ELSEVIER

Cardiovascular Research 49 (2001) 634–646

Cardiovascular Research

www.elsevier.com/locate/cardiores
www.elsevier.nl/locate/cardiores

Unorthodox angiogenesis in skeletal muscle

S. Egginton[a,*], A.-L. Zhou[a], M.D. Brown[b], O. Hudlická[a]

[a] *Angiogenesis Research Group, Department of Physiology, The Medical School, University of Birmingham, Birmingham B15 2TT, UK*
[b] *School of Sport and Exercise Sciences, University of Birmingham, Birmingham B15 2TT, UK*

Received 6 June 2000; accepted 1 November 2000

Abstract

Objective: The morphological pattern of angiogenesis occurring in mature, differentiated skeletal muscle in response to chronically increased muscle blood flow, muscle stretch or repetitious muscle contractions was examined to determine (a) whether capillary neoformation follows the generally accepted temporal paradigm, and (b) how the growth pattern is influenced by mechanical stimuli. **Methods:** Adult rats were treated for a maximum of 14 days either with the vasodilator prazosin, to elevate skeletal muscle blood flow, or underwent surgical removal of one ankle flexor, to induce compensatory overload in the remaining muscles, or had muscles chronically stimulated by implanted electrodes. Extensor digitorum longus and/or extensor hallucis proprius muscles were removed at intervals and processed for electron microscopy. A systematic examination of capillaries and their ultrastructure characterised the sequence of morphological changes indicative of angiogenesis, i.e., basement membrane disruption, endothelial cell (EC) sprouting and proliferation [immunogold labelling after bromodeoxyuridine (BrdU) incorporation]. **Results:** Capillary growth in response to increased blood flow occurred by luminal division without sprouting or basement membrane (BM) breakage. In stretched muscles, EC proliferation and abluminal sprouting gave rise to new capillaries, with BM loss only at sprout tips. These distinct mechanisms appear to be additive as in chronically stimulated muscles (increased blood flow with repetitive stretch and shortening during muscle contractions) both forms of capillary growth occurred. Endothelial cell numbers per capillary profile, mitotic EC nuclei, and BrdU labelling confirmed cell proliferation prior to overt angiogenesis. **Conclusions:** Physiological angiogenesis within adult skeletal muscle progresses by mechanisms that do not readily conform to the consensus view of capillary growth, derived mainly from observations made during development, pathological vessel growth, or from in vitro systems. The temporal and spatial pattern of growth is determined by the polarity of the mechanical stimulus, i.e., by intra-luminal (increased shear stress) or abluminal (external stretch) stimuli.

Keywords: Angiotensin; Blood flow; Capillaries; Electron microscopy; Extracellular matrix; Remodeling

1. Introduction

The process of angiogenesis is thought to involve a stereotypical cascade of events, based mainly on study of development, pathologies and on extensive literature based on in vitro systems. It is considered to be initiated by the proteolytic breakage of the basement membrane (BM) surrounding an existing capillary. The normally quiescent [1] endothelial cells (ECs), somehow released from their contact inhibition, migrate through the BM gap into the interstitium where their subsequent mitosis supports the elongation of abluminal sprouts. These develop central lumens and, eventually, contact with existing vessels to create functional anastomoses or capillary loops [2,3]. The use of in vitro systems has enabled identification of specific stimuli, such as soluble growth factors and cytokines, in relation to particular aspects of EC behaviour throughout the angiogenic cascade. However, EC proliferation and/or migration observed in tissue culture are not necessarily synonymous with angiogenesis, and the ability

*Corresponding author. Tel.: +44-121-414-6902; fax: +44-121-414-6919.
E-mail address: s.egginton@bham.ac.uk (S. Egginton).

Time for primary review 30 days.

0008-6363/01/$ – see front matter
PII: S0008-6363(00)00282-0

to isolate putative mechanisms may be achieved at the expense of the contextual complexity that exists in vivo. It is assumed, but has not been established, that this cascade holds true for physiological angiogenesis. In addition, there is evidence that different patterns of angiogenesis can occur in vivo when the mechanical environment, both inside and outside vessels, is changing. During development, angiogenesis by intussusceptive growth has been described, where extracellular material penetrates and divides vessels [4,5]. This specific pattern may be driven as much by growth and remodelling of surrounding tissue as by endothelial cell-specific stimuli. Furthermore, ECs are sensitive not only to mechanical strain imposed by the surrounding tissue but also to intravascular mechanical stimuli, such as shear stress as a result of flowing blood [6] and there are many observations of the importance of high flow rates for capillary growth in vivo [7–10].

The increase in capillarity that occurs in skeletal muscle in response to endurance exercise training [11,12] is a prime example of physiological angiogenesis in a mature, differentiated tissue. Under these conditions, skeletal muscles have increased blood flow during contractions and capillaries are also exposed to repeated shortening and elongation due to changes in sarcomere length. We have studied the process of angiogenesis in this tissue using rat models that incorporate specific mechanical component stimuli that are identifiable during muscular training. These were vasodilation, induced by administration of the α_1 receptor antagonist prazosin [13,14], stretch induced by overload of an ankle flexor due to extirpation of agonist muscle [15] and indirect electrical stimulation that increased both muscle contractile activity and blood flow [16]. We have shown that specific mechanical stimuli acting primarily at the EC luminal (vasodilation/shear stress) or abluminal surface (muscle overload/stretch), or a combination of both (electrical stimulation) would modify this pattern. When the stimulus acted via the capillary lumen, growth occurred by division of capillaries by cytoplasmic processes [17], whereas mechanical factors acting predominantly from the abluminal side initiated growth by sprouting and EC migration [18]. These distinct mechanisms appear to be additive as angiogenesis in chronically stimulated muscles (increased blood flow with repetitive stretch and shortening during muscle contractions) occurred by both sprouting and splitting [19,20]. We hypothesised that the sequence of capillary growth described for developmental and pathological situations would not apply to physiological angiogenesis in adult skeletal muscle. We therefore extended our studies by examining morphology of the capillary bed using quantitative electron microscopy, to provide a temporal analysis of features of the angiogenic cascade, i.e., basement membrane breakage, EC proliferation, that may vary in response to mechanical stimuli. Our findings challenge the assumption that angiogenesis can be described by a common paradigm.

2. Methods

2.1. Animals

Experiments were performed on male adult rats, mass 250–300 g at the time of the final experiments, in accordance with the guidance in the United Kingdom Animals (Scientific procedures) Act of 1986. Where appropriate, animals were treated postoperatively with analgesics and antibiotics (0.1 ml Temgesic and Engemycin, s.c.).

2.2. Models of angiogenesis

2.2.1. Prazosin administration

Sprague–Dawley rats ($n=6$) were given prazosin (gift from Pfizer Ltd., Sandwich, UK) ad libitum in their drinking water (50 mg l^{-1} in distilled water) for 7 or 14 days. Consumption based on volume was approximately 2 mg day^{-1}, a dose shown previously to increase skeletal muscle blood flow threefold on acute administration [13].

2.2.2. Stretch-induced overload

Wistar rats ($n=5$) underwent unilateral surgical removal of the tibialis anterior (TA) under aseptic conditions and inhalation anaesthesia (2% halothane in oxygen). Rats were taken into the final experiment 7 or 14 days after operation, when extensor digitorum longus (EDL) muscles showed hypertrophy of 20% [15].

2.2.3. Chronic muscle stimulation

Under inhalation anaesthesia (above), stainless steel multi-stranded teflon-insulated wire electrodes were implanted into Sprague-Dawley rats ($n=6$) in the vicinity of the common peroneal nerve, to indirectly stimulate ankle flexors, connected to a programmable stimulator. Commencing 1 day after surgery, muscles were stimulated at a frequency of 10 Hz, 0.3 ms pulse width, and with supramaximal voltage as in previous experiments [21]. Animals were taken into final experiments after 2 or 7 days stimulation.

2.2.4. Controls

Control age- and weight-matched rats were taken either after drinking distilled water for durations matching prazosin administration, or with no surgical intervention as controls for overload and stimulation.

