CAM ASSAY

Shell-less embryo culture

Fertilized white leghorn chicken eggs (SPAFAS Inc., Norwich, CT) were received at day 0 and incubated for 3 days at 37°C with constant humidity. On day 3, eggs were rinsed with 70% ethanol and opened into 100 mm2 tissue culture coated Petri dishes under aseptic conditions. The embryos were then returned to a humidified 38°C incubator2 for 7-9 additional days.

Mesh assay

Substrates

Vitrogen (Collagen Biomaterials, Palo Alto, CA) was diluted to a concentration of 1.46 mg/ml with an equal volume of DMEM (HEPES buffer, no phenol red, GIBCO BRL, Gaithersberg, MD). This 1:1 mixture made up half of the total volume to be pipetted onto the mesh (final concentration = 0.73 mg/ml). Matrigel (Becton Dickinson, Bedford, MA) was diluted to a final concentration of 10 mg/ml with DMEM and directly pipetted onto meshes. Nylon mesh with 250 μm2 openings were cut into 4 mm x 4 mm squares and autoclaved. In preparation for polymerization, meshes were placed, under aseptic conditions, onto a non-binding surface (*i.e.*, bacteriological Petri dish). The polymerization conditions for each substrate were identical; after mixing the growth factors and/or compounds, 40 μl were pipetted onto each mesh in a bacteriological Petri dish as described above. The Petri dish was placed in a humidified 37°C incubator with 5% CO2 for 30 minutes to allow polymerization followed by an incubation at 4°C for 2 hours.

Growth Factors

VPF/VEGF165 (Peprotech, Rocky Hill, NJ) was resuspended at 100 ng/μl in HBSS (Sigma, St. Louis, MO). For each mesh containing VPF/VEGF, 250 μg was used.

Placement of meshes

In a tissue culture enclosure, meshes were placed onto the periphery of the CAM of a day 12-14 embryo, excluding areas containing major vessels. The embryos were then returned to the humidified 38°C incubator with 3% CO2 for 24 to 48 additional hours.

Visualization of vessels

Embryos were removed from the incubator and meshes were viewed under a dissecting microscope for gross evaluation. For injection, borosilicate glass capillaries (OD 1.0 mm, ID 0.75 mm; Sutter Instrument Company, Novato, CA) were prepared with P-87 micropipette puller (Sutter Instrument Company). Needles were connected with Tygon tubing (ID 1/32", OD 3/32", wall 1/32") to a 3cc syringe with a 20-gauge needle. The syringe was attached to an infusion pump (Harvard Apparatus, South Natick, MA). Injection of 400 μl FITC dextran, MW 2,000,000 (Sigma, St. Louis, MO), into the umbilical vein was performed at a rate of 200 μl per minute. The FITC dextran was allowed to circulate for 5 minutes and 3.7% formaldehyde in PBS was applied directly on each mesh. The embryos were then incubated at 4°C for 5 minutes and the meshes were dissected off the CAM and fixed in 3.7% formaldehyde for 10 minutes to overnight.

Quantification of vessels

After fixation, meshes were mounted on slides with 90% glycerol in 1 X PBS and visualized on an inverted fluorescence microscope. A Nikon Diaphot with a Sony DXC-151A camera attached to the side port was used for capture of images, which were transferred to a Power Macintosh 7100/66AV. NIH Image 1.61 (public domain program available on the Internet at http://rsb.info.nih.gov/nih-image/) was used to capture and analyze images. For each mesh, 5 random staggered images (approximately 600 μm each) were captured using "Capture Frames," followed by "Make Montage" in order to display all of the frames at once. The areas of high intensity were highlighted using "Density Slice" with (LUT selected at 105) and measured using the "Measure" function. When density slicing is used in an image, the "Measure" function calculates the areas of highlighted pixels.