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Preferential activity of Tie2 promoter in arteriolar endothelium

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Abstract

The tyrosine kinase Tie2/Tek (the receptor for angiopoietins) is considered one of the most reliable markers of the endothelial phenotype, across organisms, organs, and developmental stages. However, endothelium is intrinsically heterogeneous in origin, composition and function, presenting an arteriolar/venular asymmetry. In this regard, the expression of Tie2 along the vascular tree, although thought to be homogenous, has not been systematically investigated. Therefore we questioned whether the activity of Tie2 promoter is uniform in the microvascular endothelium. To this end, we analyzed *in situ* the expression of the markers β-galactosidase [LacZ(Tie2)] and green fluorescent protein (GFP) [GFP(Tie2)], placed under the Tie2 promoter in transgenic mice, in whole mount tissue samples, which allow the simultaneous evaluation of its relative distribution in various microvascular compartments. In the mesenteries of LacZ(Tie2) and GFP(Tie2) mice, we found that the activity of Tie2 promoter is asymmetrically distributed, being much stronger in arteries and arterioles than on the venular side of the vascular tree. This observation was replicated in the diaphragm of LacZ(Tie2) mice. The capillaries presented a mosaic pattern of Tie2 promoter activity. Stimulation of angiogenesis either by wounding, or by intraperitoneal injection of Vascular Endothelial Growth Factor (VEGF), revealed that the arteriolar/venular asymmetry is established at endothelial cellular level early during new capillary formation, even before the starting of the microvascular blood flow. In conclusion, a strong Tie2 promoter activity qualifies as a novel marker of the arteriolar phenotype in microvascular endothelium.

Keywords: Tie2 • endothelium • asymmetry • arterioles • venules • angiogenesis • mesentery • adipose tissue

Introduction

The model of angiogenesis as self-multiplication of microvascular structures by sprouting from parental endothelium is currently confronted with evidence of a multiplicity of other mechanisms, some involving the contribution of endothelial progenitor cells, and transdifferentiation of non-endothelial precursors (for a review, see [1]). Thus, the need for a practical definition of what is an 'endothelial' cell, and how this cell type can be identified *in situ* based on the expression of reliable molecular markers,

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became of critical importance. This task is complicated by the fact that in normal circumstances the endothelium represents a heterogeneous cellular phenotype, displaying specific molecular composition and functions in almost each organ and state [2].

One aspect of this heterogeneity is the arteriolar/venular asymmetry of endothelial cells properties [3]. This asymmetry might be established onthogenically, or derived from local factors, as for instance microfluidic factors and the supportive cellular elements such as the smooth muscle cells, which incur arterial identity [4]. However, the recruitment of smooth muscle cells to previously formed capillaries is in itself dependent on the pre-established arteriolar nature of the microvessel [5]. In addition, in several angiogenic fields the arterioles tend to develop before [6], or independent [7] of the capillary network.

Among the markers that differentiate arteriolar versus venular endothelium is the ephrin system. The transmembrane ligand ephrinB2 is predominantly expressed in arteriolar endothelium, while its receptor tyrosine kinase ephrinB4 is expressed on venules [8]. Using LacZ as a reporter gene under ephrinB2 promoter, it was shown that these distinct molecular identities are established early in embryogenesis, and are maintained during adult life both in quiescent and in angiogenic conditions [9,10]. Furthermore, these molecular markers showed that capillary endothelium seems to have a pre-formed arterial or venular phenotype, at least in the microvascular beds analyzed [9].

Other markers asymmetrically distributed between the arteriolar and venular endothelial cells are VEGF receptors [11], the Notch ligand D114 [12], the tyrosine kinase Bmx [13] and, in adult animals, the tyrosine kinase Tie1 [14]. For example, in the heart endocardium, brain capillaries and veins, and in sinusoidal capillaries of the liver, β-galactosidase placed under Tie1 promoter was barely detectable [14].

Tie2, the receptor tyrosine kinase for angiopoietins [15] was considered so far (based mostly on immunocytochemistry on cross-sections) one of the most reliable endothelial indicators across the whole vascular tree [16,17] and the whole animal lifespan [17,18,19,]. This supposition was seldom contested, without an experimental evidence for the contrary [20].

