4D Microvascular Analysis Reveals That Regenerative Angiogenesis in Ischemic Muscle Produces a Flawed Microcirculation

SUPPLEMENTAL MATERIAL

DETAILED METHODS

Histology and Immunostaining

Extensor digitorum longus (EDL) muscles were dissected, fixed in either Tris-buffered zinc or 4% paraformaldehyde (PFA) overnight and embedded in paraffin. Five-μm thick cross-sections of EDL muscle were stained with hematoxylin and eosin. Near adjacent sections were subjected to heat-mediated antigen retrieval with citrate buffer and immunostained for endothelial cells using biotinylated rat monoclonal anti-mouse CD31 antibody (1:100, BD Biosciences 553371) and DAB Substrate (Vector). Nuclei were visualized by counterstaining with hematoxylin. For immunofluorescence detection of endothelial cells, five-μm thick sections of EDL muscle were subjected to antigen retrieval and immunostained using rat monoclonal anti-mouse CD31 antibody (1:20, Dianova, Clone SZ31) or rat monoclonal anti-mouse endomucin antibody (1:100, Santa Cruz, Clone V.7C7, sc-65495) and detected using Alexa Fluor-488 conjugated goat anti-rat IgG (1:100 for CD31, 1:200 for endomucin, Life Technologies). Endothelial cells were also stained using biotinylated isolectin-b4 (1:100, Sigma, BSI-B4, L2140), detected using Dylight 488-conjugated streptavidin (1:200, Vector) and immunostained for Von Willebrand factor (vWF). In the latter instance, EDL muscles were fixed in 4% PFA at room temperature for two hours, subjected to cryoprotection by immersion in 15% sucrose at room temperature for two hours then 30% sucrose at 4°C overnight, frozen in OCT compound (Tissue-Tek), and stored at -80°C. Ten-μm thick frozen sections were post-fixed with 4% PFA for ten minutes and immunostained using rabbit polyclonal anti-vWF antibody (1:50 in 0.1% Triton-x 100, Millipore AB 7356) and biotinylated goat anti-rabbit IgG (1:100 in 0.1% Triton-X 100, Vector) and Dylight 488-conjugated streptavidin (1:200 in 0.1% Triton-X 100, Vector). Nuclei were visualized with DAPI Fluoromount-G (SouthernBiotech, 0100-020). Sections were imaged by widefield microscopy (Olympus BX-51) and photomicrographs captured with Northern Eclipse (EMPIX Imaging Inc.) software.

Capillary density and capillary-to-muscle fiber ratio were quantified in 10 equally spaced high-powered (60x objective) fields from a 50-μm thick zone at the muscle periphery using ImageJ (NIH). Muscle fiber density and cross-sectional area were quantified in the mid-zone of the native and regenerated EDL muscle under high-powered view (40x objective) using ImageJ (NIH). For infarct analysis of all muscles in entire the hindlimb, the PFA-fixed hindlimb was decalcified by immersing in 6.5% EDTA solution (pH 7.0) for 10 days and then divided into five blocks that were embedded in paraffin, sectioned, and stained for hematoxylin and eosin.

Detection of Muscle Hypoxia

Muscle hypoxia was assessed by immunohistochemical detection of pimonidazole adducts, following intraperitoneal injection of pimonidazole hydrochloride (60 mg/kg, Hypoxyprobe™-1Kit, Hypoxyprobe, Inc.) into mice 28 days following femoral artery excision. Forty minutes after injection, mice were sacrificed by isofluorane overdose, perfused sequentially with PBS and 4% PFA via left ventricle cannulation at physiological pressure, and both injured and contralateral EDL muscles were dissected. Five-μm thick paraffin-embedded cross-sections were immunostained with a monoclonal mouse anti-pimonidazole antibody (Hypoxyprobe-1 mAb, 1:50) and the signal detected according to the manufacturer’s protocol with DAB substrate (Vector). Injured EDL muscles dissected from mice 28 days following femoral artery excision that were not injected with pimonidazole hydrochloride served as a technical control. Sections were counterstained with hematoxylin and hypoxia signal was quantified from entire EDL muscle mid-zone cross-sections using ImageJ, by isolating DAB via the colour deconvolution plugin¹ and measuring the raw integrated signal density following thresholding, using an identical grey
scale (61-255) for all samples.