2.3. Administration of bromodeoxyuridine (BrdU)

In four rats with stretch-induced overload for 7 days and four rats stimulated for 2 or 7 days, bromodeoxyuridine (BrdU) was administered to label cells in the S-phase of mitosis. Animals received two intraperitoneal (i.p.) injections of BrdU (Boehringer Mannheim) in saline, 40 mg kg^{-1} body mass (concentration 10 mg ml^{-1}), given 14 h

apart. Two hours after the second injection, rats were anaesthetised with sodium pentobarbitone (50 mg kg^{-1} body mass i.p.) and received a 2-h infusion via a jugular venous cannula of BrdU at the same concentration, delivered at 0.02 ml min^{-1}, followed immediately by sacrifice (pentobarbitone overdose) and muscle removal and fixation (see below). Capillary EC proliferation was evaluated on the basis of quantification of BrdU incorporation and immunostaining by light microscopy in relation to capillaries localised in serial sections.

2.4. Preparation of muscles for histology and electron microscopy (TEM)

At sacrifice, the middle portion of the EDL muscle was fixed by immersion, while the extensor hallucis proprius (EHP) muscle (mass ca. 20 mg) was fixed by superfusion in situ with 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2 at 20°C; osmolarity ca. 350 mOsm) for ca. 5 min, then carefully sliced into strips of 2 mm minimum dimension, pinned onto dental wax at resting length, covered with fixative for 5 min until hardened, and subsequently immersed in fixative for 30 min at 4°C. Samples were then trimmed into four blocks with cut faces of 1–2 mm^2, transferred into fresh fixative for a further 24 h at 4°C, post-fixed in 1% osmium tetroxide for 1 h, and vacuum embedded in epoxy resin.

One block per muscle from each animal was randomly selected and 1 μm semithin sections were cut and stained with toluidine blue, to orientate the muscle fibres and capillaries for transverse section (TS), and then rotated by 90° to give longitudinal sections (LS), for estimation of a branching index (BI) using the ratio of capillary counts in TS and LS, at a magnification of 400. Silver (60–70 nm) ultrathin sections were double stained with methanoic uranyl acetate (30%) and aqueous lead citrate (2%), viewed at a magnification between 5800 and 29,000, as appropriate for the structure concerned. Some sections were stained with 0.1% tannic acid (Electron Microscopy Science, UK) for 7 min at room temperature prior to routine staining, to enhance contrast of the extracellular matrix and BM around blood vessels.

2.5. Tissue preparation for immuno-electron microscopy (IEM)

In order to preserve antigenicity, small (<1 mm^3) blocks of muscle were briefly fixed [22] by immersion in 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C (10–60 min on ice). Dehydration was performed in an ascending series of alcohols using a progressive lowering of temperature method, in 30 min steps as follows: 30% ethanol (0°C; on ice), 55% ethanol (−15°C; cryostat chamber), 70% ethanol (−30°C), 100% ethanol (−50°C×2; Reichert CS Auto) followed by infiltration with Lowicryl HM20 at −50°C by progressive replacement of ethanol over 18 h. Blocks were embedded in fresh resin and polymerised with UV light at −50°C for 48 h, then +20°C for 24 h. Ultrathin (60 nm) sections were collected on nickel grids for post-embedment labelling. Non-specific binding was blocked by incubation with 1% BSA and goat serum in phosphate-buffered saline (PBS; 45 min at room temperature, pH 8.2–8.5). Primary antibody (mouse monoclonal anti-BrdU; Amersham) was applied overnight at 4°C, followed by washing in blocking buffer and immersion in secondary antibody (goat anti-mouse IgG) conjugated with colloidal gold (5 nm diameter particles) for 90 min at room temperature. Silver enhancement (Silver Enhance, Biocell) increased visibility by an order of magnitude using ultrapure water, followed by counter-staining with 1% uranyl acetate.

2.6. Quantitative analysis

From tissue prepared for TEM, semithin sections were used to estimate capillary tortuosity, by calculating the ratio of counts in longitudinal and transverse sections [23]. This BI enables a quantitative description of the degree to which the capillary path follows that of the fibre axis; low values means the orientations are closely parallel, while high values indicate capillary branches that deviate from the long axis of the muscle. Subsequently, ultrathin sections were taken and at least two grids from each block were examined, involving more than 150 microvessels per muscle. A systematic examination of all microvessels present in each muscle cross-section was used to score the frequency of different types of capillary morphology for evidence of angiogenic activity. Signs of endothelial cell activation were also noted. In addition, from stretched muscles, forty capillaries in each cross-section were selected in a systematic–random manner and photographed at an EM magnification of 1700–2500 for further quantitative analysis. For IEM samples, ultrathin transverse sections were sampled in a systematic manner to examine all microvessels in each section, examining only 30–100 capillaries per animal as a result of the small block face required for optimal fixation.

2.7. Statistical treatment

Treatment effects were determined by factorial ANOVA, with intergroup comparisons assessed by post-hoc tests (Fisher's PLSD), and significance was set at $\alpha = 0.05$. Data are presented as means±S.E.M.

3. Results

3.1. Capillary supply and pattern

Based on histochemical detection of capillary endothelial alkaline phosphatase in frozen sections, values for

capillary per fibre ratio (C:F) after 14 days treatment with prazosin [17], 14 days stretch-induced overload [18] or 7 days of stimulation [24] were similar (prazosin 1.93±0.07, overload 2.07±0.13, stimulation 2.09±0.08 vs. control values of 1.52±0.07, 1.38±0.06 and 1.44±0.06, respectively). The pattern of capillary growth, however, was quite different. The BI indicates that, after prazosin, new vessels were orientated preferentially along the long axis of muscles fibres, whereas in both overload and stimulated muscles, the capillary bed was extended, at least in part, by lateral sprouts or capillary anastomoses (Fig. 1). These data confirm previous qualitative descriptions of the three-dimensional structure of the capillary bed in these models based on confocal microscopy (prazosin [17]; overload [25]; stimulation [19]). Since these distinct patterns represent the accomplished growth outcome, we focused attention on ultrastructural changes occurring prior to this, when sequential signs of angiogenesis that may account for the different patterns could be followed.

3.2. Changes in the basement membrane

Breakage of the BM was completely absent at any time in prazosin-treated muscles (Fig. 2a) and capillaries were indistinguishable from control vessels, which have complete integrity of this structural support, showing quite clearly that it is not a prerequisite for capillary growth. In stretched muscles after 7 and 14 days, the frequency of BM breakages was increased to 6.0±1.0 and 9.5±2.3%, respectively (Fig. 2a), but this was always associated with sprouts and not the parent vessel. A similar appearance was noted in muscles stimulated for 3 and 7 days, although the increase was less, to 1.3±0.7 and 2.5±1.4%, respectively ($P<0.05$ vs. control). BM disruption was seen where processes from individual endothelial cells were protruding

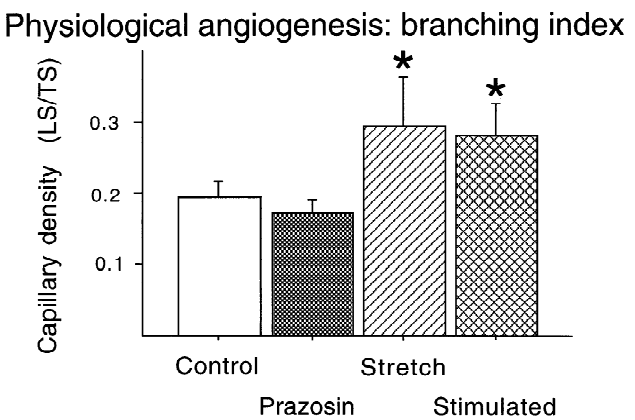

Fig. 1. Effect of different forms of physiological angiogenesis on the branching index, used as an indication of the degree to which the expanded capillary bed maintains a preferential orientation along the muscle's long axis. Significant deviations from the anisotropy displayed in control muscle (open bars) is evident following both 14 days overload (hatched) and 7 days stimulation (cross-hatched), but not 14 days prazosin treatment (stippled). *$P<0.05$ vs. control.