Tie2 was found generally associated with cells lining a vessel or in circulating cells, although there are several known exceptions. In addition, Tie2 is systematically expressed in *bona fide* endothelial progenitor cells (EPC) [21], but not by smooth muscle cell progenitors [22]. Tie2 was found upregulated in areas of active angiogenesis, consistent with a role in blood vessel maturation and maintenance [23]. Nevertheless, the uniformity of Tie2 expression is a matter of controversy in wounds [24], as well as in psoriatic versus uninvolved skin and in chronic spongiotic dermatitis skin [25]. In other studies, highly variable levels of mRNA for Tie2 (up to 150 fold) were found among the endothelial cells of *corpus luteum* [26], but the high/low expressors were not clearly assigned to one or the other side of microcirculation. Tie2 expression also displayed heterogeneity in the brain [15]. Some reports [17,27] indicate that Tie2 would be homogeneously expressed throughout the vasculature, but closer examination of the published images seem to indicate a lower intensity of immunostaining in veins [16]. In avian embryos, Tie2 was strongly expressed in veins, but only weakly transcribed in most arteries, while neuropilin-1 was expressed by arterial endothelium and wall cells, but absent from veins [28].

In order to assess the activity of Tie2 promoter in quiescent and angiogenic tissues of adult mamals, as well as the suitability of Tie2 as a pan-endothelial marker, we took advantage of the availability of transgenic mice that express green fluorescent protein (GFP) [29] and β-galactosidase (LacZ gene) [17] under the Tie2 promoter, thus labeling the endothelium of the whole animal *in vivo*. We used en face analysis of the mesenteric microvasculature of these mice both in unstimulated and in angiogenic settings. Here we show that Tie2 promoter activity is unevenly distributed in the endothelium of quiescent as well as of angiogenic mouse tissues.

Material and methods

Animals

Transgenic mice expressing LacZ gene under the endothelial Tie2 promoter [TgN(Tie2LacZ)182Sato],

and GFP (Tg(TIE2GFP)287Sato/J), as well as their respective wild-type controls, were obtained from Jackson Laboratories (Bar Harbor, ME). All experiments were performed in accordance with the guidelines of the Committee for Animal Research of the Ohio State University. For the surgical procedures, the mice were anesthetized with intraperitoneal injection of 100 mg/kg body weight ketamine and 10 mg/kg body weight xylazine.

Visualization of microvasculature

In order to analyze the microvascular architecture in its most natural disposition, we avoided the cross sectioning of the tissue, and we used the mesenteries and the diaphragms of the mice for en face imaging of the microvessels, as follows:

a) In GFP(Tie2) mice, the fluorescence was examined by confocal microscopy (Zeiss LSM 510 confocal multiphoton microscope) on freshly obtained, unfixed and unstained mesenteric specimens. For detection of microvascular lumen, the mice were first injected with 200 µl/animal of a 10 mg/ml suspension of Rhodamine-dextran (Molecular Probes, Eugene, OR) through the tail vein just prior to mesentery exposition.
b) For detection of β-galactosidase activity in the endothelium of LacZ(Tie2) mice, mesenteries or diaphragms were displayed on SuperFrost slides, let to dry for 20 minutes, fixed in 2% formaldehyde and 0.2% glutaraldehyde for 30 min and stained for LacZ activity using the X-gal substrate (Gibco-BRL, Gaithersburg, MD). The specimens were incubated overnight in an X-gal solution (0.1 % X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS, pH 7.0), as described [17]. This extensive incubation was necessary in order to make sure that the detection of β-galactosidase positive cells was not limited by the sensitivity of the assay. Special attention was paid to the pH of the incubation medium, as to avoid the artefactual staining of endogenous, "senescence-type" β-galactosidases, known to be active at more acidic pH (below 7.0) [30]. For easier identification of the microvascular lumens, the blood was not washed out.

Nuclear counterstaining of the mesentery was performed using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes, Eugene, OR), after β-galactosidase staining, following manufacturer's guidelines. Specimens were mounted in Fluoromount and analyzed with a Nikon Eclipse 800 fluorescence microscope in dual (transmission and fluorescence) mode.

c) Specific lectin staining of the microvascular endothelium was done by *in vivo* injection of biotin-labeled *Lycopersicon esculentum* lectin (Vector Laboratories, Burlingame, CA), and detection by a peroxidase-diaminobenzidine (DAB) based method, as described [31].

