Numerous laboratories are focusing efforts on delivering gene products to induce or prevent the development of new blood vessels in adults, with the hope of rescuing ischemic tissues, circumventing cardiac bypass surgery, or inhibiting tumor growth. Current approaches to the assessment of vascular continuity involve the introduction of either dyes or fluorescent microspheres to track blood flow. However, dyes and dextrans are subject to leakage when vessels are hyperpermeable, a situation that may occur in studies of tumor vasculature and during efforts to stimulate therapeutic angiogenesis. Furthermore, the microspheres that are used for flow studies do not allow a comprehensive visual analysis of vascular continuity. Here we report a method for the visual assessment of microvascular continuity in mouse muscle under circumstances in which vessels are leaky. The approach involves perfusion of the vasculature with fluorescent beads that are much smaller than those used for flow studies. The suspension behaves like a fluid and completely fills the vessels, yet the beads do not leak from VEGF-permeablized capillaries and remain localized in histological sections. Use of beads with the proper fluorescence emission wavelengths allows immunofluorescent colocalization with vessel-specific markers. We compare this improved method with other methods for tracking vascular continuity involving dextrans and larger beads. This approach should aid in the dynamic study of tumor angiogenesis and the evaluation of efforts to deliver angiogenic factors.

Key Words: angiogenesis; beads; dextran; fluorescent microspheres; permeability; tracer dye; vascular continuity.
method for retaining the marker within the vasculature would have broad applications.

One method commonly in use to analyze blood flow to different tissues involves the intravascular injection of radioactive, colored, or fluorescent microspheres. These microspheres are typically about 7–16 μm in diameter and are designed to lodge in the capillary bed, allowing the subsequent quantitation of microsphere sections or extracts of the tissue of interest (11, 12). However, because they are intended to become stuck in the capillaries, these microspheres do not furnish a way to completely assess continuity from one group of vessels to another and do not offer a visual picture of the vasculature being studied.

To assess microvascular continuity in skeletal muscle under conditions of vascular hyperpermeability, we devised a method that overcomes the limitations of both of the above approaches and capitalizes on the advantages offered by each. In this approach, mice are perfused immediately after they are sacrificed with a suspension of 0.2-μm fluorescent polystyrene beads. Because the beads are much smaller than those used for standard flow studies, the suspension behaves like a fluid and completely fills up the vascular space. By immunostaining sections of the resulting muscles with antibodies that identify vascular endothelium, capillaries could be detected and the continuity with the preexisting vessels could be assessed. By contrast, under these same conditions, large fixable fluorescent dextran were not retained within the vessels. This approach should prove useful for other studies aimed at determining the effects of a number of factors that have potential in inhibiting tumor angiogenesis or inducing therapeutic angiogenesis.

**Materials and Methods**

**Perfusion**

Tetramethylrhodamine-conjugated dextran (lysine fixable; 2,000,000 M_\text{r}) was obtained from Molecular Probes (Eugene, OR) and used either undiluted or diluted 1:10 in PBS. Fluorescent carboxylate-modified polystyrene beads (FluoSpheres, Molecular Probes) conjugated to either Nile red dye (535-nm excitation maximum, 575-nm emission maximum) or “blue” dye (365-nm excitation maximum, 415-nm emission maximum) were used either undiluted or diluted 1:6 with PBS (concentration as supplied was 2% solids in water). The beads were 1.0 μm in diameter for some experiments and 0.2 μm in diameter for other experiments.

Male SCID C.B-17 mice were obtained from the Stanford Department of Comparative Medicine. All animal procedures were carried out in accordance with the guidelines of the Stanford University Administrative Panel on Laboratory Animal Care.

Mice were preanesthetized with Metofane and then deeply anesthetized with an intraperitoneal injection of Nembutal (sodium pentobarbital, Abbott Laboratories, North Chicago, IL). In some experiments, Tridil (nитрогlycerин, Abbott Laboratories) was included in the Nembutal at 50 μg/ml to allow maximal vasodilation of the peripheral vasculature. The chest was opened through a midline sternotomy. The left ventricle was identified and the ventricular cavity was entered through the apex with a 27-gauge needle. The right ventricle was easily identified and an incision was made in the free wall to exanguinate the animal and to allow the excess perfusate to exit the vascular space. The animal was first perfused with approximately 2–6 ml of PBS at approximately 10 ml/min and then with the fluorescent marker of interest. Care was taken to avoid air bubbles.

After perfusion was complete, entire hindlimbs were removed at the knee, the tibia was removed as described in Springer et al. (27), and the legs were snap frozen in freezing isopentane/liquid N\textsubscript{2}. Sections were cut on a cryostat at either 14 or 10 μm.

**Implantation of VEGF-Expressing Myoblasts**

Myoblasts that had been retrovirally transduced to express VEGF were implanted into the tibialis anterior and the lateral gastrocnemius as described previously (26).