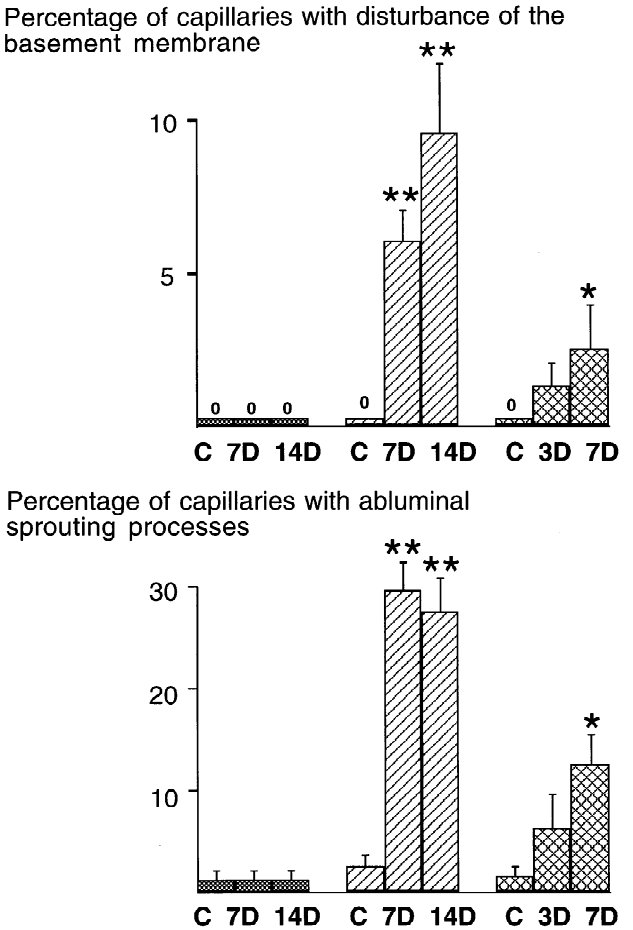

Fig. 2. (a) The proportion of capillaries with a disrupted basement membrane. Note that in control and prazosin treated muscles, this is never observed, whereas it is significantly increased in both overloaded and stimulated muscles. (b) The proportion of capillaries with abluminal protrusions, showing a pattern that is qualitatively similar to that of BM disruption. Abbreviations: 3D, 3 days; 7D, 7 days; 14D, fourteen days. *$P<0.05$, **$P<0.01$ vs. control.

abluminally, i.e., sprouting, into the interstitium, with the BM being absent only at the sprout tips (Fig. 3).

3.3. Abluminal sprouts

Endothelial cells were defined as sprouting if they showed cytoplasmic processes projecting into the interstitium. The appearance of these varied from small protrusions of individual endothelial cells, to solid sprouts with no lumen, to small capillaries with visible lumen. Abluminal sprouts were absent after prazosin treatment, but were seen in almost one third of all capillaries in muscle subjected to stretch for 7 days, and occurred in around 12% of capillaries after 7 days of stimulation (Fig. 2b). These higher frequencies suggest that while all breakages in the BM were associated with abluminal processes, any disruptions may be quickly repaired since an intact BM was seen around many EC sprouts. Fig. 3 shows an example of a relatively mature capillary sprout with a

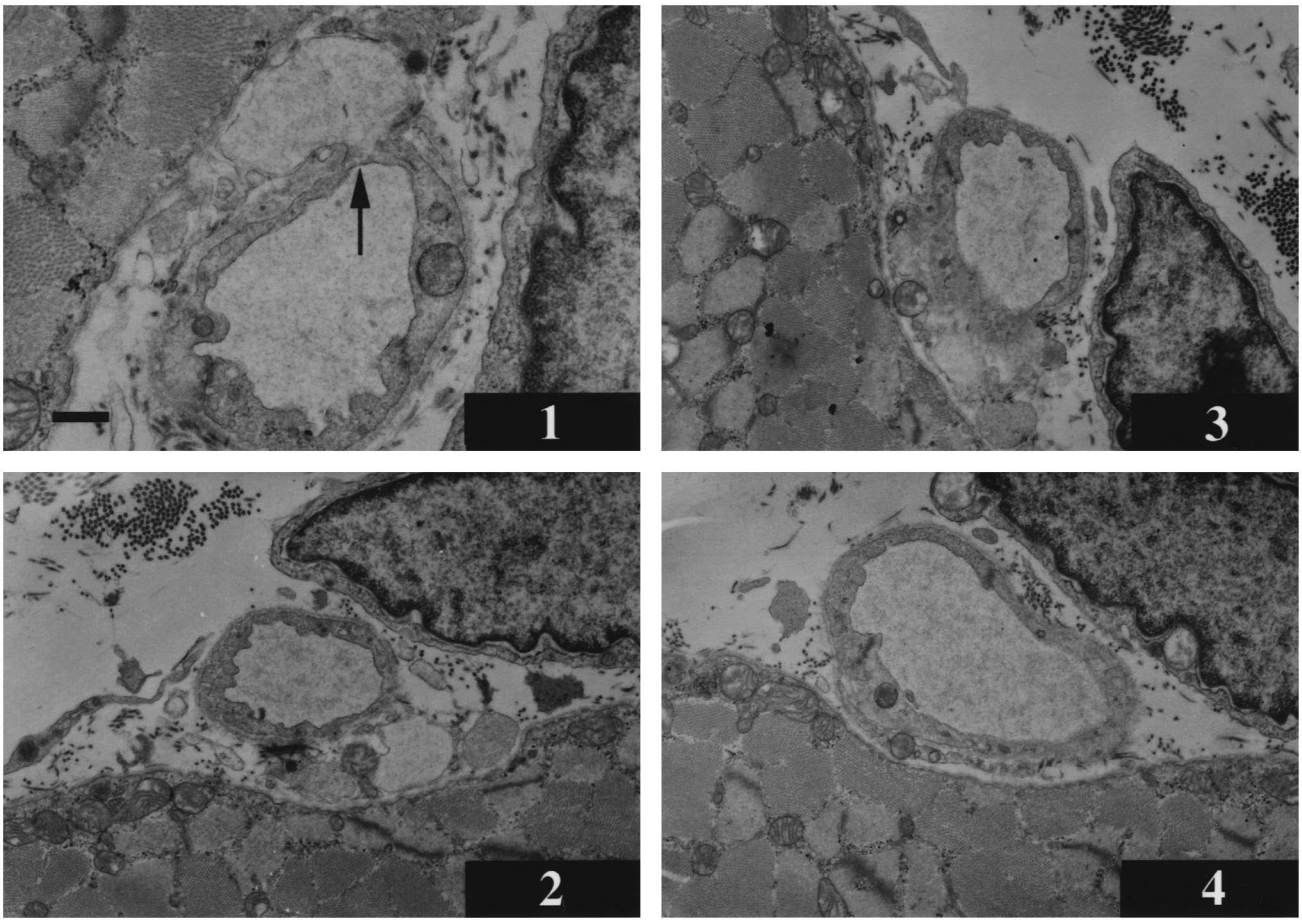

Fig. 3. Three day stimulated rat EHP (tannic acid stain, periodic sections). (1) A sprout tip with a gap in the endothelium (arrow) showing possible extrusion of plasma protein; (2–4) distal sections covering ca. 1.6 μm, showing a doubling of sprout cross-sectional area. Scale bar length=0.45 μm in (1) and 0.9 μm in (2–4).

lumen in 3-day-stimulated muscle, with serial sections showing the gradation in size approaching the sprout tip. Such sprouts were typically composed of one or two ECs with the lumen formed either by intracellular canalisation or between apposed cells (Fig. 4). Even at this early stage of development, signs of both patterns of capillary growth can be observed (Fig. 4).

3.4. Luminal processes

Fine endothelial cell processes were frequently observed protruding into the capillary lumen, and forming septa that eventually fused with opposing cytoplasm to divide the lumen into two, a luminal division that gives rise to the low branching index described above. The frequency of these luminal processes increased from 16.7±3.9% in control muscles to a peak of 47.5±6.2% after 7 days of treatment with prazosin ($P<0.01$) and was still high (34.2±5.5%, $P<0.05$) after 14 days. In contrast, stretch overloaded muscles showed no significant increase over control values in the proportion of capillaries with luminal processes. The incidence of capillaries with luminal processes was significantly increased after 3 and 7 days of stimulation, but to a lesser degree than with prazosin (Fig. 5), and again was not associated with disturbance of the BM. Fig. 6 shows clear evidence from serial sections of a 3-day-stimulated muscle for a double lumen forming within one capillary by such a process.