Induction of Mesenteric Angiogenesis

Was done by two methods:

a) Mechanical irritation. Mice were anesthetized as described, and the abdomens were opened following the median line. A light irritation was made by gently touching the mesenteric window with a sterile cotton swab, or by slightly pressing the fat tissue with sterile tweezers. The mice were then closed back with a few sutures, and let to recover. After 7 days, mice were sacrificed and the wounded part of the mesentery was stained for LacZ as indicated [17].
b) Intraperitoneal injection of angiogenic factors [32,33,34]. Mouse VEGF (R&D Systems, Minneapolis, MN), 10 ng/ml in 1 ml, was injected twice a day, for 5 days. After two additional weeks, the mice were sacrificed, and the mesenteries were collected on microscopic slides, dried in air for 30 minutes and then processed as described for LacZ detection.

Results and discussion

Heterogeneity of GFP fluorescence in GFP(Tie2) transgenic mouse mesentery

We chose to analyze Tie2 promoter activity in the mouse mesenteric tissue, since it has several advantages: (a) The microcirculation of adipose tissue is particular within the vascular system, because of its capacity to grow throughout most of adult life [35] *i. e.*, it maintains an angiogenic character. (b) Adjacent to the mesenteric fat, the mesentery contains avascular windows, which can become vascularized upon stimulation, and

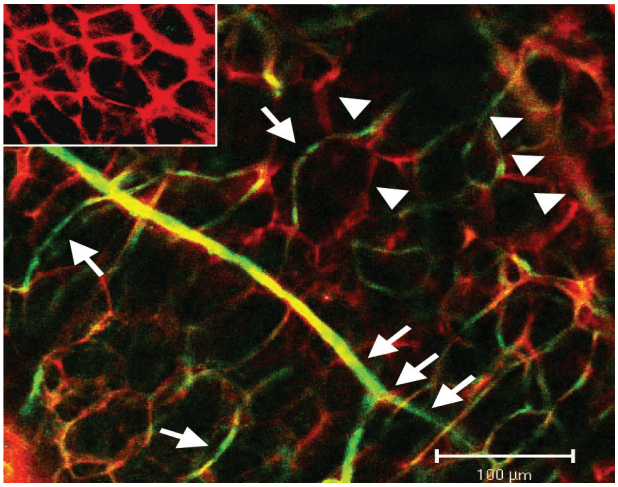

Fig. 1 Distribution of GFP fluorescence in mesenteric fat of GFP(Tie2) transgenic mice. Mice were perfused with Rhodamine-dextran (red) for labeling of all microvessels. GFP (green) fluorescence was present in arterioles and selected capillaries (arrows), and much weaker in the microvessels of the venular side (arrowheads). Inset: In a non-transgenic control mouse, imaged on both (green ad red) channels, the green fluorescence was absent in larger as well as in the smaller microvessels, making unlikely that the green fluorescence in GFP-Tie2 mice is the autofluorescence. Confocal microscopy. Bar, 100 µm.

thus offering a remarkable opportunity for the study of the architecture of spontaneous or induced angiogenesis in an essentially two-dimensional system [32,33,34].

To determine whether the activity of Tie2 promoter is uniform in various compartments of the microvascular tree, first we analyzed *ex vivo* the pattern of Tie2 promoter activity, in the non-perfused mesentery of transgenic GFP(Tie2) mice, by assessing the amount of GFP fluorescence present in microvessels from freshly isolated mesenteric fat tissue.

In transgenic mice expressing GFP(Tie2) under the Tie2 promoter, the green fluorescence of the GFP marker was abundantly present, together with the red fluorescent Rhodamine-dextran microvascular tracer. Hoverer, its distribution was very inhomogeneous, with about half of the microvessels of all calibers being labeled, while the other half barely presented this marker at all (Fig. 1). In the wild type controls we found the red fluorescent labeling of lumens of microvessels of various calibers, but no green fluorescence was detected (Fig. 1, *inset*). The smallest microvessels, most of them surrounding adipocytes (and thus being the capillaries), also presented a bimodal distribution (Fig. 1), suggesting that the large vessel phenotype of endothelium, at least with regard to GFP(Tie2) expression, reaches the capillary size range. Thus, Tie2 promoter is unevenly distributed in the mesenteric fat microvasculature of mice.