**Histological Analysis**

Muscle tissue sections were fixed in 1.5% formaldehyde in PBS for 15 min and then permeabilized and blocked with 0.1% saponin and 0.5% BSA in PBS (SBPBS) for 30 min. The slides were incubated for 2 h at room temperature with a rat monoclonal antibody against PECAM-1 (clone MEC 13.3; PharMingen, San Diego, CA; 1:100 dilution). Sections were rinsed in SBPBS and then incubated for 1-1.5 h at room temperature with goat anti-rat secondary antibody conjugated to either Texas Red or Alexa488 (Molecular Probes) at 1:100 dilution. Slides were then rinsed and mounted in PBS.

**Results**

**Injection of Fluorescent Dextran; Space-Filling but Leaky**

To assess the utility of a fluorescent tracer for following the continuity of the microvasculature, the tracer was injected into the circulation, the animal sacrificed, and the muscle cryosectioned, and the sections were stained with antibodies coupled to a different dye, thereby allowing the simultaneous identification of capillaries. In this way, the extent to which the tracer dye had collected in the capillaries could be compared to the total number of capillaries identified by immunolabeling. Tetramethylrhodamine-conjugated dextran of 2,000,000 M_\text{r} was injected into the circulation of mice via several different routes. This dextran was lysine fixable, so that when the sections were fixed and immunostained, the dextran would not diffuse away. We initially attempted to inject a small volume, ranging from 50 to 400 μl at 5 mg/ml, into the circulation of living, completely anesthetized mice directly through the femoral artery, into the aorta, or into the left ventricle. The mice were then sacrificed and leg muscle was harvested and frozen immediately for cryosectioning. However, none of these approaches resulted in significant amounts of dextran in the capillaries.

Much better results were achieved by whole-body perfusion of dead mice with the dextran. The mice were first perfused with 2 ml of PBS (approximately the blood volume) and then perfused with 2 ml of a 1:10 dilution of dextran (0.5 mg/ml). The leg muscle was then isolated and processed. This resulted in visible dextran fluorescence in most of the capillaries (Fig. 1). Many capillaries, identified by green immunostaining for the endothelial marker PECAM-1 (CD31), contained the red dextran, although in a few cases the dextran either leaked out of the vessels before the muscle was frozen or diffused away after sections were cut. This leakage was greatly exacerbated by the expression of recombinant VEGF by nearby muscle.
fibers after the implantation of genetically engineered myoblasts (26), rendering the histology uninterpretable (data not shown). The success of perfusion in all cases was variable, as some regions of muscle were well-perfused and other regions completely lacked detectable fluorescent dextran.

Injection of 1-μm Fluorescent Beads; Nonleaky but Inadequate for Visualization of Vessels

Similar approaches were taken with the fluorescent microspheres. Instead of the ~15-μm beads used for standard flow studies (11, 12), we first tried 1-μm-diameter beads that were conjugated to Nile red. As with dextran, injections of 50–400 μl of bead suspension (undiluted 2% solid stock suspension) into the bloodstream of living animals did not result in a significant number of beads in the muscle. When the animals were perfused with 2 ml of PBS followed by 2 ml of a 1:6 dilution of beads, as was the case with the dextran perfusions, fluorescent beads were seen in many capillaries and larger vessels in the muscle (Fig. 2).

These beads suffered from three problems. First, the Nile red dye, originally chosen for its wide excitation spectrum to ensure adequate fluorescence with standard rhodamine fluorescence microscope filter sets, was so bright in the beads that its emission spilled over into all other fluorescence channels. Hence, double-labeling for capillaries could not be achieved because the beads also showed up as bright green in the fluorescein channel and were even visible with UV excitation filters. This was solved by use of 1-μm “blue” fluorescent beads (365-nm excitation maximum, 415-nm emission maximum), which were an intense blue-white in the UV channel but were completely invisible in the rhodamine channel. This allowed double-labeling of PECAM in capillaries using antibodies conjugated to Texas Red (not shown; see colors in Fig. 3). The second problem was that while the beads were small enough that they could travel freely through the capillaries, they were still large enough to show up as round spheres that did not completely fill the lumens of the microvessels (Fig. 2), unlike the space-filling dextran (Fig. 1). Finally, perfusion was still unreliable and variable.
FIG. 2. Perfusion of vessels with 1.0-μm red fluorescent beads. Both panels (two different fields) show muscle cross sections (14 μm) taken from mice that were perfused with red beads. (A) Double exposure of the red bead fluorescence and standard differential interference contrast (Nomarski) optics, allowing the muscle fibers to be visualized. (B) Single exposure of simultaneously viewed bead fluorescence and Nomarski optics; thus, the background appears red because of the red fluorescence filter. Beads are visible between muscle fibers but do not accurately depict the shape or extent of the capillaries because of the beads’ relatively large size; therefore, capillary lumens are not directly observable. Bar = 100 μm.

FIG. 3. Colocalization of immunostained capillaries (red) and injected 0.2-μm bead suspension (blue–white). (A–D) Mice were perfused with 0.2-μm “blue” fluorescent beads, muscle was sectioned at 10 μm, and capillaries were immunofluorescently stained with antibodies against PECAM-1 (red). A vast majority of capillaries were completely filled with bead suspension, and there was no sign of leakage (the blue halo is caused by glow from the bright fluorescence and does not represent leakage from the vessels). (E, F) A cluster of capillaries at the site of expression of VEGF in the muscle. The blue fluorescent bead suspension has not leaked out of capillaries within and adjacent to the implantation region. Bar for A and B = 200 μm; bars for C–F = 100 μm.
from region to region of the muscle, even when the perfusion volume was increased to 4 ml.

