-QDs, $6.0 \pm 0.4 \times 10^4$; VEGF-QDs, $7.7 \pm 0.6 \times 10^4$) (Figure 3E, F, I). These results demonstrate that VEGF-QDs bind specifically to VEGF-R.

**In vivo** distribution of VEGF-R labeled with VEGF-QDs

To determine the distribution of VEGF-R labeled with VEGF-QDs during angiogenesis, we performed two **in vivo** fluorescence imaging techniques. In these imaging protocols, we examined ischemic model mice at 4, 9, and 14 days after operation to analyze in detail the changes in VEGF-R distribution over time. Imaging performed with the IVIS® spectrum system has the advantage of imaging the fluorescence of the entire body of the mouse, although the spatial precision of this technique is low. To simultaneously observe the fluorescence of both ischemic and control legs after injection of unconjugated QDs or VEGF-QDs into the model mice, the IVIS Spectrum system was used (Figure 4A-D). Individual mice exhibit different degrees of autofluorescence. The relative ratio of fluorescence between the ischemic and control leg in each mouse was therefore calculated (ischemic leg fluorescence / control leg fluorescence). In model mice at 4 days after operation, the relative fluorescence ratio resulting from injection of unconjugated QDs was $0.71 \pm 0.01$, and the ratio following injection of VEGF-QDs was $0.72 \pm 0.03$ (Figure 4E). These results indicate that VEGF-QDs did not selectively accumulate in the ischemic legs at the time of measurement. In contrast, at 9 and 14 days after operation, the relative ratios resulting from injection of unconjugated QDs were $0.65 \pm 0.02$ and $0.76 \pm 0.05$, respectively, while the observed ratios following injection of VEGF-QDs were $0.94 \pm 0.06$ and $1.00 \pm 0.04$, respectively (Figure 4F, G), suggesting that VEGF-QDs accumulated in the ischemic leg to a greater degree than in the control leg at the time of measurement.

In addition to using the IVIS® system, we also conducted **in vivo** single-particle imaging. For this protocol, we developed surgical fixation methods of mice beyond that in our previous imaging method. In particular, we designed a polyvinyl chloride plate with a window fit to the shape of the gastrocnemius. The skin around the gastrocnemius was bound to the plate with suture thread and superglue (Figure 5). These improvements eliminated background oscillations due to the heartbeat and respiration, enabling us to observe the physiological angiogenesis that sustains blood flow following ischemia (Supplementary Movie 1, 2). We
observed the distribution of unconjugated QDs or VEGF-QDs after injection of the respective probe. The results indicate that a large number of VEGF-QDs were specifically localized on the vessel walls in ischemic legs (Figure 6A-H), whereas fluorescence following injection of unconjugated QDs was very weak (Supplementary figure 2A-D). We measured the total fluorescence intensity due to all QDs on the vascular wall (total-QD-value) and the fluorescence intensity of single QD (single-QD-value) as gray values. The total-QD-value was then divided by the single-QD-value, and the number of QD particles per 10 µm of the vascular wall was calculated (Figure 6I, Supplementary figure 2E). These analyses revealed that QD fluorescence on the vascular wall was weak in both ischemic and control legs in mice injected with unconjugated QDs (Supplementary Figure 2E). When VEGF-QDs probe were injected at 4 days after operation, the number of VEGF-QDs on the vascular walls of ischemic legs was similar to the number observed in control legs. Moreover, there was no difference in number of particles on the walls of branched or linear vasculature in ischemic legs (Figure 6C, D, I). In contrast, the number of VEGF-QDs in the branched vasculature in ischemic legs was 3.4-fold greater than the number of VEGF-QDs in the linear vasculature 9 days after operation (Figure 6E, F, I) and 4.5-fold greater than the number of VEGF-QDs in the branched vasculature in control legs (Figure 6B, F, I). Fourteen days after operation, the number of VEGF-QDs on the walls of the branched vasculature in ischemic legs was 3.3-fold greater than the number of particles on the walls of the linear vasculature (Figure 6G, H, I) and 4.3-fold greater than the number of VEGF-QDs in the branched vasculature in control legs (Figure 6I). These results demonstrate that our single-particle imaging method is able to quantitatively describe the *in vivo* distribution of VEGF-R labeled with VEGF-QDs during angiogenesis in ischemic legs.
Discussion

In vivo molecular imaging using high spatial precision in ischemic model mice is highly effective for the quantitative description of the molecular dynamics of VEGF and VEGF-R during angiogenesis. This information can be applied to the development of treatments for ischemic disease. Previously described mouse models suffered from surgical limitations. These difficulties involved 1) the induction of angiogenesis without arteriogenesis, an effect of arteries that remains following surgery, and 2) the induction of inflammation and edema during surgery. We focused on the gastrocnemius, which is located deep in the thigh, and developed a new ischemic mouse model consisting of the ligation of three pairs of vessels: the superficial femoral, popliteal, and saphenous arteries and veins (Figure 1). We confirmed that angiogenesis was effectively induced in the model mice using conventional evaluation methods, histological capillary density measurements and laser Doppler imaging (Figure 2D, K).