Transcript Analysis by RT-PCR

Total RNA from uninjured and regenerated EDL muscles 28 days following femoral artery excision was extracted with Trizol (Life Technologies) and RNEasy (Q IAGEN) and subjected to reverse transcription as described previously.2 The mRNA abundance of adrenomedullin, lysyl oxidase (LOXL2), procollagen lysyl hydroxylase 2 (PLOD2), and VEGF-A was quantified using quantitative RT-PCR and SYBR Green chemistry and a ViiA 7 Real Time PCR System (Life Technologies). Gene expression was normalized to the mouse 18S signal using the \( \Delta \Delta \)Ct method. Primers pairs (Origene) employed were: Adrenomedullin, 5’-GCCAGATAGTCTCTCGCAGTT-3’ and 5’-GCGACTACTCTTGGTGACGAGTT-3’; LOXL2, 5’-TTCTGCCTGGAGGACACTGAGT-3’ and 5’-CGGTCACCTATCTGTAGTGGCT-3’; PLOD2, 5’-CATCCGAGGTTCCATTGGCTCCAG-3’ and 5’-CATGGTGACTTTTCTCTGCACG-3’; VEGF-A 5’-CTGCTGTAACGATGAAGCCCTG-3’ and 5’-CCTCTACTCGAAGATGTGCG-3’; ANGPT1 5’-AACCGAGGCTTACACAAGTACG-3’ and 5’-GATCCCTCGTGTGATCG-3’; and ANGPT2 5’-AATCTCGCTTCTGGAGATACG-3’ and 5’-TTCCGAGCAGTCTCTGGAAGTG-3’.

Intravital Video Microscopy

RBC hemoglobin O\(_2\) saturation in capillaries near the EDL surface was assessed by white light transillumination IVVM, as described previously.\(^3,4\) For this, a silk suture was attached to the distal EDL tendon. The tendon was severed distal to the ligaure, and the EDL was reflected onto the microscope stage (Olympus IX81) and secured at approximately its in situ resting length and orientation. The EDL was kept moist with Plasma-Lyte 148 (Baxter International) at 37°C, and covered with polyvinylidene chloride film (Saran Wrap, Dow Chemical) and a glass coverslip to isolate it from room air. The EDL was transilluminated with a 100-W xenon lamp, and transmitted light was captured with 10x and 20x objectives (Olympus UPlanSapo) and a parfocal beam splitter (DualCam) fitted with 442-nm (an O\(_2\) sensitive wavelength for oxy- and deoxyhemoglobin)\(^5\) and 454-nm (an isosbestic or O\(_2\) insensitive wavelength) band pass filters for dual video cameras. The beam splitter passed 442-nm light to one cooled coupled device camera (Rolera-XR, QImaging) and 454-nm light to a second camera, and dual video recordings (696x520 pixels, 21 images/s) were simultaneously obtained using custom capture software (Neovision). Images from both cameras were registered using the beam splitter and the capture software ensured synchronized frame-by-frame acquisition from both cameras. A minimum of 20 fields of view (10 at the arteriolar end, and 10 and the venular end of a capillary network) were randomly selected and recorded for 60 seconds in each EDL muscle. Video sequences were stored as uncompressed PNG files for post-processing using in-house software written in the MATLAB (Mathworks) programming environment. O\(_2\) saturation in capillaries was obtained by analysis of PNG files using custom image analysis software written in Matlab, as described by Ellis et al.\(^6,7\) and Japee et al.\(^8,9\). Briefly, in-focus capillary segments were selected, and the location of the vessel centerline was used to extract light intensity values from every video frame and generate space-time images for both the 442- and 454-nm wavelengths. The light intensity values were analyzed frame-by-frame to identify the location of each RBC and plasma gap within the capillary. The mean optical density (OD) of each RBC at both wavelengths, was computed as OD=\log(I_o/I_m) were I_o is the measured light intensity of the plasma gaps (intensity of incident light) and I_m is the measured light intensity values for RBCs. The mean RBC hemoglobin O\(_2\) saturation was determined from the ratio of OD(442)/OD(454), which is linearly related to O\(_2\) saturation, i.e. SO2 = a + b * ODratio. The slope and intercept were determined from an in vivo calibration.

