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Experimental and theoretical modelling of blind-ended vessels within a developing angiogenic plexus

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ABSTRACT

Angiogenic sprouts at the leading edge of an expanding vascular plexus are recognised as major regulators of the structure of the developing network. Early in sprout development, a vascular lumen is often evident which communicates with the parent vessel while the distal tip is blind-ended. Here we describe the temporal evolution of blind-ended vessels (BEVs) in a small wound made in the *panniculus carnosus* muscle of a mouse viewed in a dorsal skin-fold window-chamber model with intra-vital microscopy during the most active period of angiogenesis (days 5–8 after injury). Although these structures have been mentioned anecdotally in previous studies, we observed BEVs to be frequent, albeit transient, features of plexus formation. Plasma leakage into the surrounding extracellular matrix occurring from these immature conduits could play an important role in preparing hypoxic tissue for vascular invasion. Although sprout growth is likely to be regulated by its flow environment, the parameters regulating flow into and through BEVs have not been characterised *in situ*. Longitudinal data from individual animals show that the number of BEVs filled with plasma alone peaks at day 7, when they can exceed 150 μm in length. Additionally, BEVs greater than 40 μm in length are more likely to be filled with stationary erythrocytes than with plasma alone. Using a mathematical model, we show how the flux of 150kD fluorinated (FITC-) dextran through an individual plasma-filled BEV is related to its geometry being determined primarily by its surface area; by fitting theoretical intensity values to experimental data we assess the permeability of the vessel to FITC-dextran. Plasma skimming provides a mechanistic explanation for the observation that BEVs with larger surface area are more likely to recruit erythrocytes.

Introduction

Establishment of a circulatory system is a key stage in the embryonic growth of vertebrates, which allows organ growth and the subsequent differentiation of tissue types. The maintenance of an appropriate vascular supply in the adult is a tightly regulated process, which is often temporally restricted. This is particularly evident in the hormonally-driven cyclical control of vascular growth in the female reproductive organs (particularly the ovary, corpus luteum and uterus) during the menstrual cycle, as well as in the intense vascular growth and remodelling that occur during physiological wound healing.

There are at least 5 distinct morphological processes in which vascular plexi are established, maintained or their structural complexity is increased: vasculogenesis (Risau and Flamme, 1995), angiogenesis (Carmeliet and Jain, 2000), intussusception (Burri, 1992), elongation (Gargett and Rogers, 2001) and integration of circulating endothelial precursors (Kopp et al., 2006). Although vasculogenesis is largely restricted to embryogenesis and extra-embryonic tissues (such as the yolk-sac and placenta), all of the other processes have been described in either normal physiological or pathological conditions in the adult. The most intensively studied of these processes is angiogenesis, whose defining characteristic is the initial sprouting of solid vessel cords from pre-existing vasculature.

Angiogenesis is a multi-step process, starting with the activation of endothelial cells (ECs) by a local preponderance of pro-angiogenic factors over inhibitors, previously described as the angiogenic switch (Hanahan and Folkman, 1996). Activated ECs loosen their homotypic interactions, produce extracellular matrix-degrading enzymes and migrate towards the angiogenic stimulus. Specialised endothelial "tip

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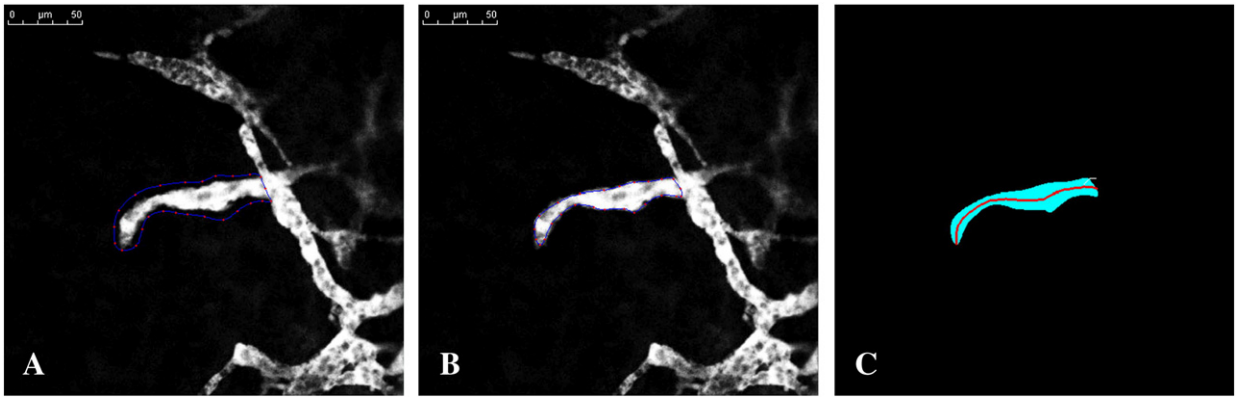