Variable Tie2 promoter activity in the microvessels of mesenteries and diaphragms of LacZ(Tie2) mice

To rule out that the inhomogeneity of GFP(Tie2) distribution could be an artifact of optical confocal sectioning of the reporter gene (GFP), we also analyzed the pattern of Tie2 promoter activity in the mesentery of transgenic LacZ(Tie2) mice, using β-galactosidase staining. For microvessels visualization, we used two approaches: (a) We collected the mesenteries form LacZ(Tie2) mice without prior washing of the microvasculature, and stained them for β-galactosidase activity. (b) We first injected the mice with *L. esculentum* lectin which has specific affinity for mouse endothelial cells [31], and then the mesenteries were collected, mounted on slides, fixed, stained for both β-galactosidase and the lectin, and visualized in transmission microscopy.

In the first case, we found that after β-galactosidase staining the arteries and arterioles of the specimens turned heavily blue. However, the residual blood retained in the venular compartment revealed, within the same microscopic fields, the presence of many other LacZ negative microvessels of comparable diameters. (Fig. 2A). This observation was further supported by the analysis of mesenteric microvascular beds after injection with the endothelium-specific lectin *L. esculentum* (Fig. 2B). This approach revealed both large and small microvessels, including the peri-adipocyte capillaries, which were constantly negative for β-galactosidase. The β-galactosidase-positive domain of arterioles was sometimes continued by single or aggregate LacZ(Tie2) positive cells, likely to be arteriolar-type domains (or bridges) amongst the capillaries (Fig. 2B). Alternatively, these isolated cells could be EPC, since it is known that EPC are strong expressors of Tie2 [21], and are also abundantly present in the mesenteric fat [36]. Their numbers

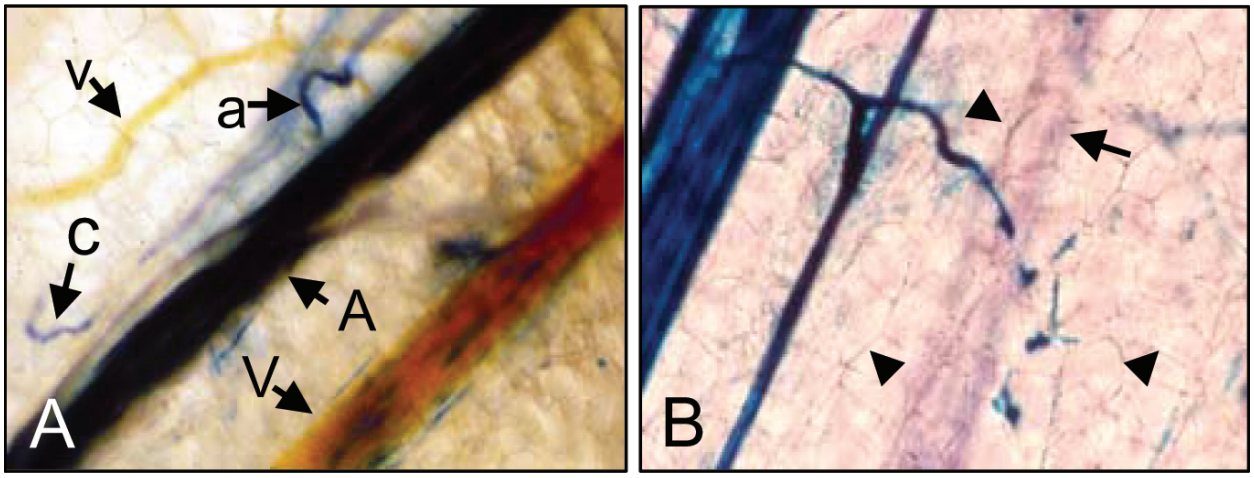

Fig. 2 β-galactosidase staining in the mesenteric fat of LacZ(Tie2) transgenic mouse. A. The arteriolar sides were intensely LacZ positive, while the venular branches and LacZ(Tie2) negative capillaries were detectable mostly due to their yellow-brownish color of leftover blood. A - artery; V - vein; a-arteriole; v - postcapillary venule; c - capillary. B. LacZ(Tie2) distribution in a mouse perfused with *L. esculantum* lectin (brown). Note the intense β-galactosidase staining of the arterioles, and virtual absence of staining in vein (arrow) and most capillaries (arrowheads), except in scattered cell clusters at the end of an arteriole. Phase contrast microscopy. Original magnifications: A, x 200, B, x 120.

were largely increased in angiogenic mesenteries (Anghelina *et al.*, in preparation).

