Angiogenesis-related ultrastructural changes of capillaries in human skeletal muscle in response to endurance exercise

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Abstract

The ultrastructure of capillaries in skeletal muscle was morphometrically assessed in vastus lateralis muscle (VL) biopsies taken before and after exercise from 22 participants of two training studies. In study-1 (8-weeks of ergometer training), light microscopy revealed capillary-fiber (C/F)-ratio (+27%) and capillary density (+16%) to be higher ($P \leq 0.05$) in the post- than the pre-exercise biopsies of all 10 participants. In study-2 (6-months of moderate running), C/F-ratio and capillary density were increased (+23%/+20%; $P \leq 0.05$) in VL biopsies of 6 "angiogenesis responders" (AR) after training, while 6 "non-angiogenesis responders" (NR) showed non-significant changes of these structural indicators (-4%/-4%). 40 capillary profiles per participant were evaluated by point and intersection counting on cross-sections after transmission electron microscopy. In study 1, volume density ($V_v$) and mean arithmetic thickness ($T$) of endothelial cells (ECs; +19%/+17%) and pericytes (PCs; +20%/+21%) were higher ($P \leq 0.05$), while $V_v$ and $T$ of the peri-capillary basement membrane (BM) were -23%/-22%-lower ($P \leq 0.05$) in the post-training biopsies. In study 2, exercise-related differences between AR- and NR-groups were found for $V_v$ and $T$ of PCs (AR: +26%/+22%, both $P \leq 0.05$; NR: +1%/-3%, both $P > 0.05$) and BM (AR: -14%/-13%, both $P \leq 0.05$; NR: -9%/-11%, $P = 0.07/0.10$). $V_v$ and $T$ of ECs were higher (AR: +16%+18%, NR: +6%+6%, all $P \leq 0.05$) in both groups. The PC-coverage was higher (+13%, $P \leq 0.05$) in VL biopsies of AR but non-significantly altered (+3%, $P > 0.05$) in those of NR after training. Our study suggests that intensified PC-mobilization and BM-thinning are related to exercise-induced angiogenesis in human skeletal muscle, while training per se induces EC-thickening.
Introduction

It is well established that skeletal muscles have the potential to adapt in structure and function to endurance exercise. In humans, most studies dealing with this plasticity of skeletal muscle have focused on the adaptive capacity of the fibers per se, which increase their volume density (Vv) of mitochondria (20) and store more lipid droplets (29) and glykogen (23) after a period of endurance exercise. Likewise, the microvascular system in skeletal muscle of humans may respond to a training stimulus, in particular by increasing the numerical density of the capillaries (1, 19, 22). This process is referred to as angiogenesis, which is a frequently occurring phenomenon in differentiated skeletal muscle to enhance performance (21).

Deduced from their ultrastructural features, two modes of capillary growth have been identified in skeletal muscle of rats and mice: sprouting and splitting angiogenesis (the later mode appears to be similar or identical to intussusception (10)). Both angiogenic modes differ significantly in their initiation and implementation.

Sprouting is triggered by the paracrine activation of capillary ECs (36), which induces their proliferation and the expression of higher levels of matrix metalloproteinases (MMPs). These MMPs catalyze the degradation/loosening of the surrounding basement membrane (BM), thereby permitting the abluminal outgrowth of new capillary segments (27). The process of outgrowth is arrested when the sprouts make contact and merge with existing capillaries (11).

Splitting/intussusceptive angiogenesis, on the other hand, is achieved by the intraluminal splitting of capillaries (15, 35). It is triggered by the increase in shear stress that follows upstream arterial vasodilation (9). In response to the hemodynamic stimulus, ECs send cytoplasmic projections into the capillary lumen (35). When two such projections extending from opposite sides of the lumen meet, a transluminal pillar is formed, which increases in girth by the deposition of connective
tissue until it separates the vascular segment into two independent microvessels. In contrast to abluminal sprouting, intraluminal splitting does not immediately depend on proliferation of the capillary ECs (17).