Fig. 1. (A) A B-spline curve is drawn around a plasma-filled BEV with nodes shown (red). (B) After applying several energy minimisation steps, the nodes describing the B-spline approximate more accurately the shape of the BEV. (C) The inner line of the final shape (see previous step) is extracted (red) and grey level measurements are performed at regular intervals along this inner line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells" direct the advancing cord and the cells at the base of this structure are able to proliferate, providing a pool of cells for expansion of the growing sprout (Gerhardt, 2003). These solid EC cords initially develop a lumen, forming blind-ended vessels; the tips of these vessels may join with other segments, establishing blood flow through the loop, as recently shown in the zebrafish gastrula (Vogeli, 2006). The transport of oxygen and nutrients though these newly formed vessels is mediated by well-characterised non-linear rheological effects (such as the plasma-skimming, Fahraeus and Fahraeus–Lindqvist effects (Popel and Johnson, 2005)), which influence both the spatial and temporal haematocrit distribution in microvascular networks (Pop, 2007). In particular, the phase-separation (plasma-skimming) effect acting at divergent bifurcations (Pries et al., 1996), in which plasma is drawn down low-flux branches in preference to suspended red blood cells, is likely to influence strongly the flow environment in BEVs.

Features of individual BEVs have been elegantly described both ultrastructurally and also in an *in situ* model of juvenile rat mesentery (Rhodin and Fujita, 1989), where rouleaux (clusters of erythrocytes), platelet aggregation and circulatory stasis were recorded. As BEVs represent the leading edge of the primary vascular plexus and therefore regulate its initial patterning, knowledge of their structure, temporal development and physiological properties is integral to the understanding of how a primary plexus develops. Angiogenic sprouts, a proportion of which are BEVs, represent the first evidence of functional circulation at the leading edge of vascular growth into ischaemic tissue. New vessels, including BEVs, which have yet to form functional basement membranes or attract peri-vascular support cells, are characteristically leaky, allowing large molecular-weight proteins access to the interstitium. These proteins include platelet degranulation products (Italiano, 2008), matrix-degrading enzymes (Roy et al., 2006) and components of the haemostatic cascade, which serve as a scaffold for blood vessel ingress and wound healing (Laurens et al., 2006).

In this study, the temporal development of BEVs in relation to vascular closure in a small healing wound is presented and from this data a mathematical model relating geometry to function of these critical intermediary structures is proposed. Relatively few previous mathematical models of the microcirculation have considered flow in permeable vessels (Baish et al., 1997; Pozrikidis and Farrow, 2003); unlike these studies, the proposed model specifically considers non-uniform vessels that are closed at one end. The model enables quantification of the transport of plasma labelled with the passive tracer 150 kDa FITC-dextran along the BEV and out into the interstitium, which in turn allows the permeability of the BEV walls to be determined. The model gives insight into the details of the transport properties that are inaccessible experimentally *in situ*. Combining theoretical and experimental techniques, the hypothesis that plasma skimming and vessel geometry determine the degree to which erythrocytes fill the BEV is investigated.

Materials and methods

Animals and surgery

Window-chamber surgery was performed as previously described (Lehr, 1993). Briefly, adult (10–12 week old) male CD1 mice (Harlan, UK) were anaesthetised with Hypnorm and Hypnovel and the hair on the dorsum was shaved with clippers. The skin was prepared for surgery by application of depilatory cream followed by 3 successive applications of Hibiscrub and Isopropanol. Titanium window chambers were implanted on the mid-dorsum and a small circular wound was created on the *panniculus carnosus* with a hot probe (1mm diameter, 70 °C for 3 s). The preparation was imaged after injury with a Nikon Eclipse E600FN epi-fluorescent microscope using a variety of objective magnifications. Imaging of the vasculature was facilitated by the intra-venous (*i.v.*) injection of 50 μl of 5% 150 kDa FITC-dextran (Sigma-Aldrich, UK). A total of 3 regions of interest (ROIs) around the wound area were imaged, beginning at the surviving rim of the lesion. Successive images were taken at the leading edge of the vasculature (at higher magnifications) between days 5–8 when the greatest numbers of BEVs were observed.

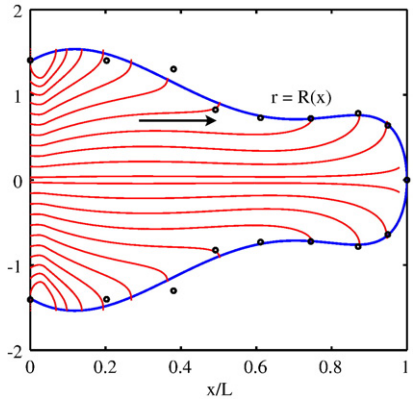

Fig. 2. Measurements of BEV radius $R(x)$ (scaled on the characteristic radius R_0) versus axial distance x/L (points) fitted with a polynomial (solid, blue). Internal lines show streamlines, computed for $\alpha = 1$. The flow as indicated by the arrow is from left to right.