In both analyzed models we found that the expression of β-galactosidase under the control of Tie2 promoter is unevenly distributed in the adult mesenteric microvessels. The order of expression of the staining intensity was arteries > arterioles>> veins > venules. The capillaries again displayed a mosaic staining, similar to the expression of arteriolar marker ephrinB2 [9]. Thus, in accordance with the GFP(Tie2) results, the expression of LacZ under the Tie2 promoter was drastically reduced in the post-arteriolar portion of microvasculature, with fainter and sometimes patchy distribution in larger veins, even after the extended incubations with the substrate for β-galactosidase. We also noted that instead of following a looped pattern as expected from the continuous blood conduits, the β-galactosidase positive vessels often terminated in non-stained, 'dead' ends, indicating an abrupt transition in Tie2 activity between the arteriolar and downstream endothelial domains. This was particularly clear in the diaphragm (Fig. 3), where the existence of bipolar microvascular fields (BMFs, [37]) facilitates the en face observation of transition between the arteriolar an venular domains.

The frequency of the observed Tie2 positive cells in microvessels was lower than when using GFP(Tie2) model, indicating that the histochemical method is less sensitive and may produce false negatives among the low expressing cells. However, both animal models and all tissues we examined so far showed the same type of variability of the reporter gene (implying Tie2 promoter) activity. For example, the same pattern of β-galactosidase staining was found in the muscular tissue of the diaphragm (Fig. 3), indicating that the uneven pattern of Tie2 distribution in adult mouse microvasculature is a more general feature.

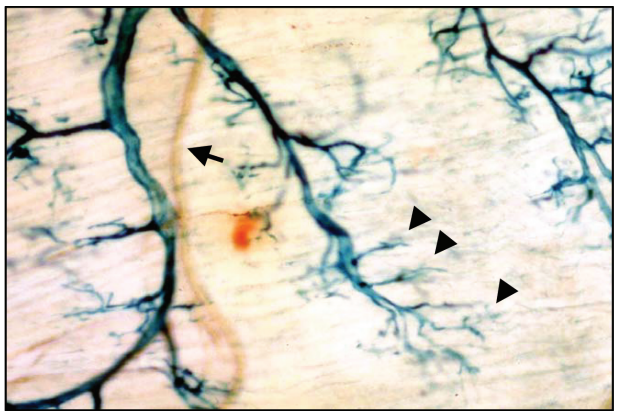

Fig. 3 β-galactosidase staining in the diaphragm of a LacZ(Tie2) mouse. Note the 'dead end' type distribution of the staining, indicating the low level of Tie2 expression in the venular loops of this microvascular bed. Original magnification: x 120.