Several experimental set-ups have been developed to induce excessive angiogenesis in skeletal muscles of laboratory animals (15, 21). Muscle overload (e.g. induced by the surgical extirpation-induced overload of a muscle agonist (17) or in response to chronic electrical stimulation (36)) elevates the contractility of the muscle fibers, which triggers the paracrine-mediated modulation of sprouting angiogenesis. Peripheral vasodilation (e.g. initiated by the administration of the alpha1-adrenergic receptor antagonist prazosin) leads first to functional hyperemia and then to an increase of shear stress in the capillaries (9). The strength of the force thereby generated is somehow registered by ECs of the capillaries in the skeletal muscles and controls the degree of splitting/intussusceptive angiogenesis (3, 35).

Although sprouting and splitting angiogenesis in skeletal muscle have been thoroughly documented in these experimental set-ups, it surprisingly has not yet been studied how physiological angiogenesis in response to endurance exercise is realized in this tissue in either laboratory animals or humans.

Our group has characterized the changes in ultrastructure of skeletal muscle in humans in response to endurance exercise for many years (19, 20, 28, 30). In these investigations, however, the assessment of the ultrastructure of the capillaries was neglected. We thus have now subjected the ultrathin sections of vastus lateralis muscle (VL) biopsies from 22 persons before and after a training period of strenuous cycling (8-weeks) or moderate, long-lasting running (6-months), which were derived from two of these earlier studies (28, 30), to a second morphometric analysis with the distinct focus on the ultrastructural phenotype of capillaries.
The primary aim of this investigation was to verify the hypothesis that the ultrastructure of the capillaries in human skeletal muscle changes in response to endurance exercise. Because light microscopy analysis of the muscle biopsies revealed that only one half of the study group 2 had undergone skeletal muscle angiogenesis after training while the other half did not, we had the opportunity to investigate which of the training-induced alterations of the capillary ultrastructure are related to angiogenesis in skeletal muscle of humans. Furthermore, in order to figure out whether splitting or sprouting is/are involved in angiogenesis in humans muscles, the outcome of study-2 was compared with the morphometrical findings described in papers using rodents (15, 21), in which the concept of splitting or sprouting angiogenesis had been established.
Materials and Methods

Study participants and protocols

For this analysis, sections from biopsies of the vastus lateralis muscle (VL) of humans were used, which were originally collected in two training studies conducted earlier by our group (28, 30). Informed consent was obtained in each case previous to the study begin. These investigations fulfilled all criteria and ethical guidelines for the treatment of human participants valid at the time of study execution.

Study 1:
10 male, untrained participants exercised on an ergometer for 8 weeks (5x week for 30 min). As it has been previously described in detail (28), the training intensity was judged by the heart rate achieved at the end of each training session, which typically reached 90-95% of the participants maximum. In addition, the training intensity was periodically checked by continuous recording of heart rate, oxygen uptake, and plasma lactate levels. The work loads were set to allow each subject to work at peak power output maintainable for 30 min and increased periodically to keep the training intensity at the same relative level throughout the training period.
The mean age of the participants was 30.5 ± 5.0 years and their mean height of 178.0 ± 7.1. The mean body mass of 70.8 ± 5.4 kg altered only non-significantly ($P > 0.05$) during the training period. As determined by an incremental performance test on the ergometer, the mean VO$_2$-max increased significantly ($P \leq 0.05$) from 51.6 ± 3.2 ml/kg*min to 58.4 ± 2.1 ml/kg*min.

Study 2:
Although 20 male non-trained subjects participated originally in this study (30), sections from only 12 randomly selected participants were prepared for
morphometry. These 12 participants had a mean age of 36.3 ± 6.3 years, a mean height of 181.0 ± 7.1 cm, a mean body weight of 85.6 ± 16.0 kg and a mean body-mass index of 26.2 ± 4.0 kg/m².