3.5. Endothelial cell proliferation

Signs of EC activation, such as cell thickening and increased content of endoplasmic reticulum, Golgi apparatus, ribosomes and mitochondria, were rare with prazosin treatment, and there were no significant changes in the numbers of ECs per capillary cross-section. In stretched muscles, EC activation was widespread and the mean number of ECs increased from 1.78±0.03 to 2.14±0.07 ($P<0.01$) after 14 days. Likewise, in stimulated muscles, angiogenesis was associated with activated ECs and with an increase in the average number of ECs per capillary cross section after 7 days, from 1.57±0.07 in control muscles to 1.84±0.08 ($P<0.05$). The slight difference between control values may be due to these being

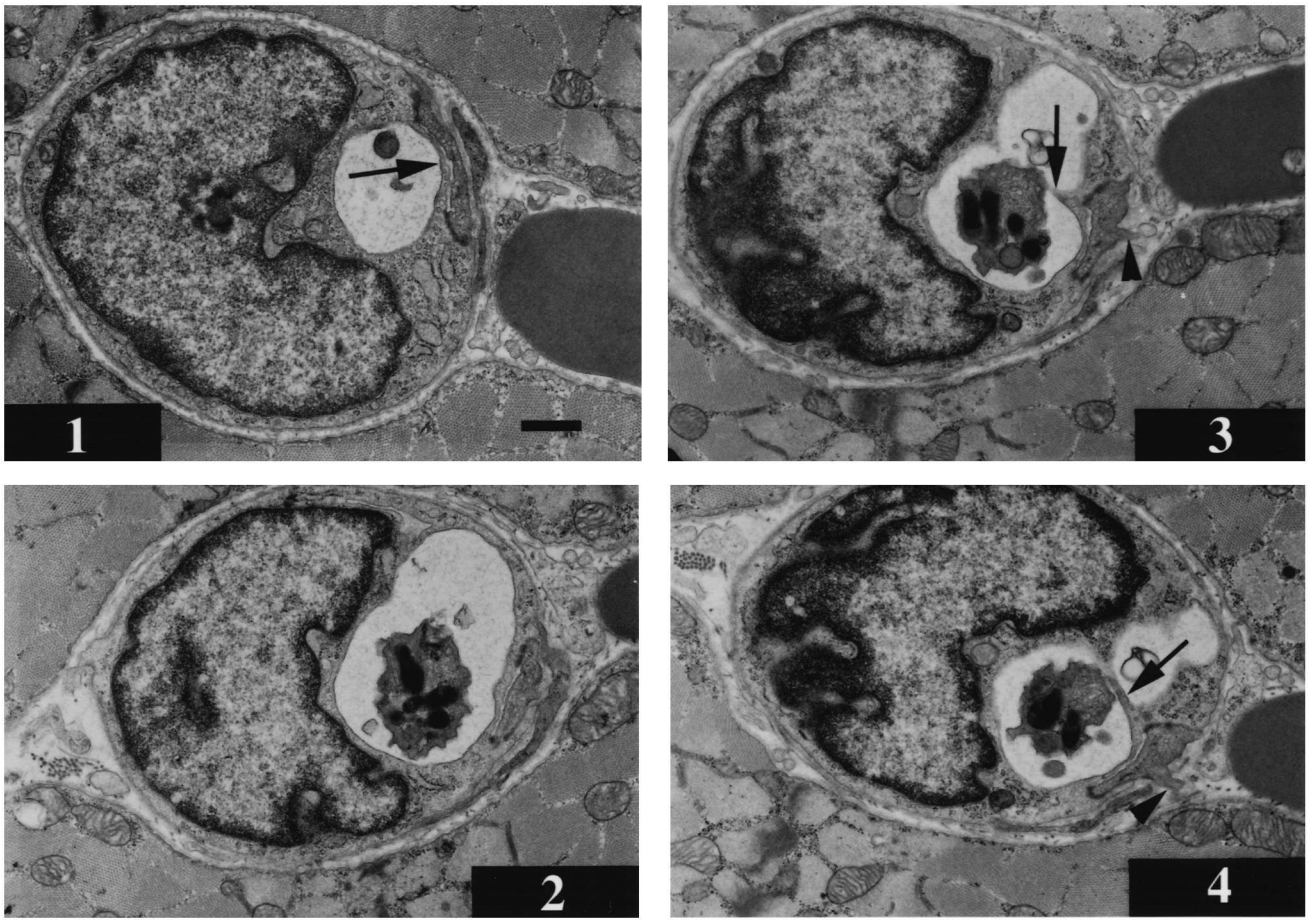

Fig. 4. Three day stimulated rat EHP (tannic acid stain, periodic sections). (1) A capillary with a regional double layer of endothelium (arrow) and a small lumen; (2) a section ca. 0.6 μm distal to that above showing that the lumen area has increased by >50%; (3–4) sections 0.2 and 0.3 μm distal to that above, respectively, showing the development of a septum that divides the lumen into two (arrow), as well as an abluminal endothelial cell projection (arrowheads). Scale bar=0.9 μm.

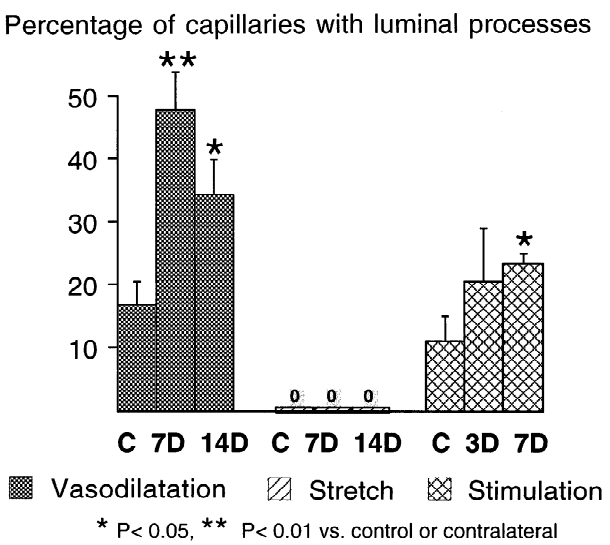

Fig. 5. The proportion of capillaries with luminal protrusions, showing a reciprocal pattern to that seen for abluminal processes (see Fig. 2b).

from different strains of rats. Changes in population means was a result of a distinct rightward shift in the distribution, away from capillary profiles consisting of only one or two ECs and an order of magnitude increase in those consisting of four or more ECs (Table 1).

In muscles treated with prazosin for 7 days, there was no evidence of any significant BrdU labelling of capillaries (Fig. 7 and [28]). In stretched muscles, BrdU labelling was increased nearly threefold after 7 days (Fig. 7), co-incident with the highest frequency of sprouts (Fig. 2b), and returned to control values after 14 days, even though sprouting remained high. BrdU incorporation was not confined to capillary locations because other cell nuclei lying within the interstitium also showed increased labelling in stimulated and stretched muscles (Fig. 7). We have previously evaluated capillary EC proliferation on the basis of BrdU immunostaining, which showed that, in stimulated muscles, capillary-linked proliferation was increased as much as fourfold after 2 days, maintained at this level after 7 days [26], and was still increased after 14 days [27]. Thus, very early proliferation occurred even before the