Analysis of Microvascular Network Architecture

Length density and bifurcation density of EDL muscle microvascular networks were quantified from RBC transit maps, which effectively displayed a micro-angiogram of all flowing microvessels, using ImageJ. RBC transit maps were generated from IVVM video sequences using custom MATLAB software.
as described. From the videos generated by ultraviolet light epi-illumination, RBC transit maps were generated from “minimum” (MIN) images, which displayed the minimum light intensity value and therefore RBC transit at a given pixel during the duration of the video sequence. From the videos generated by blue light epi-illumination, RBC transit maps were generated from “sum of all differences” (SAD) images, which displayed the cumulative sum of the square of differences in light intensity values at each pixel between consecutive video frames, thus generating a single map of all microvessels perfused with RBCs and plasma. Length density (microvasculature length [μm] / EDL area [μm²]) was quantified at each time-point via manual tracing of all vessel centerlines and normalizing total network length to EDL area. Bifurcation density was determined by manual point counting of all vessel bifurcations and normalizing to total network length. Arteriole-capillary-venule microcirculatory units and AV malformations were identified from blue light epi-illumination videos and corresponding SAD images.

**Analysis of Capillary Red Blood Cell Velocity and Distribution**

Red blood cell velocities ($V_{RBC}$ [μm/sec]) in individual capillaries exhibiting characteristic single-file RBC transit were quantified from video files using space-time images. Briefly, 1-second (21 frames) interval mean red blood cell velocities were calculated using custom MATLAB software, generating a 2D gray scale plot of RBC location change with time. This enabled an unbiased quantification of RBC transit velocities within capillaries throughout the entire muscle. RBC velocity was determined from a total of 1658 one-second intervals in 115 uninjured capillaries, and from 2269 one-second intervals in 138 neocapillaries, 28 days following femoral artery excision. Histograms of mean $V_{RBC}$ determinations from each uninjured capillary and neocapillary were generated to define the spatial heterogeneity of red blood cell velocity within the microvasculature. The number of non-flowing capillaries and neocapillaries was determined from blue light epi-illumination videos, cross-referencing with corresponding SAD images, evident as either empty channels of plasma or capillaries with stationary RBCs for at least 15 seconds duration.

**Analysis of Capillary Network Hemodynamic Resistance**

To assess possible changes in capillary network resistance, three control and three regenerated networks (all at day 28) were reconstructed using 10x IVVM data from fields of view acquired to determine RBC flow and network architecture, as described above. Each network was reconstructed into a collection of nodes and connecting cylindrical vessel segments using our previously developed software. Two-phase (RBCs and plasma) steady-state blood flow simulations were performed using an established computational model with a fixed pressure difference (DP) between all inflow and outflow nodes. The resistance for each network was calculated as DP divided by the total flow ($Q_{tot}$) through (i.e., into or out of) the network, $R=DP/Q_{tot}$. Flow was mainly unidirectional (e.g., in at bottom and out at top of network), but for two networks flow was bidirectional indicating a major source (arteriole) or sink (venule) within the network. For these networks, resistances were calculated for upward and downward flowing portions separately and added to approximate the resistance of a single network with unidirectional flow. For all networks resistance was normalized by dividing by length (L) in the main flow directional and multiplying by width (W) in the perpendicular direction, so that $R_{norm}=WxR/L$. This was done to account for variability in network lengths and widths produced by the capture and reconstruction procedure, since resistance is proportional to vessel length and total flow should be proportional to width of a network.