Prior to the training, the participants had not been engaged in any endurance activities. The moderate home-based training regime consisted of four 30-min jogging sessions per week for 6 months, with a heart-rate corresponding to a maximal oxygen uptake (VO₂-max) of 75%. The heart rate of the subjects participating in this study was continuously recorded during the training with a portable heart rate monitor as previously reported (30).

VO₂-max was determined in a continuous incremental exercise test on a bicycle ergometer (start at 75 W, followed by increments of 30 W every 2 min) to voluntary exhaustion. The mean VO₂-max increased from a pre-training value of 38.3 ± 6.0 ml/kg*min to a post-training one of 40.3 ± 6.3 ml/kg*min, but the difference was not significant (P > 0.05).

**Biopsies**

VL biopsies, 50–100 mg in weight, were obtained using a Bergström fine needle 48 h after the last training session, as previously described (28, 30).

For each participant, biopsies of the VL were taken before and after the training period from the leg using Bergström needles. The specimens were chemically fixed in a 6.25% (v/v) glutaraldehyde solution buffered with 0.1 M sodium cacodylate-HCl (pH 7.4) for several days at 4°C

**Light Microscopy**

After chemical fixation, VL biopsies were divided into 2-3 pieces, each with a volume of approximately 0.5 mm³, after which they were post-fixed in 1% (w/v) OsO₄,
stained *en bloc* in 0.5% uranyl acetate, dehydrated in increasing concentrations (70-100%) of ethanol and embedded in Epon 812 (Fluka, Buchs, Switzerland).

One-micrometer semithin sections were cut on an ultramicrotome (LKB Ultrotom III, Bromma, Sweden) using a diamond knife (Diatome, Biel Switzerland) and stained with Toluidine Blue (4). For the morphometric analysis, transverse or slightly oblique sections through the muscle (volume of approximately 1 mm³) were cut from two randomly selected Epon blocks. A systematic sampling strategy was implemented to acquire 10 photographs of each section at a magnification of x630 in a Leica DMR light microscope (Leica Microsystems, Heerbrugg, Switzerland). Using these light micrographs, the number of capillary profiles and that of muscle fibers were quantified taking into account the forbidden line rule (33). The mean cross-sectional fiber area (MCSFA) was estimated by point counting, and capillary profile density and capillary-to-fiber (C/F) ratio were calculated as previously described (4).

**Transmission electron microscopy**

Ultrathin sections (70-80 nm in thickness) of the Epon blocks were prepared with an Ultracut ultramicrotome (LKB Ultrotom III, Bromma, Sweden), using a diamond knife (Diatome, Biel Switzerland). The sections were floated on 200-mesh parlodion coated copper grids (Plano, Wetzlar, Germany) and contrasted with 0.5% uranyl acetate and 3% lead citrate, as previously described (4, 19). The inspection was carried out using a transmission electron microscope (Morgagni M268; FEI, Brno, Czech republic).

**Morphometric analysis**

Ultrathin sections from two randomly selected Epon-embedded blocks of each VL biopsy were utilized for the morphometric analysis.
20 randomly selected capillaries on each section were photographed in the transmission electron microscope at a final magnification of x8.900 (corresponding to a 11.1 µm x 7.9 µm 'field of view') using a MORADA digital camera (OSIS, Münster, Germany). Capillaries with a length-to-width ratio larger than 2 were considered to be too obliquely sectioned and excluded from the morphometric analysis since the boundary length of the capillaries was used as reference structure in the morphometric analysis (see below). The electron micrographs were overlaid with a grid consisting of 10 x 8 perpendicular test lines at a distance (d) of 1.2 µm in the final magnification. The line crossings were defined as the test points (totally 80), each representing an area of 1.44 µm² (= 1.2 * 1.2).

The number of points (P) landing on capillary lumen, endothelial cells (ECs), EC nucleus, pericytes (PCs) and the basement membrane (BM) were counted and expressed relative to the number of all reference points on the capillaries, thereby yielding unbiased estimates of the mean volume densities (Vv) as Vv = P_{compartment} / P_{capillary} and the mean cross-sectional area (A) of each compartment as A = P_{compartment} * d².