We have previously described the development of in vivo single-particle imaging using QDs with a spatial precision of 7-9 nm to clarify the molecular mechanisms of a anti-HER2 antibody-based drug delivery system and cancer metastasis in tumor-bearing mice. In previous studies, imaging with high spatial precision was not applied to the visualization of angiogenesis. For this application in the current studies, we further modified our surgical fixation method (Figure 5). For this imaging technique used here, we designed a polyvinyl chloride plate with a window fit to the shape for the gastrocnemius. This window enabled us to observe the physiological angiogenesis during active blood flow. We observed the in vivo molecular distribution of VEGF-QDs using this improved imaging in ischemic mice at 4, 9, and 14 days after a surgery in which angiogenesis in the gastrocnemius was effectively induced. The results demonstrate that a large number of VEGF-QDs specifically localized to the vessel wall in ischemic legs (Figure 6A-H), whereas fluorescence due to unconjugated QDs at the wall was very weak (Supplementary figure 2A-D). To quantitatively analyze the molecular distribution of VEGF-QD-labeled VEGF-Rs, we measured the total number of QDs (Figure 6I, Supplementary figure 2E). Because QDs with the same fluorescence wavelength are uniform in size, the fluorescence intensity of the QDs is proportional to the number of particles. This property enabled us to determine relative VEGF-R expression levels with a very high degree of accuracy. In mice 4 days after operation, there were no significant differences between the branched vasculature and the linear vasculature (Figure 6C, D, I). Furthermore, the IVIS spectrum data from this time point did not show a significant difference in the accumulation of unconjugated QDs or VEGF-QDs in ischemic legs (Figure 4E). These data indicate that any VEGF-R redistribution induced by hypoxic stimulation in ischemic legs occurs only at low levels at this time point. In mice at 9 days after operation, the data demonstrate a 3.4-fold greater number of VEGF-QDs on the walls of branched vasculature in ischemic legs than on the walls of linear vasculature in these legs and a 4.5-fold greater number than on the branched vasculature of control legs in
model mice (Figure 6I). In mice at 14 days after operation, the data revealed that the number of VEGF-QDs on the walls of branched vasculature in ischemic legs was 3.3-fold greater than that on the linear vasculature in ischemic legs and 4.3-fold greater number than the number of VEGF-QDs on the branched vasculature in control legs in model mice (Figure 7I). These results demonstrate that the data acquired from mice at 9 and 14 days following operation using the single particle imaging technique was similar to data acquired using the IVIS spectrum system (Figure 4F, G). From the data, it appears that the VEGF-R protein expression on the branched vasculature in ischemic legs increases gradually between day 4 and day 9 via hypoxic stimulation, peaks at approximately day 9 and remains steady from day 9 to day 14. Histological capillary density data reveals that capillary densities in ischemic legs increase gradually over 14 days (Figure 2D). It is very interesting, therefore, that the peak of VEGF-R expression occurred approximately 9 days following the procedure. Furthermore, these data suggest that only a several fold increase in the expression level of VEGF-R on endothelial cells is critical for angiogenesis in ischemic tissues.

During angiogenesis, tip cells, stalk cells, and phalanx cells control vessel sprouting. Tip cells are located at the forefront of the sprouting vessel, stalk cells are located behind the tip cell at the branch, and phalanx cells are found in the unbranched endothelial layer. Previous ex vivo studies reported that VEGF-R expression in the tip cell is high in order to sense the VEGF concentration gradient in the extravascular area. In stalk cells, which control elongation of a new branch, VEGF-R levels are lower than in the tip cells. In phalanx cells, which normalize the endothelial cell layer, VEGF-R levels are lower than in the stalk cells. However, these are qualitative data, and a quantitative analysis of the VEGF-R distribution required to regulate the behavior of these cells has not been carried out in vivo. Additionally, previous ex vivo data revealed that the amount of VEGF-R protein involved in angiogenesis in ischemic tissue is 13-fold greater than in normal tissues. In this report, however, because the total amount of VEGF-R in both the vessel wall and the extravascular area was measured, the detailed quantitative distribution of VEGF-R was not known. Our results suggest that only an approximately 3-fold difference in VEGF-R distribution on the vascular wall is involved in the formation of branched vasculature from linear vasculature during angiogenesis (Figure 6I). The increase in VEGF-R levels we observe following ischemia may quantitatively reveal a gradient of VEGF-R expression levels between the stalk cells and pharynx cells.