**Statistics**

All values passing D’Agostino and Pearson omnibus normality testing are presented as mean ± SEM. Those not passing the normality test are presented as median and IQR. Comparisons among normally distributed variables were made by t-test or analysis of variance with Bonferroni’s post hoc test. Comparisons among hypoxia RBC measurements, width of inter-process gaps, and $V_{RBC}$ were made by Mann-Whitney test or Kruskall-Wallis test with Dunn’s post hoc test. Comparisons among percent of arterioles with AV malformations, percent of capillaries depicting a greater than 5-fold hyperemic
hypoxia response, and percent of capillaries sustaining a hyperemic hypoxia response were made by Fisher’s exact contingency test. Mean $V_{\text{RBC}}$ histograms were compared using a two-way Kolmogorov-Smirnov test in MATLAB custom software.
Online Figure I  Loss of Capillaries Following Ischemic Injury to the EDL Muscle

A-B. Hematoxylin and eosin-stained sections of EDL muscle before (A) and three days following femoral artery excision (B). The native muscle fibers are surrounded by capillaries and discrete endothelial cell nuclei can be seen (A, arrows). Following ischemic injury, the skeletal myocytes have lost their nuclei. Capillaries are no longer evident, with only the occasional fibrous ghost structure without nuclei (B, arrowheads).
Online Figure II  Capillary Network Hemodynamic Resistance

A-B. Three-dimensional microvascular network reconstructions of IVVM data collected from native (A) and regenerated (B, 28 days) EDL muscle. Each reconstruction is a collection of nodes and cylindrical vessel segments. Color coding denotes depth separation of the capillary segments. C. Graph showing capillary network hemodynamic resistance ascertained by simulating two-phase (RBCs and plasma) steady-state blood flow through three-dimensional network reconstructions (n=3 native and regenerated network reconstructions, *p=0.032).
Online Figure III  Capillary RBC Hemoglobin O₂ Saturation

A. Box and whisker (min-max) plot of capillary RBC hemoglobin O₂ saturation in native capillaries in control EDL muscle and regenerated capillaries in EDL muscle 120 days after ischemic injury. Data are from 24 capillaries from 3 native EDL muscles and 56 neocapillaries from 3 regenerated muscles. *p=0.0003. Plots in B and C depict RBC hemoglobin O₂ saturation selectively in post-arteriolar capillary segments (*p=0.005) and in pre-venular capillary segments (*p=0.002).
SUPPLEMENTAL REFERENCES


LEGENDS FOR VIDEO FILES

**Online Video I:** RBC transit in the native EDL muscle microvasculature visualized by blue light epi-illumination real-time video microscopy. RBCs are seen in relief against the bright plasma that has been labeled with high molecular-weight FITC-labeled dextran.

**Online Video II:** Real-time video microscopy sequence showing no RBC transit in the EDL muscle one day following femoral artery excision.

**Online Video III:** Real-time video microscopy sequence showing RBC transit in the early (10 day) regenerated EDL muscle microvasculature.

**Online Video IV:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 28 days after femoral artery excision.

**Online Video V:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 56 days after femoral artery excision.

**Online Video VI:** Real-time video microscopy sequence showing RBC transit in the regenerated EDL muscle microvasculature 120 days after femoral artery excision.

**Online Video VII:** Real-time video microscopy sequence showing a neo-arteriole (top of field) in the late-stage regenerated EDL muscle microvasculature that bifurcates into daughter vessels with strikingly unequal lumen diameters. RBCs are visualized by ultraviolet light epi-illumination.

**Online Video VIII:** Real-time video microscopy sequence of the microvasculature 28 days after femoral artery excision showing halted RBC transit.

**Online Video IX:** Real-time video microscopy sequence showing RBC transit (demarcated by FITC-labeled dextran) through an arteriole-capillary-venule microcirculatory unit in the native EDL muscle. A normal microvascular hierarchy is evident with divergence of an arteriole into discrete capillary meshes that drain into venules.

**Online Video X:** Real-time video microscopy sequence showing RBC transit through an aberrant microcirculatory unit in the regenerated (28 day) EDL muscle microvasculature. An arteriolar-venular malformation (AV-connection) with high RBC flux can be seen to directly connect the neo-arteriole to a venule, bypassing a capillary mesh.

**Online Video XI:** Real-time video microscopy sequence showing RBC transit through an AV-connection in the 28-day regenerated EDL muscle microvasculature, visualized by ultraviolet light epi-illumination. A direct connection between the neo-arteriole and a venule is present.

**Online Video XII:** Real-time video microscopy sequence of a native EDL microvasculature showing an increase in RBC velocity and supply rate in response to a local hypoxia challenge. The video sequence displays the identical field of interest during hyperoxia (12% O₂) and 120 seconds after converting to hypoxia (2% O₂).