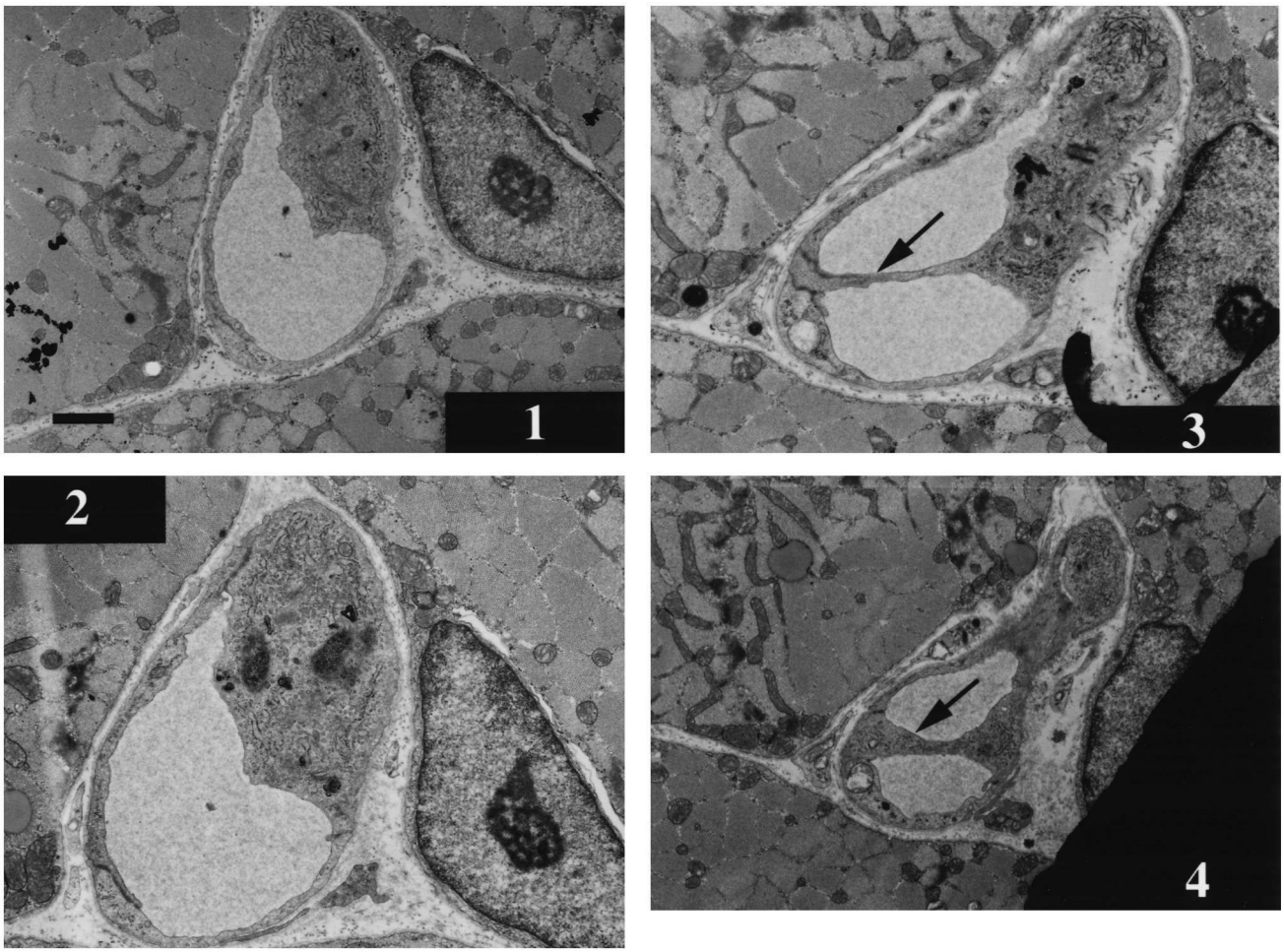

Fig. 6. Three day stimulated rat EHP (tannic acid stain, periodic sections). (1) A capillary with thickened endothelium and dense organelles; (2) a section ca. 0.2 μm distal to that above, showing endothelial cell mitosis; (3) a section ca. 0.7 μm distal to that above, showing a thin septum (arrow) that divides the lumen; (4) the septum becomes thicker only 0.3 μm distally, indicative of a point close to separation of daughter vessels. Scale bar=0.9 μm.

Table 1
Proportion (%) of capillary profiles with different numbers of endothelial cells in cross sections following overt angiogenesis

	Number of endothelial cells					
	1	2	3	4	5	6
Prazosin						
Control	45.0±6.1	51.3±5.1	3.8±1.3	0.1±0.1	0	0
7 days	43.8±2.3	49.2±4.4	6.3±2.6	0.8±0.5	0	0
14 days	40.0±3.4	50.0±4.6	7.5±3.5	2.5±0.9*	0	0
Overload						
Control	33.8±2.9	55.6±3.1	9.4±3.1	1.3±0.7	0	0
7 days	23.5±2.8*	50.0±4.2	17.5±3.3	8.0±1.8*	0.5±0.5	0.5±0.5
14 days	4.5±1.7**	18.0±5.0**	59.0±4.8**	13.0±3.5**	4.5±2.4	0
Stimulated						
Control	46.2±5.5	51.3±5.0	4.2±1.3	0.1±0.1	0	0
7 days	23.0*±7.6*	53.2±4.9	14.4±1.0**	1.8±0.7	1.2±0.7	0

* $P<0.05$.
** $P<0.01$ vs. control.

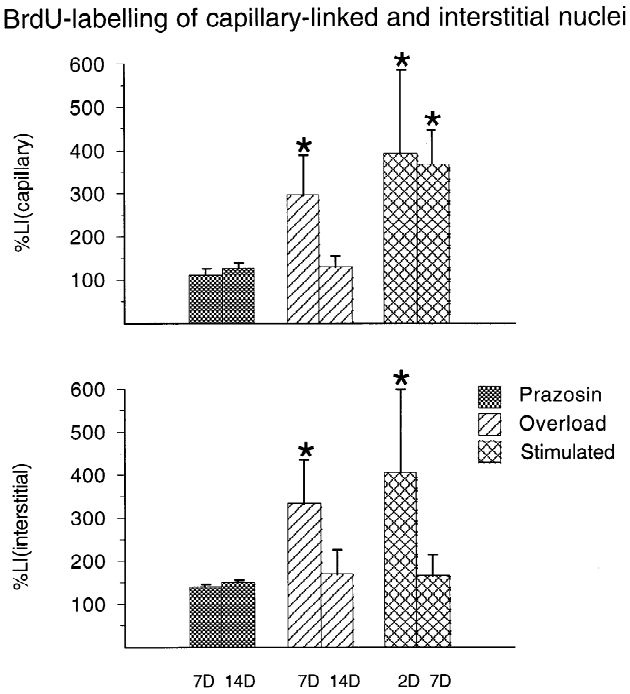

Fig. 7. Labelling indices from light microscopy, which are significantly increased following both overload (hatched) and stimulation (cross-hatched), but not prazosin treatment (stippled). Abbreviations: 2D, 2 days; 7D, 7 days; 14D, fourteen days. *$P<0.05$ vs. control.

highest incidence of sprouting, which was seen after 7 days (Fig. 2b).

Since light microscopy cannot distinguish between labelling of endothelial cells or peri-capillary cells, e.g., pericytes and fibroblasts, we used the resolution of electron microscopy to determine the specificity of BrdU labelling. Data from IEM showed that EC labelling is increased in muscles after both 7 days overload (Fig. 8a), and 2 and 7 days stimulation (Table 2). Endothelial mitosis occurred in capillaries that showed no obvious signs of either luminal division or abluminal sprouting, in contrast to previous reports of mitosis occurring only during EC migration [3].

3.6. Peri-capillary cells

In overloaded muscles, the proportion of capillaries with adjacent fibroblasts increased from 84.9±2.8% in controls to 89.9±2.8% at 14 days (n.s.), with a similar trend for the extent of EC–fibroblast attachment, from 16.1±3.7 to 25.0±6.1%, respectively. Despite these changes, only EC mitosis was significantly increased after 7 days overload, with no concomitant increase in fibroblast labelling (Table 2). In 2 day stimulated muscle, there was increased labelling of adjacent fibroblasts in close contact with capillaries (Fig. 8b), which declined after 7 days. After 2 days of stimulation, ECs and fibroblasts were equally labelled, whereas after 7 days, mainly ECs were labelled (Table 2). Proliferation of other cell types associated with capillaries, such as pericytes, was not seen and they could thus be excluded as sites of proliferation contributing to labelling.