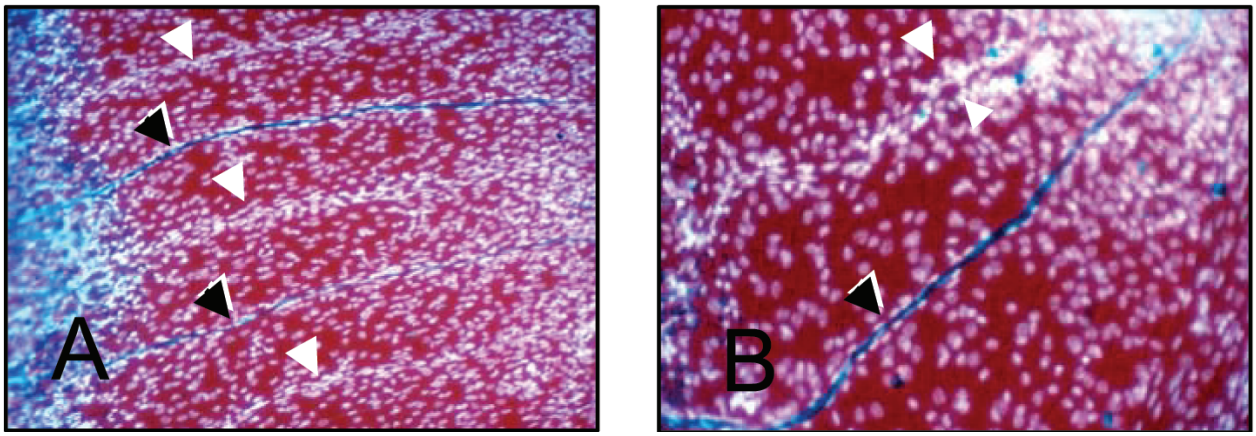

Fig. 4 Distribution of LacZ(Tie2) in a mesenteric angiogenic field induced by intraperitoneal VEGF administration. Clusters of mononuclear cells, containing isolated LacZ(Tie2) positive cells appeared in the window (right side of the image), connected *via* vascular cellular cords with the nearby mesenteric fat (left side). These cords displayed an alternate distribution of LacZ(Tie2) positive (black arrows) and negative (white arrow) staining. LacZ(Tie2) positive cords consistently displayed a well organized, microvessel like pattern, while the LacZ(Tie2) negative cords were more diffusely assembled, suggestive for a venular nature.Nuclei were counterstained with DAPI. overlay of fluorescence and dim direct illumination (transmission). Original magnification: x 120.

These findings are different from the original description of the LacZ(Tie2) mouse, where a relatively uniform distribution of the expressed LacZ in both embryonic tissues and in adult animals was reported [17]. Although it was not excluded the possibility that some minor populations of vascular beds (as a whole) would not express the reporter gene, no such examples were given. Specifically, regarding the intestine, the authors stated that the mesenteric microvasculature is LacZ positive [17]. A thorough histological study of mouse endothelium showed that large vessels always stained first and more intensely than microvessels, but this phenomenon was explained as a technical artifact [38]. The same study also indicated the limited reliability of the anti-β-galactosidase immunostaining for comparing the actual levels of the Tie2 protein of expression in situ.

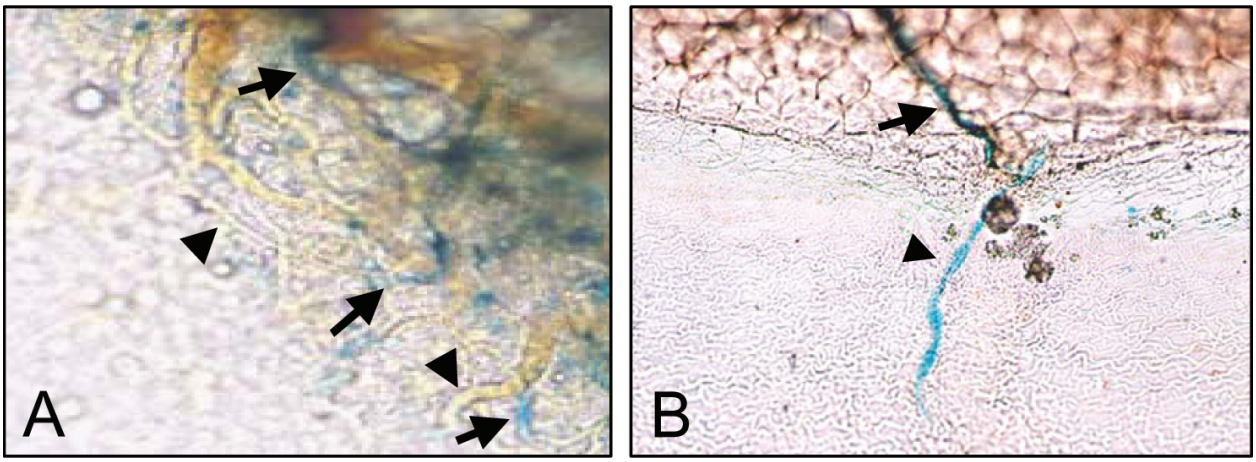

Fig. 5 Interface between the mesenteric fat and nearby angiogenic windows. A. Outgrowth of LacZ(Tie2) positive (arrows) and LacZ(Tie2) negative (arrowheads, indicating the presence of residual blood) capillaries in the avascular window of a mouse mesentery, after mild mechanical irritation. B. Half-positive LacZ(Tie2) capillary loop (arrowhead) emerging from the mesenteric fat stimulated with VEGF in the continuation of a double LacZ(Tie2) (blue) and lectin (brown) positive, perfused microvessel (arrow). Original magnifications: A, x 200; B, x 120.