The counting of line intersections (I) that overlapped with the abluminal side of the capillary ECs (= EC/BM transition) permitted an estimate of the mean boundary length (B) of this border. It was calculated as B = 0.5 * d/2 * π * I (33). The mean arithmetic thickness (T) of each compartment was calculated as the cross-sectional area (representing the profile of its volume) divided by the boundary length (representing its surface density) computed as T = P_{compartment} * d / (0.25 * π * I).

The nucleus volume density in EC was calculated as P_{EC nucleus} / (P_{EC nucleus} + P_{EC}). The PC coverage of the capillaries was expressed as the number of test lines crossing simultaneously both the EC/BM transition and a PC surface relative to the total number of test lines hitting the EC/BM transitions without PC contact. The
intraluminal filopodia proportion was calculated as the number of test lines crossing a projection surface relative to the total number of test lines hitting lumen EC/transitions.

Due to their localization within the BM around capillaries, which is easily recognized as sharply demarcated layer on electron micrographs, we are sure to have included only PC in the morphometry of PC features. Other cell types present in the skeletal muscle endomysium (mast cells, macrophages, lymphocytes, satellite cells, fibroblasts, telocytes and stem cells) are not found inside the peri-capillary BM. Smooth muscle cells are located in the peri-vascular BM but surround exclusively arterioles, arteries and veins in skeletal muscle as a characteristic closed cell ring accompanied by an internal and external elastic lamina.

It should be borne in mind that all morphometric indicators are 2D-approximations and do not represent unbiased estimators in 3D.

Statistics

Numerical data are expressed as mean values together with the standard deviations. Parameters pertaining to the morphometric analyses of study-1 were compared using the Student’s T-test.

In study-2, the significances of differences between the AR and NR as well as pre- and post-training groups were assessed by a one-way ANOVA (repeated measures). In cases of significant interactions, differences in the morphometric indicators between the groups were identified using pairwise the Bonferroni correction method. The level of statistical significance was set at p ≤ 0.05.
Results

In order to study the capillarity in skeletal muscles of humans after endurance exercise, we studied the capillary supply by means of light microscopy and the ultrastructure of the capillaries by transmission electron microscopy (TEM) in combination with morphometry.

Capillary supply

Light microscopy was performed to quantify the capillary supply in the 44 VL biopsies of 22 participants of the two training studies collected before and after endurance exercise training (Tab. 1). In both studies, the mean values for C/F-ratio and capillary density were higher in the biopsies obtained after the exercise period than in those obtained before the training. These differences in capillary supply were significantly higher ($P \leq 0.05$) in all 10 participants of study-1 (C/F-ratio: +27%; capillary density: +16%), while both parameters for capillary supply were not significantly varying ($P > 0.05$) in the 12 participants of study-2 (C/F-ratio: +9%; capillary density: +6%). By contrast, MCSFA was not significantly different ($P > 0.05$) between the VL biopsies obtained pre- and post-exercise in both studies (study-1: +8%; study-2: +3%).

An increased C/F-ratio in skeletal muscle is characteristic for “angiogenesis responders” (AR), while “non-angiogenesis responders” (NR) comprise the subjects that do not show a higher C/F-ratio in response to an angiogenic stimulus. In accordance to this definition, closer analysis of the VL biopsies revealed all participants of study-1 to be AR. In study-2, C/F-ratio was increased after training (23%; $P \leq 0.05$) in VL biopsies of 6 AR, while 6 NR showed only non-significant changes (-4%; $P \geq 0.05$) of this indicator (Tab. 1).