Injection of 0.2-μm Fluorescent Beads; Space-Filling and Nonleaky

The limitations of dextran and the 1-μm beads were obviated by the perfusion of the animals with a suspension of smaller 0.2-μm blue beads. These beads were small enough that the suspension behaved like a fluid, filling up capillary lumens as the dextran did, but they remained completely localized to the vessels in tissue sections like the larger beads did (Fig. 3). Furthermore, the beads remained localized to vessels in the presence of VEGF, which had caused the dextran to leak (Figs. 3E and 3F). Therefore, in contrast to the methods shown in Figs. 1 and 2, the 0.2-μm bead suspension acted as a fluid but nonleaky tracer.

Two additional modifications to the procedure solved the earlier problem of unreliable perfusion: (i) The vasodilator nitroglycerine was added to the Nembutal used to euthanize the animals, ensuring that the blood flow to certain muscle regions was not reduced as the animals expired. (ii) Care was taken to avoid deforming or applying pressure to the lower legs during the procedure; that is, the hindlimbs of the mice were left unrestrained by pins or tape to avoid interfering with access to regions of the hindlimb muscles. Both of these modifications, as well as a 4-ml perfusion volume, were used to generate the results shown in Fig. 3. The modified perfusion regimen similarly increased the access to capillaries of the 1-μm beads, but they were still too large to successfully delineate the outlines of the microvessels. The 0.2-μm bead suspension was not only present in the vast majority of capillaries identified by PECAM staining, but was also completely space-filling such that the bead fluorescence was nearly identical to the PECAM fluorescence and was still not observed to leak from the vessels before or during processing of the tissue.

DISCUSSION

The use of extremely small 0.2-μm fluorescent beads shown here combines the advantages of both categories of tracer dyes currently in use for studies of blood flow and continuity. Dextran and other soluble dyes have been advantageous for many applications because they are a fluid and fill up the lumen of a blood vessel, thus giving an accurate outline of the vessel by microscopy. We observed rare instances of dextran leakage from normal vessels (shown in Figs. 1E and 1F), but these instances of leakage were associated with longitudinally cut capillaries, suggesting that much of the leakage was caused by the sectioning itself. However, even large-molecular-weight dextrans can leak under conditions of vascular permeability. In contrast, 7- to 16-μm fluorescent microspheres do not leak out of vessels and have been used in many studies of blood flow, but they are designed to lodge in capillaries rather than outline them. Smaller, nonoccluding fluorescent beads (1.3 μm in diameter) have been employed for video measurement of travel through microvessels (22). However, as we have shown here, even 1.0-μm beads are too large to accurately visualize the vessels. The suspension of 0.2-μm fluorescent beads that we used is best, as it combines the best attributes of the previously used methods. The individual beads are still too large to leak from vessels, but the suspension appears and behaves as a fluid, yielding pictures of capillaries that are essentially indistinguishable from immunostaining of the capillaries themselves.

One notable difference between this approach and previously reported methods that involve soluble dyes is that total perfusion of the vasculature, involving complete replacement of the blood, is much more effective than the injection of dye into the circulation of living animals. Thus, the beating of the heart and the active circulation of the blood are not available to ensure complete distribution of the tracer throughout the entire vascular tree. Therefore, extra measures had to be included to help the tracer gain access to the entire microvascular compartment. The use of nitroglycerine as a vasodilator helped to prevent vessels from being constricted as the animals died. Likewise, instead of tightly pinning the animals down to a board, as is customary during dissection, only the forepaws were pinned and the rest of the animal was left in a natural position. The combination of these two methods was critical to our results, as they allowed complete perfusion, whereas our earlier efforts frequently resulted in complete lack of bead suspension in large regions of the muscle.

The ability to analyze leaky blood vessels is likely to become increasingly important as interest grows in the delivery of permeability-enhancing angiogenic factors like VEGF. Furthermore, tumor microvessels exist in a highly permeable state, and analyses of both their growth and origin can be aided by the use of nonleaky tracers. The need for such analysis is especially important in light of recent observations that normal and tumor-associated blood vessel formation appears to involve circulating endothelial precursor cells (1-3, 24, 28). The assessment of vascular continuity between new vascular structures and the preexisting vasculature can help to determine if the new structures are derived from the preexisting vessels or if they have arisen de novo. This approach is serving as the basis for ongoing studies of the response to high-level VEGF gene expression (M. Springer, unpublished). Furthermore, the recent intriguing observation that some tumors may be vascularized in part by nonendothelial channels (16) serves as a reminder of how much remains to be learned about tumor vasculature. The system described herein provides a way of studying tumor vessels and new vessels formed in response to angiogenic factors delivered directly and by gene therapy. Thus, this approach should have applications for both tumor biology and induction of therapeutic angiogenesis.
REFERENCES