4. Discussion

A widely held paradigm is that angiogenesis begins by disturbance of the endothelial basement membrane, followed by migration of the endothelial cells outside of the existing capillary structure, with mitotic activity beginning at the base of the sprouts that support their elongation [2]. Growth factors can provide the initial signal for angiogenesis, and we cannot exclude their involvement in our models. Although there was no change in the expression of fibroblast growth factor 2 (FGF-2) in either overloaded [15] or stimulated [29] muscles, higher levels of a low-molecular-weight angiogenic factor (ESAF) were present in prazosin-treated [28], overloaded [15] and stimulated [24] muscles. In addition, there is evidence for a higher proportion of capillaries expressing VEGF in muscles of both prazosin-treated and stimulated muscles [30]. However, we hypothesise that alterations in the local mechanical environment in the three models are important in initiating distinct growth patterns by acting as differential stimuli initiating angiogenesis, either directly via mechanotransduction by ECs or indirectly by activation of metalloproteinases [27], upregulation of VEGF receptors [35] or VEGF expression [30], or a secondary release of growth factors leading to tissue remodelling.

A similar degree of capillary growth can be achieved in adult skeletal muscles under three different conditions involving growth with or without breakage of the BM, and with or without endothelial cell proliferation, depending on the type of differential stimulus applied. Thus far, a distinction has been made between non-sprouting angiogenesis by capillary splitting in response to prazosin treatment, and sprouting but not splitting growth in stretched muscles. Stimulated muscles display examples of both sprouting and splitting types of angiogenesis, neither being as prevalent as in the other models with only a single type of growth. Combining our previous data [17,19] with those of the current study produces a summary of the response to perturbation of the local mechanical environment as follows. Long-term administration of prazosin results in an elevated C:F after 7 and 14 days without any evidence of basement membrane breakage, which is the supposed first stage in the angiogenic cascade. Although ECs showed signs of activation (increased proportion of capillaries with large cytoplasmic vacuoles, thickened endothelium, and irregular luminal surface), there was no increase in labelling by BrdU either in endothelial or other interstitial cells. The increased presence of intra-luminal processes that divided capillaries into two lumens sharing a common EC implies the likely mode of capillary growth to be by luminal division, giving rise to the bifurcating vessels viewed by confocal microscopy of lectin-stained

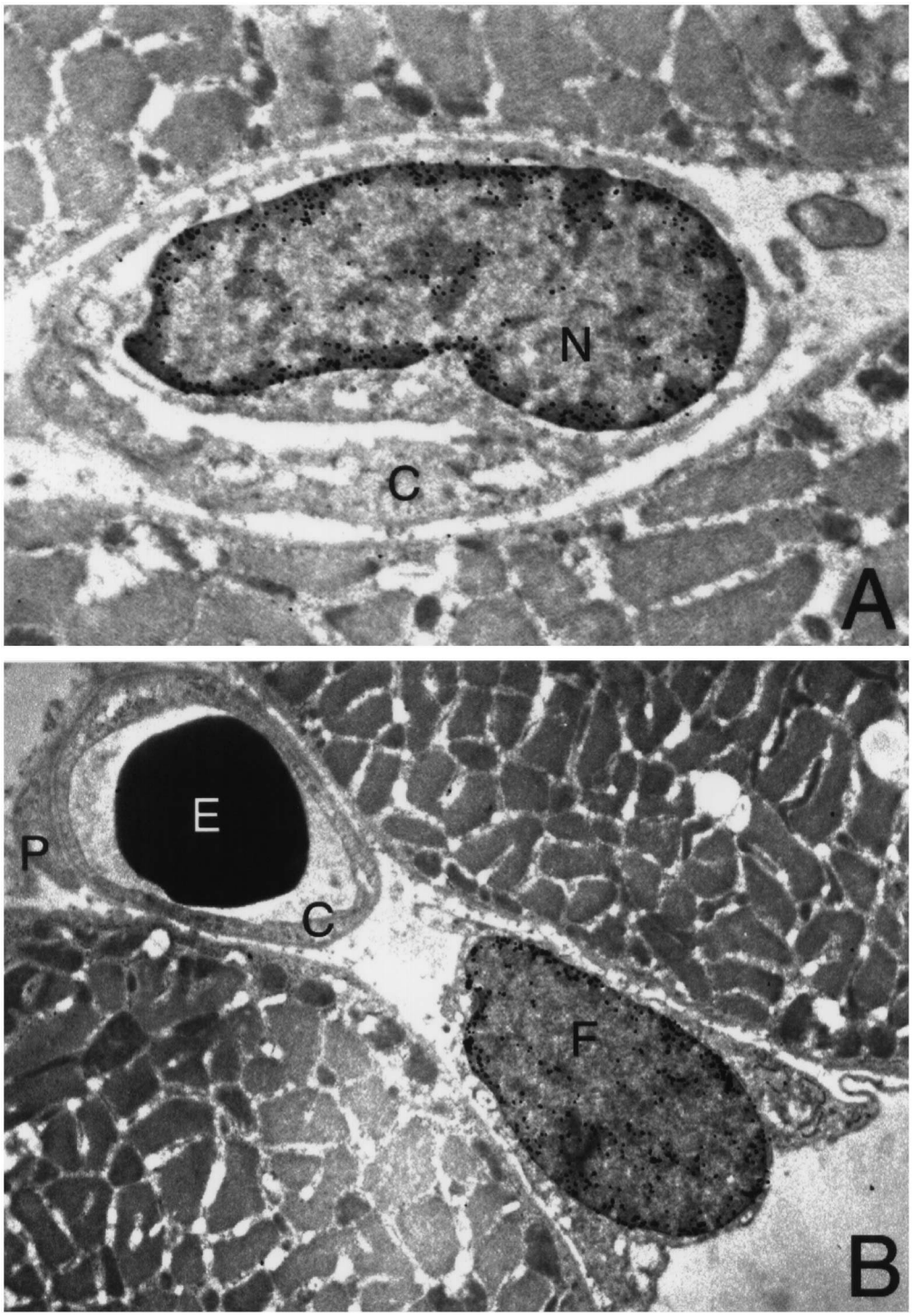

Fig. 8. Immuno-electron microscopy of BrdU labelling (black dots) of (A) endothelial cell nucleus following 7 days overload and (B) fibroblast following 2 days stimulation of rat EHP (negatively stained sections, magnification ×12,500). C, capillary; E, endothelial cell; F, fibroblast; N, nucleus; P, pericyte.

whole-mounts [17], and a low BI. In muscles overloaded by stretch, a progressive increase in C:F was coincident with evidence of basement membrane breakage, the appearance of abluminal sprouts and increased endothelial cell mitosis. Consistent with these findings, there was an increased number of lateral branches per unit capillary length (high BI). Chronic electrical stimulation increased C:F more quickly than did either of the other treatments, involving both luminal division and abluminal sprouting, with occasional disruption of the BM around capillaries with activated ECs. Endothelial cell proliferation was observed preceding the increase in sprout appearance and

Table 2
Proportion (%) of labelled nuclei observed in endothelial cells and fibroblasts

	Control	Overload 7 days	Stimulated 2 days	Stimulated 7 days
Number of capillaries	200	156	72	196
Proportion of capillaries with EC nucleus	31.1±1.5	30.0±1.4	36.1±0.8	39.8±0.8**
Proportion of labelled EC nuclei	0	14.9±5.7**	7.1±3.6**	9.0±3.8**
Capillaries with adjacent fibroblast nuclei	4.5±1.5	6.7±2.6	7.3±1.0	6.1±1.9
Proportion of labelled fibroblast nuclei	8.3±4.1	5.0±2.5	83.3±16.7**	18.8±6.3**
Capillaries with adjacent pericyte nuclei	4.0±0.9	8.1±1.7	4.1±1.2	4.6±1.6
Proportion of labelled pericyte nuclei	0	0	0	0
Other cell types	0.5±0.5	1.0±1.0	5.3±1.2	4.6±1.6

** $P<0.01$vs.control.

capillary supply. Confocal microscopy revealed an intricately branching capillary network, with numerous loops and lateral branches [19], confirmed by an increased BI.