There were no significant differences ($P \geq 0.05$) in anthropometric data between the participants belonging to either the AR or the NR groups of study-2: age (AR: 36.2 ±
5.3 y; NR: 37.8 ± 8.3 y), VO₂-max before training (AR: 39.2 ± 6.6 ml/min/kg; NR: 39.8 ± 5.6 ml/min/kg), VO₂-max after training (AR: 41.2 ± 7.4 ml/min/kg; NR: 41.5 ± 6.5 ml/min/kg), body mass (AR: 90.2 ± 22.3 kg; NR: 80.9 ± 6.0 kg), height (AR: 182.1 ± 6.6 cm; NR: 180.7 ± 5.4 cm), BMI (AR: 26.3 ± 6.0 kg/m²; NR: 25.7 ± 0.7 kg/m²) and mean training performance/week (AR: 13.0 ± 4.8 km and 77 ± 21 min; NR: 16.8 ± 3.2 km and 110 ± 42 min).

Electron microscopy

For the qualitative and morphometric analysis of their ultrastructure in VL biopsies, 40 capillaries (20 from each of two blocks) in each of the 44 biopsies (22 pre-exercise and 22 post-exercise biopsies) were randomly assessed by TEM. Hence, TEM sections of 1760 capillary profiles were evaluated in total.

Qualitative TEM analysis

In each case, the capillary profile manifested the same characteristic ultrastructural features: the lumen was mantled with ECs, the abluminal surface of the ECs of which was skirted by a dense BM containing the profiles of one or more PCs (Fig. 1A,B). In the ECs of all capillaries, cytoplasmic vacuoles were often observed. In many of the capillaries of all participants, one or more protrusions projected from the ECs into the capillary lumen often in close neighborhood to tight junctions (Fig. 1C). In some instances, the ECs of capillaries, particularly in those of post-exercise biopsies, were strongly enlarged and thickened (Fig. 1D).

We made use of the micrographs to study the spatial relationship between ECs and PCs in human VL biopsies. Consistently, the PCs profiles were separated by a peri-capillary BM from the abluminal surface of the EC (Fig. 1). Frequently, PC projections entered the ECs (Fig. 2A,B). At those sites where these PC projections
invaded the ECs either EC bridges (Fig. 2C) or intracellular EC pockets and holes (Fig. 2D-F) were seen, as far as it is possible to assess this on 2D photo-images. These features of PCs invading ECs were seen in about 50% of all capillary profiles detected in VL biopsies taken before and after exercise (data not shown).

Relatively infrequently (in 2 pre-exercise and 6 post-exercise capillary profiles out of 1760 assessed ones), we detected abluminal projections outgrowing from the EC towards the endomysium, which we assume to be sprouts (Fig. 1E,F). Remarkably, the entire capillary circumference with the abluminal projections was surrounded continuously by an intact peri-capillary BM. Serial sections enabled us to characterize one of these presumptive sprouts in more detail (Fig. 3): it became detached from the abluminal surface of capillary EC (Fig. 3C) and ended lastly as small EC clot without detectable lumen (Fig. 3D).

A cytoplasmic vacuolization was seen to a various extent in the capillary ECs: some ECs contained only a few membranous vesicles, while others were completely filled by these organelles (Fig. 3A versus 3D). However, apparent differences in the degree of vacuolization in one of the experimental groups were not evident to us.

**Morphometry of the capillary ultrastructure**

**Study-1:** As shown in Fig. 4, the values for the mean arithmetic thickness (T) of EC (+17%) and PC (+21%) were significantly higher ($P \leq 0.05$), while T of BM was significantly lower (-22%, $P \leq 0.05$) in the post-exercise VL biopsies than the pre-exercise ones. T of lumen did not differ significantly (-1%, $P > 0.05$) between the biopsies. Accordingly, the mean cross-sectional capillary area (A) including BM and PC (+5%) and the mean arithmetic circumference (+8%) differed only non-significantly ($P > 0.05$) between pre- and post-exercise biopsies. The relative proportion of the compartment T resembles the absolute estimations: the volume
densities (Vv) of EC (+19%), PC (+20%) and BM (-23%) but not the lumen (-5%) were significantly different (P ≤ 0.05) between the post- and the pre-exercise biopsies. As shown in Fig. 4, the nucleus density in EC (-9%) and the enlargement of the luminal EC surface by projections (+7%) were only non-significantly altered (P > 0.05) in the capillaries of the VL biopsies after the training period. In contrast, the PC coverage of the capillaries, which reflects the proportion of abluminal EC surface that is covered by PC, was significantly increased (+17%, P ≤ 0.05) after exercise.