Uneven distribution of Tie2 receptor between the two microvascular types may have functional reasons. When angiopoietin-1, its activating ligand, is overexpressed in mice, the proportion of venules increases dramatically [39]. Therefore, in normal animals it would be anticipated that a balanced phenotype of the two microvascular types requires a down-regulation of Tie2 expression in the venular side of the microvasculature.

Asymmetric distribution of Tie2 in the angiogenic microvasculature of mesenteric windows.

A main function of Tie2 in microvascular biology is during embryogenesis. Disruption of Tie2 function in mice resulted in embryonic lethality with defects in embryonic vasculature, suggesting a role in blood vessel maturation and maintenance [40]. Moreover, Tie2 was upregulated and activated in the endothelium of rat ovary and in healing rat skin wounds, both areas of active angiogenesis [23]. We were therefore interested to assess the distribution of Tie2 promoter activity in newly formed microvessels in a model of mesentric angiogenesis. We induced angiogenesis by repeated i.p. injection of VEGF and by aseptic mechanical irritation of the mesentery. To reveal all microvessels, as well as their perfusion status, animals were injected before sacrifice with the *L. esculentum* lectin.

The cellular architecture of VEGF-induced angiogenic fields, as related to the local epithelial cellularity in the avascular windows of mouse mesentery, was clearly revealed by nuclear staining with DAPI and fluorescence microscopy (Fig. 4). On the surface of the mesenteric windows of VEGF-treated animals, we found random colonies of mononuclear cells. These colonies were often connected to the nearby microcirculation of the mesenteric fat by two types of blood conduits, LacZ-positive and -negative, respectively (Fig. 4). Based on the general pattern of LacZ(Tie2) expression described so far, on the occasional continuity with microvessels in the adjacent fat, as well as on their diameters [more compact comparatively with the LacZ(Tie2) negative ones (Fig. 4B)], we consider that these LacZ(Tie2) positive neovessels are arterioles. No evidence of blood perfusion at this stage was apparent, although this cannot be excluded. Interestingly, the regular pairing of LacZ(Tie2) positive with LacZ(Tie2) negative cellular cords (Fig. 4A) suggests a pre-assigned arteriolar/venular circulatory role of these cellular cords.

Minimal aseptic irritations of the mesenteries of LacZ(Tie2) mice also produced at the periphery of the adjacent fat, after 7 days, evidence of an ongoing angiogenic process. New capillaries, some of them judged to be functional based on their blood content, emerged from the existent microvessels in the otherwise avascular mesenteric window. Their LacZ staining was, again, highly variable (Fig. 5A), thus confirming the previous observations on quiescent microvasculature, and further supporting the notion that the asymmetry of Tie2 extends to the level of smallest branches, namely to the capillaries.

In Fig. 5B, obtained from a VEGF-treated animal, we show that in the mesenteric window although there was no lectin staining (indicating no perfusion, as opposed to the nearby mesenteric fat), the tip (or loop) derived from a perfused Tie2 positive microvessel did advance in the window. The continuity with the vessel present in the fat tissue, likely to be an arteriole based on its LacZ(Tie2) positivity, indicates that: (1) selected capillaries can achieve strong Tie2 expression even before they become perfused, suggesting that an arteriolar-type identity could be established in the capillary endothelium before the blood starts to flow; and (2) the neovessels could originate from arterioles (the classical sprouting model would indicate an origin in the post-capillary venules). Both these observations were also present in mice expressing LacZ under the ephrinB2 (arteriolar specific) promoter [9,10].

In conclusion, this study revealed that the arteriolar and venular components of the microvasculature of mesentery and diaphragm of adult mice, both in quiescent state and as effect of angiogenic stimulation, are asymmetric with regard to the activity of the endothelial promoter Tie2, with a stronger activity on the arteriolar side. Moreover, we have shown that this asymmetry is present in a fraction of capillaries and is established even before becoming functional, *i.e.*, before blood starts flowing through these conduits. This confirms the existence of distinct arteriolar/venular phenotypes in the endothelium of capillaries.

Acknowledgements

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