It was previously shown that prazosin administration increased skeletal muscles' blood flow [13] and that prolonged vasodilation led to an increased capillary shear stress [14]. The resultant mode of capillary growth is in contrast to the accepted angiogenic cascade and represents an unorthodox form of angiogenesis initiated by elevated shear stress. This type of growth involves a direct response of ECs per se, leaving the abluminal surface of capillaries essentially intact. It is distinct from the intussusceptive growth described during development in which an invagination of the capillary wall is followed by infiltration of the resulting endothelial pillar by interstitial cells [4,5], but has similarities with the longitudinal splitting demonstrated in developing hearts [31]. This form of angiogenesis should result in an extended capillary bed with an orientation broadly similar to that observed in control muscles, and it is clear from the BI that this is indeed the case. In muscle overload, which increased sarcomere length, it was postulated that a significant signal for capillary growth could be stretch of the surrounding tissue [15]. Indeed, changes in blood flow were relatively small and only increased after the appearance of new capillaries [15]. This predominantly abluminal signal resulted in endothelial sprouting with breakage of basement membrane only at the tip of the sprouts [17]. This again shows that BM breakage is not necessarily the first stage of angiogenesis, that EC mitosis occurs in otherwise intact capillaries, that abluminal sprouts were formed by both inter- and intra-cellular canalisation, and suggests a significant deviation from the 'normal' pattern of capillary growth by sprouting angiogenesis.

It is evident that ECs exhibit a polarity of response, being responsive to mechanical stimulation from both luminal and abluminal directions. An important question is what happens when these distinct mechanical stimuli are combined, and whether either angiotype becomes dominant. Intensive muscle activity induced by chronic electrical stimulation increased both blood flow and shear stress [6] and repeatedly distorted the abluminal side of capillaries due to intermittent contraction and relaxation of the muscle fibres. This resulted in reorganisation of the whole capillary bed, displaying both luminal and abluminal growth patterns [17–19]. Our findings that the focal discontinuity of the BM was limited to the tip of abluminal sprouts is consistent with this stage being mediated by in situ activation of matrix metalloproteinases. Indeed, there is elevated expression of the metalloproteinases MMP-2 and MT1-MMP after 3 days of stimulation, and inhibition of their activity in vivo prevented angiogenesis [27]. Stimulation of skeletal muscle is the most potent initiator of physiological angiogenesis yet described, showing EC proliferation after 2 days and overt capillary growth by 4 days. The greater number of lateral anastomoses predicted from abluminal sprouting leads to an increase in BI, and expansion of the capillary bed consistent with measurements of functional hyperaemia and muscle endurance, suggesting that the increased exchange capacity is utilised in an integrated and highly controlled manner. Our observations suggest that differential mechanical stimuli may act through different signalling cascades at the same time to initiate two distinct forms of angiogenesis in close proximity.

In an analogous manner to BM breakage prior to abluminal sprouting, luminal division may be preceded by disruption of the overlying glycocalyx [32]. Indeed, this may be a clue to the origin of the observed differential growth. Shear stress induces the upregulation of EC vitronectin receptors in vitro, possibly including the angiogenic marker $\alpha_v\beta_3$ [33], while β_3 integrin distribution was not affected by cyclical strain [34]. However, transendothelial signalling is reported, with shear transducing signals through activation of focal adhesion molecules [35], suggesting that a polarity of signal need not necessarily be translated into a polarity of response. For example, the BM and glycocalyx present physical barriers that may inhibit or impair the formation of abluminal sprouts and luminal processes, respectively. If abluminal protease activity is not induced by increased shear stress, disruption of the luminal glycocalyx may offer a pathway of least resistance for EC outgrowth initiated by an angiogenic stimulus. Similarly, mechanical stretch appears to have little effect on the glycocalyx (unpublished data), but invokes focal disruptions in the overlying BM.

A final difference between physiological angiogenesis and the accepted paradigm is the timing of EC proliferation. The angiogenic cascade proposes that endothelial cells forming sprouts that migrate outside the existing vessels will undergo proliferation to extend the sprouts. Evidence of endothelial proliferation was found in both overloaded and stimulated muscles but was absent from the control and prazosin-treated animals. It is assumed that migration precedes mitosis, although we found evidence of EC mitosis in capillaries with no obvious signs of either luminal division or abluminal sprouting. This is consistent with an increase in labelling index following BrdU incorporation into capillary-associated nuclei in stimulated muscle, but not following prazosin treatment [28]. This may be a consequence of EC elongation coupled with a substantially reduced rate of mitosis. Proliferation of ECs prior to sprouting angiogenesis in stimulated muscle has been indicated by BrdU incorporation, although labelling of perivascular cells suggests a role for these in modulating the angiogenic response. However, the coverage of capillaries by pericytes was diminished in stimulated muscles [36], and we failed to find any evidence for an overt inflammatory response [21]. The higher resolution offered by electron microscopy (EM) and immunogold staining with BrdU labelling (IEM) showed not only mitotic activity and labelling of ECs but also proliferation of fibroblasts, some of which were in close contact with capillaries both in overload and stimulated muscles, but little evidence for proliferation of other types of cells associated with capillaries, e.g., pericytes or macrophages.

Other studies have also shown differences from the presumed angiogenic cascade. In the cornea, capillary sprouting has been described in the absence of EC proliferation [37], and proliferation of ECs prior to their interstitial migration has also been noted [3]. Some studies have suggested that multiple mechanisms of vascular angiogenesis may occur in the same tissue [38,39], although, apart from the present study, there is relatively little known about the role of mechanical factors in determining the pattern of capillary growth in vivo. However, Branemark [40] suggested that oscillatory movements of erythrocytes are important in the 'opening up' of newly formed sprouts, and Rodbard [41] stressed the importance of the mechanical forces not only in growth but also in the regression of blood vessels after parturition. Consistent with these findings are the early observations of Clark [7], who described sprouting in tadpole tails preferentially from capillaries with high flow while those with low flow regressed. A similar pattern was observed in an implanted ear chamber in rabbits, where capillaries with high flow gradually changed into venules or arterioles [8].

5. Conclusions

We have shown that the EC response during physiological angiogenesis within skeletal muscle is potentially sensitive to the direction of mechanical stimulus, leading to either luminal division or abluminal sprouting following elevated internal shear stress or external stretch, respectively. Although capillary growth can be induced without breakage of the basement membrane by luminal division, proteolysis of the BM is critically important for in vivo angiogenesis by abluminal sprouting. Importantly, EC proliferation may precede the formation of capillary sprouts. Furthermore, it indicates for the first time in vivo that two different mechanisms may operate in the same tissue and we propose that these responses are additive, leading to capillary growth that combines elements of both processes when stimuli of different polarity are applied concurrently (Table 3). Fibroblasts may control angiogenesis either by paracrine secretions modifying the extracellular matrix or by acting as a source of newly differentiated pericytes. We conclude that the context of any stimulus is crucial to the type of angiogenesis observed. While in vitro stimulation of ECs may produce a wide array of genes that are upregulated, in vivo, these cells are subject to mechanical and chemical constraints of the local environment, capable of inducing a differential response to common angiogenic stimuli.

Table 3
Changes in capillary morphology during angiogenesis, relative to those in control muscles[a]

	Prazosin		Overloaded		Stimulated	
	7 days	14 days	7 days	14 days	3 days	7 days
Capillary supply	+	+	±	+	±	+
Luminal processes	+	+	±	±	+	+
Intra-luminal thin septa	+	+	−	−	+	±
Abluminal processes	−	−	+	+	+	+
Breakage of BM	−	−	+	+	+	+
Sprouts without a lumen	−	−	+	+	+	±
Endothelial mitosis	−	−	+	+	+	−

[a] The proportion of capillaries with intra-luminal endothelial cytoplasmic processes, abluminal endothelial cell protrusions, displaying disruption or absence of capillary basement membrane, and the presence of ECs undergoing mitosis within the normal capillary profile. Abbreviation: BM, basement membrane. Symbols: −, not observed/altered; ±, sometimes present/increased; +, always present/increased.

Acknowledgements

This work was supported by the MRC and the Rowbotham Bequest, and benefited from the collaboration of Dr F.M. Hansen-Smith.

