Antibodies and growth factors

Recombinant EphB4/Fc, recombinant ephrinB2/Fc, the polyclonal anti-mouse ephrinB2 antibody and recombinant human monocyte chemoattractant protein-1 (MCP-1) were purchased from R&D Systems (Wiesbaden, Germany). The monoclonal anti-human CD31 (clone: JC70A) antibody was from DAKO (Glostrup, Denmark). The polyclonal anti-human CD31 antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

Animal models

All animal studies were performed with permission of the Regional Council Karlsruhe and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Five to 6 months old male C57/B6 mice were anesthetized with isoflurane and the femoral artery was ligated just distal to the origin of the deep femoral artery. On day 7 after surgery, the mice were euthanized and the left ventricle of the heart was cannulated and perfused for 2 min at 100 mm Hg with Ringer solution containing 0.1% adenosine plus 0.05% BSA (w/v) at 37°C followed by zinc fixative containing a colored pigment (HKS Gouache 318; Schmincke, Germany) that cannot pass the capillary system. The muscles containing the growing collateral arteries from the ligated and sham operated hind limbs were excised and processed for histological examination.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from freshly collected umbilical cords of newborn and cultured in medium M199 (Invitrogen, Karlsruhe, Germany) containing 20% fetal bovine serum (Life Technologies, Karlsruhe, Germany), 50 U/ml penicillin, 50 µg/ml streptomycin, 5 mmol/l HEPES supplemented with ECGS (Promocell, Heidelberg, Germany) on plastic dishes or BioFlex™ Collagen type I 6-well plates (Flexcell, Hillsborough, NC) coated with gelatin (2 mg/ml gelatin in 0.1 M HCl for 30 min at ambient
temperature). Human smooth muscle cells (HSMC) were isolated from human thymus glands after thymectomy and cultured in DMEM (Invitrogen) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal bovine serum. The phenotype of these cells was confirmed by an α-smooth muscle actin and desmin specific immunofluorescence. The isolation of both HUVEC and HTSMC was approved by the local ethics committee (Heidelberg, Germany) and conformed to the principles outlined in the Declaration of Helsinki (1997). Human aortic endothelial cells and human saphenous vein endothelial cells were purchased from Promocell and cultured in culture medium according to the manufacturer’s instructions. Cells were cultured at 37°C, 5% CO₂ and 100% humidity. Only cells cultured up to passage 4 were used for the experiments. Monocytes were freshly isolated from human buffy coat by a Ficoll and Percoll gradient density centrifugation and cultured for 24 hours in F12 medium containing 10% FCS and 5 µg/ml gentamycin before use. The identity and purity of these cells was verified by positive CD31 immunofluorescence.

Visualization of the arterial system

Perfused mouse hind limbs were postfixed in zinc fixative (18 hours) and dehydrated using a series of alcohol and isopropanol following standard protocols. Tissues were then incubated in a mixture of benzyl alcohol and benzyl benzoate having the same refractive index of the tissue for at least 18 hours. This procedure induces transparency of the tissue and allows detailed analysis of the pigment loaded arterial system. Diameter of collateral arterioles was determined using the morphological analysis software Cell^R from Olympus (Hamburg, Germany).

Morphological analysis

Immunohistochemical and immunofluorescence staining for ephrinB2 was performed on 4-µm paraffin sections by using the polyclonal goat anti-mouse ephrinB2 antibody or - as control antibody - a goat anti-rabbit antibody (DAKO) in combination with an enhanced detection method (Envision™, DAKO) and 3,3-diaminobenzidine (DAB) or tyramide-Cy3 (Perkin Elmer, Rodgau, Germany) according to the manufacturers’ instructions. Nuclei were visualized by
Mayer’s hemalaun counter stain or the Hoechst dye 33258. EphrinB2 staining intensity was determined by using the Cell^R software analyzing at least two different sections of two different collateral arterioles per experimental group and animal. Exposure times during digital imaging were kept constant.

*Image acquisition and processing*

Generation of fluorescence images: Slides were viewed with an Olympus IX81 confocal microscope (Olympus Europe Holding, Hamburg, Germany) using a UPlanApo lens at 20x/0.70 and 40x/0.90 and Prolong Gold Antifade reagent (Molecular Probes, Eugene, USA). Images were acquired using a black and white CCD camera (Hamamatsu, Germany) model ORCA-ER, and were processed with the Cell^R imaging software (Olympus).

Generation of colored or phase contrast images: Slides were viewed with an Olympus CKX41 inverted research microscope using a LCAch lens at 20x/0.40 PhP and a CAch lens at 10x/0.25 (Olympus) and DePeX Mount medium (Roth, Karlsruhe, Germany). Images were acquired using a Olympus digital camera model Camedia C-5050 Zoom, and processed with the Cell^R imaging software (Olympus).

*Transmigration assay*

PAEC were seeded onto 6-well cell culture inserts with 8 µm pore size (BD Biosciences, Heidelberg, Germany). Transmigration of 1 x 10^6 THP-1 cells or human monocytes seeded into the upper chamber was enhanced by supplementing the medium in the lower chamber with 100 ng/ml MCP-1. Transmigration was quantified by counting the number of THP-1 cells which had moved into the lower chamber within 18 hours. At the end of the experiment, the culture inserts were washed three times in Hank’s buffered salt solution (HBSS), fixed in buffered 4% p-formaldehyde and subsequently stained for human CD31 to detect the THP-1 cells lodged in the endothelial cell monolayer. Staining was performed by using a monoclonal anti-human CD31 antibody (clone: JC70A, DAKO) in combination with a Cy2-labeled secondary antibody. Only the human THP-1 cells but not the PAEC are visualized by this method.
In a separate series of experiments, HUVEC were seeded onto 24-well cell culture inserts with 3 µm pore size (BD Biosciences, Heidelberg, Germany). Transmigration of monocytes seeded into the upper transwell chamber was induced by supplementing the medium in the lower chamber with 100 ng/ml MCP-1. In some experiments, ephrinB2 binding to its receptors was blocked by adding 4 µg/ml recombinant ephrinB2 (GenWay Biotech, San Diego, USA) to the medium in the upper chamber. Transmigration was quantified by counting the number of monocytes which had migrated to the lower chamber within 18 hours.

**RT-PCR and decoy oligodeoxynucleotide technique**

Reverse transcription (RT) and polymerase chain reaction (PCR) for human ephrinB2, GAPDH and ribosomal protein L32 (RPL32) cDNA as well as preparation of the AP-1 decoy oligodeoxynucleotides was performed as described previously. The specific primers for ephrinB2, EphB1, EphB2, EphB3 and EphB4 were used as described before by Kim et al. Double-stranded AP-1 decoy oligodeoxynucleotides (ODNs) were prepared from complementary single-stranded ODNs (forward strand: 5'-CGCTTGATGACTCAGCCGAA-3') as described previously. HUVECs were transfected by ODN treatment (10 µM) for 4 hours without using any cationic lipid or liposomal complex.

**References**