**Study-2:** In study-2, subjects underwent a less strenuous training than the participants of study-1. In the AR-cohort, similar quantitative changes in the capillary ultrastructure as in study-1 were found in response to endurance exercise (Tab. 2). In order to figure out whether changes in the capillary ultrastructure in response to exercise are associated with the onset of angiogenesis, the morphometric findings of the AR and NR groups of study-2 were compared. Most indicators were altered to a similar extent in both groups by the training (Tab. 2).

Significant pre/post exercise differences between the AR and NR groups were found for Vv and T of PC (AR group: +26% and +22%, both P ≤ 0.05; NR group: +1% and -3%, both P > 0.05) and BM (AR group: -14% and -13%, both P ≤ 0.05; NR group: -9% and -11%, P = 0.07 / 0.10), while the EC surface enlarged by projections was significantly altered in the NR group (+24%; P ≤ 0.05) but not the AR group (+11%; P > 0.05). The PC coverage of the capillaries was significantly altered in the post-exercise biopsies of AR (+13%; P ≤ 0.05) but not the NR (+3%; P > 0.05). Vv and T of EC were significantly higher (AR group: +16% and +18%, both P ≤ 0.05; NR group: +6% and +6%, both P ≤ 0.05) in the post- than the pre-training VL biopsies of both groups suggesting that EC thickening was induced by the training stimulus as such without association with angiogenesis.
Discussion

In the present investigation, we have morphometrically assessed the ultrastructure of capillaries in 44 vastus lateralis muscle (VL) biopsies taken before and after exercise from 22 participants of two training studies. In study-1, all 10 participants underwent angiogenesis in skeletal muscle. In study-2, 6 "angiogenesis responders" (AR) were identified, while 6 "non-angiogenesis responders" (NR) did not show a significant change of the C/F-ratio after training. Transmission EM (TEM) analysis combined with morphometry of capillaries in VL biopsies of AR and NR of study-2 suggest that intensified pericyte (PC)-mobilization and peri-capillary basement membrane (BM)-thinning are related to exercise-induced angiogenesis, while training per se induced endothelial cell (EC)-thickening. Comparison of the structural indicators assessed in our analysis of human skeletal muscle biopsies with those gained in rodents, as previously published, does not provide clear indication whether exercise-induced angiogenesis in humans is implemented by sprouting and/or splitting.

Angiogenesis in skeletal muscle is defined as statistically significant increase in the values for C/F-ratio (21). Although we included two blocks (with 10 micrographs each) per biopsy in the morphometric analysis, C/F-ratio was calculated after addition of the numbers of all capillary and fiber profiles on the 20 micrographs. With only one reading of capillary supply per biopsy, however, statistical significance cannot be computed and, thus, it cannot be decided whether training-induced angiogenesis in the skeletal muscle of a particular person. In order to overcome this problem, we defined AR if the C/F-ratio was at least 10% higher in the post-exercise biopsies than in the pre-exercise ones. All other participants were assigned to the NR group. This threshold used to allocate the study-2 participants in AR and NR is mainly based on experimental experiences of our group with elite athletes and untrained volunteers (28, 30). In the pre- and post-exercise biopsies of the cohorts in
these studies, changes in the C/F-ratio lower than about 10% revealed to be statistically non-significant, as also reported by others (18). This value was then transferred as threshold to the individual analysis described here. Using this classification, we could identify 10 AR (study-1) as well 6 AR and 6 NR (study-2) that contributed to our investigation.

Although all participants of study-2 underwent an exercise training of similar intensity and duration, the changes of capillary supply in the VL were inconsistent. Heterogeneity in responsiveness to a training stimulus is a well-documented phenomenon for other parameters (e.g. VO_{2-max}), and is assumed to reflect mainly individual differences in genetic predisposition (7, 24, 25), which could be ascribed, for example, to the variable frequency of transcriptional reading of the VEGF promoter (26). The evident variances in angiogenic capacity might be likewise ascribed to the heterogeneous genetic background of the participants but might be also influenced by their health status and their distinct fitness (rather untrained those of study-1) and/or the training load (rather moderate compared to study-1) in study-2.