References

[1] Hobson B, Denekamp J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. Br J Cancer 1984;49:405–412.
[2] Cliff WJ. Observations on healing tissue: a combined light and electron microscopic investigation. Philos Trans R Soc London B 1963;246:305–325.
[3] Ausprunck DH, Folkman J. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc Res 1977;14:53–66.
[4] Burri PH, Tarek MR. A novel mechanism of capillary growth in the rat pulmonary microcirculation. Anat Rec 1990;228:35–45.
[5] Patan S, Haenni B, Burri PH. Evidence for intussusceptive capillary growth in the chicken chorioallantoic membrane (CAM). Anat Embryol 1993;187:121–130.
[6] Hudlická O, Brown MD, Egginton S. The role of hemodynamic and mechanical factors in vascular growth and remodelling. In: Lelkes PI, editor, Endothelium and Mechanical Forces, London: Harwood Academic Publishers, 1999, pp. 291–359, chapter 14.
[7] Clark ER. Studies on the growth of blood vessels in the tail of frog larvae. Am J Anat 1918;23:37–88.
[8] Clark ER, Clark EL. Microscopic observations on the extraendothelial cells in living mammalian blood vessels. Am J Anat 1940;66:1–49.
[9] Tornling G, Unge G, Adolfsson J, Ljungqvist A, Carlsson S. Proliferative activity of capillary wall cells in skeletal muscle in rats during long-term treatment with dipyridamole. Arzneim Forsch 1980;30:622–623.
[10] Mall G, Mattfeldt T, Reiger P, Volk B, Frolov VA. Morphometric analysis of the rabbit myocardium after chronic ethanol feeding — early capillary changes. Basic Res Cardiol 1982;77:57–67.
[11] Andersen P, Henriksson J. Capillary supply of the quadriceps femoris of man: adaptive response to exercise. J Physiol 1977;270:677–690.
[12] Hudlická O, Brown MD, Egginton S. Angiogenesis in skeletal and cardiac muscle. Physiol Rev 1992;72:369–417.
[13] Ziada AMAR, Hudlická O, Tyler KR, Wright AJA. The effect of long-term vasodilatation of a1-blocker prazosin on capillary density in cardiac and skeletal muscle. Pflügers Arch 1989;415:355–360.
[14] Dawson JM, Hudlická O. Can changes in microcirculation explain capillary growth in skeletal muscle? Int J Exp Pathol 1993;74:65–71.
[15] Egginton S, Hudlická O, Brown MD, Walter H, Weiss JB, Bate A. Capillary growth in relation to blood flow and performance in overloaded rat skeletal muscle. J Appl Physiol 1998;85:2025–2032.
[16] Brown MD, Cotter MA, Hudlická O, Vrbova G. The effect of different patterns of muscle activity on capillary density, mechanical properties and structure of slow and fast rabbit muscles. Pflügers Arch 1976;361:241–250.
[17] Zhou A-L, Egginton S, Brown MD, Hudlická O. Capillary growth in overloaded, hypertrophic adult rat skeletal muscle: an ultrastructural study. Anat Rec 1998;252:49–63.
[18] Zhou A-L, Egginton S, Hudlická O, Brown MD. Internal division of capillaries in rat skeletal muscle in response to chronic vasodilator treatment with α_1 antagonist prazosin. Cell Tissue Res 1998;293:293–303.
[19] Hansen-Smith F, Hudlická O, Egginton S. In vivo angiogenesis in adult rat skeletal muscle: early changes in capillary network architecture and ultrastructure. Cell Tissue Res 1996;286:123–136.
[20] Zhou A-L, Egginton S, Hudlická O. Ultrastructural study of three types of physiological angiogenesis in adult rat skeletal. In: Maragoudakis ME, editor, Angiogenesis. models, modulators and clinical applications, New York: Plenum Press, 1998, pp. 556–557.
[21] Myrhage R, Hudlická O. Capillary growth in chronically stimulated adult skeletal muscle as studied by intravital microscopy and histological methods in rabbits and rats. Microvasc Res 1978;16:73–90.
[22] Zhou AL, Egginton S. Immunogold labelling of proliferating cells during skeletal muscle angiogenesis. J Vasc Res 1998;35:386, (Abstract).
[23] Egginton S. Morphometric analysis of tissue capillary supply. Adv Comp Environ Physiol 1990;6:73–141.
[24] Brown MD, Hudlická O, Makki RF, Weiss JB. Low molecular mass endothelial cell-stimulating angiogenic factor in relation to capillary growth induced in rat skeletal muscle by low-frequency electrical stimulation. Int J Microcirc 1995;15:11–116.
[25] Hansen-Smith FM, Hudlická O, Egginton S. Patterns of capillary growth in stimulated vs. stretched rat muscle. Microcirculation 1997;4:144, (Abstract).
[26] Pearce S, Hudlická O, Egginton S. Early stages in activity-induced angiogenesis in rat skeletal muscles: incorporation of bromodeoxyuridine into cells of the interstitium. J Physiol 1995;483:146P, (Abstract).
[27] Haas TL, Milkiewicz M, Davis SJ, Zhou A-L, Egginton S, Brown MD, Madri JA, Hudlická O. Matrix metalloproteinase activity is required for adaptive angiogenesis in skeletal muscle. Am J Physiol 2000;279:H1540–H1547.
[28] Brown MD, Hudlická O, Weiss JB, Bate A, Silgram H. Prazosin-induced capillary growth in rat skeletal muscle; link with endothelial cell-stimulating angiogenic factor. Int J Microcirc 1996;16:207, (Abstract).
[29] Brown MD, Walter H, Hansen-Smith FM, Hudlická O, Egginton S. Lack of involvement of basic fibroblast growth factor (FGF-2) in capillary growth in skeletal muscles exposed to long-term contractile activity. Angiogenesis 1998;2:81–91.
[30] Milkiewicz M, Brown MD, Egginton S, Hudlická O. Shear modulation of angiogenesis and VEGF in muscles in vivo. Microcirculation, in press.
[31] van Groningen JP, Wenink AVG, Testers LHM. Myocardial capillaries: increase in number by splitting of existing vessels. Anat Embryol 1991;184:65–70.
[32] Brown MD, Egginton S, Hudlická O, Zhou A-L. Appearance of the capillary endothelial glycocalyx in chronically stimulated rat skeletal muscles in relation to angiogenesis. Exp Physiol 1996;81:1043–1046.
[33] Shyy JYJ, Chien S. Role of integrins in cellular responses to mechanical stress and adhesion. Curr Opinion Cell Biol 1997:707–713.
[34] Mills I, Sumpio BE. Endothelium and cyclic strain. In: Lelkes PI, editor, Endothelium and mechanical forces, London: Harwood Academic Publishers, 1999, pp. 249–273, chapter 12.
[35] Chen KD, Li S, Kim M, Yuan S, Chien S, Shyy JY. Mechanotransduction in response to shear stress. Role of receptor tyrosine kinases, integrins and Shc. J Biol Chem 1999;274:18393–18400.
[36] Egginton S, Hudlická O, Brown MD, Graciotti L, Granata AL. In vivo pericyte–endothelial cell interaction during angiogenesis in adult cardiac and skeletal muscle. Microvasc Res 1996;51:213–228.
[37] Sholley MM, Ferguson GP, Seibel HR, Montour JL, Wilson JD. Mechanism of neovascularization: vascular sprouting can occur without proliferation. Lab Invest 1984;51:624–634.
[38] Scoazec JY, Degott C, Reynes M, Benhamou JP, Feldmann G. Epithelioid hemangioendothelioma of the liver: an ultrastructural study. Hum Pathol 1989;20:673–681.

[39] Strek P, Litwin JA, Nowogrodzka-Zagorska M, Miodonski AJ. Microvasculature of the dorsal mucosa of human fetal tongue: a SEM study of corrosion casts. Anatomischer Anzeiger 1995;177:361–366.
[40] Branemark PI. Capillary form and function. Bibli Anat 1965;7:9–28.
[41] Rodbard S. Physical factors in arterial sclerosis and stenosis. Angiology 1971;22:267–284.