Our results indicate that VEGF-R distribution in in vivo ischemic tissues increased gradually over a specific period and remained steady at an approximately 3-fold increase. To increase the number of sprouting vessels for the operation of ischemic hypoxia, the steady several-fold increase of VEGF-R expression levels at the ischemic site may be effective for controlled angiogenesis. Previously, treatment against ischemic hypoxia using VEGF gene therapy induced only a slight increase in VEGF blood concentrations. Additionally, overexpression of VEGF produces abnormal vessels in tumor angiogenesis. These results suggest that in the
context of ischemia, it is difficult to site-specifically modulate the concentration of a VEGF-related therapeutic to an appropriate value. Therefore, next-generation therapies targeting VEGF-R, in addition to VEGF, may effectively induce site-specific angiogenesis for the treatment of ischemic disease. The novel *in vivo* imaging technique described here for the analysis of ischemic model mice may increase understanding of the mechanisms of angiogenesis and aid in the development of VEGF-R-related therapies.
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Authorship Contributions
Y.H. and K.G designed and performed the research, analyzed the data and wrote the manuscript; M.T., A.S., and N.O. performed research and analyzed data; M.W. performed research; T.Y. provided the equipment for laser Doppler perfusion imaging and performed research.

Disclosure of Conflicts of Interest
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References
Figure legends

Figure 1. Procedure for preparing the hemi-hind limb ischemic mouse model. On the right leg, we ligated the proximal end of the superficial femoral artery, the origins of the popliteal artery, and the distal portions of the saphenous artery with surgical silk. All vessels surrounding the three ligated points were excised. The left leg was not treated except for an incision in the skin of the thigh.

Figure 2. Evaluation of angiogenesis of the ischemic model mice. (A-C) Typical images of the gastrocnemius immunostained with an anti-CD31 antibody. The muscles from ischemic legs were isolated at 7, 14, and 28 days after the induction of ischemia and immunostained. Brown circles and dots indicate CD31+ capillaries. Scale bar, 50 μm. (D) Measurement of CD31+ capillary density. In the gastrocnemius of the ischemic or control leg, capillaries stained with the anti-CD31 antibody were counted. Solid and dotted lines indicate the sample derived from ischemic and control legs, respectively. n = 4. Error bars, s.e.m. (E-J) Changes in blood perfusion were assessed using laser Doppler perfusion imaging in ischemic legs of model mice. (E, F) Images prior to and soon after operation. After operation, the perfusion signal in the ischemic leg is significantly decreased. (G-J) Images from 7, 14, 21 and 28 days after operation. Blood perfusion in an ischemic leg gradually increases. (K) Change in perfusion units as determined by this imaging technique. Relative ratios of mean perfusion units between ischemic and control legs in the same mouse are shown (ischemic leg value / control leg value). n = 6. Error bars, s.e.m.

Figure 3. VEGF-R distribution in MS1 and MS1-VEGF cells and the affinity of angiogenesis factor-conjugated QDs to these cell lines. (A-D) Immunostaining of MS1 and MS1-VEGF cells with an anti-VEGF-R antibody. Scale bar, 50 μm. (E-H) Typical images of cells treated with fluorescent particles. MS1 and MS1-VEGF cells were treated with unconjugated QDs, PDGF-QDs, or VEGF-QDs. Representative images are shown of cells treated with PDGF-QDs and VEGF-QDs. Data from cells treated with unconjugated QDs are not shown. White dots in pictures indicate fluorescent QDs. White dotted lines represent the outline of the cell. Scale bar, 10 μm. (I) QD fluorescence intensity per cell. The fluorescence intensity of QD signals from the cells was analyzed as gray values. In each condition, n = 30. **P < 0.01, error bars, s.e.m.
Figure 4. *In vivo* fluorescence imaging of the ischemic model mice using the IVIS® spectrum system. (A-D) Images using IVIS pectrum. Unconjugated QDs and VEGF-QDs were injected intracardially into the model mice. Mice were illuminated with light with a wavelength of 625-655 nm. Excited fluorescence was filtered with a 690-710 nm wavelength band-pass filter. (E-G) The relative ratios of fluorescence between ischemic and control legs in mice at 4 days (E), 9 days (F), and 14 days (G) after operation. The fluorescence intensity of the ischemic leg divided by that of the control leg was calculated (ischemic leg fluorescence / control leg fluorescence). n = 5. Error bars, s.e.m. **P< 0.05.

Figure 5. Schematic of the *in vivo* single particle imaging system. An ischemic leg of a model mouse was stabilized on a polyvinyl plate using suture thread and instant superglue. The skin and hemimembranous muscle were then removed. The gastrocnemius was selectively imaged using a single particle imaging system.

Figure 6. *In vivo* imaging of unconjugated and VEGF-QDs during angiogenesis in ischemic legs. (A-H) Typical images of vasculature in control and ischemic legs. These images were generated by overlaying 100 frames (200 msec/frame), each consisting of a 192-pixel-square image, using image processing software. In model mice injected with VEGF-QDs, we observed “linear vasculature” (A, C, E, G) and “branched vasculature” (B, D, F, H) in control and ischemic mouse legs at 4, 9, and 14 days after operation. The data from unconjugated QDs are not shown (see Supplementary figure 2). White dots in the pictures indicate QD fluorescence. Scale bar, 10 μm. (I) The number of VEGF-QDs in different types of vasculature. The number of fluorescent particles per 10 μm of the vascular wall is quantified. n = 4. **P< 0.05, error bars, s.e.m.
Figure 1
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In vivo imaging of the molecular distribution of the VEGF receptor during angiogenesis in a mouse model of ischemia

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