To our best knowledge, this is the first study in which the ultrastructural phenotype of capillaries was evaluated in skeletal muscles of non-diseased, young humans after an aerobic training. Notably, a partial aspect of this topic was addressed, when it was reported that the thickness of the BM was reduced by 30-40% in the gastrocnemius muscle of elderly persons (mean of 64 years) after nine months of endurance exercise training (34).

In both studies, the VL muscle biopsies were taken two days after the last training session. Consequently, ultrastructural alterations of capillaries, which we describe here, are not caused acutely but represent chronic changes in the capillary arrangement.
In study 1, ECs and PCs have relatively (volume density; Vv) and absolutely (mean arithmetic thickness; T) increased, while Vv and T of the peri-capillary BM were lower in the post-exercise than the pre-exercise biopsies of study-1. In contrast, Vv and T of the capillary lumen were not significantly altered in the VL biopsies after training. Because the area and the circumference of capillaries *in toto* did not significantly differ between the pre- and post-exercise biopsies, quantitative changes in Vv and T in response to the training stimulus must be caused by an internal rearrangement of the capillary compartments (EC, BM, PC).

To determine whether any of these exercise-related changes of the capillary ultrastructure are specifically associated with angiogenesis or rather represent angiogenesis-unrelated reactions of the microvasculature to the exercise stimulus, we assessed and compared the phenotype of the capillaries in skeletal muscles of AR and NR of study-2 by means of morphometry. Most strikingly, higher values for Vv and T of PC and lower ones of BM as well as increased PC coverage were detected in the biopsies of the AR group but not in those of the NR indicating that higher PC coverage and BM thinning are related to exercise-dependent angiogenesis in humans. In contrast, the thickening of ECs after exercise was observed in the VL biopsies of both groups. Thus, the EC expansion appears to be unrelated to angiogenesis. Interesting, EC swelling has also been observed in rats subjected to electrical stimulation (12), which resembles an experimental procedure of endurance exercise.

Hudlicka, Egginton, Brown and colleagues have identified hallmarks for changes in the ultrastructural phenotype of capillaries associated with splitting and sprouting angiogenesis in skeletal muscles of rodents (13, 15, 35, 36). However, it is not known how this process is realized physiologically in response to endurance exercise. This lack of knowledge is at least partly due to the fact that there are no
cellular markers available for both angiogenic modes so far. Without doubt, the gold standard to address this issue is therefore still the quantitative evaluation of the capillary ultrastructure by the combination of TEM analysis and morphometry (13, 15, 32, 35, 36), which however is elaborative to perform.

During splitting angiogenesis, a higher proportion of intraluminal irregularities, projections and septa combined with extensive cytoplasmic vacuolization of EC were observed in skeletal muscle capillaries of prazosin-treated rats compared to those of control animals (35). In contrast, we found the intraluminal EC surface in capillaries to be only non-significantly altered by projections in the post-exercise biopsies of the study participants. These findings implicate that physiological angiogenesis in skeletal muscles of humans in response to endurance exercise training may not substantially manifested by splitting/intussusception.

Sprouting angiogenesis in skeletal muscle of rats induced by surgical extirpation of a synergistic muscle was associated with an increase of abluminal EC processes, higher rates of EC mitosis (detected by a higher frequency of bromodeoxyuridine incorporation) and focal breakage of the peri-capillary BM (36). In the human muscle biopsies, we observed only occasionally abluminal processes, the capillaries were continuously surrounded by an intact peri-capillary BM and the nucleus density (Vv) in capillary-associated ECs was not different between pre- and post-exercise biopsies (-9%).

During sprouting angiogenesis induced by extirpation of the tibialis anterior muscle of rats, a higher PC coverage of capillaries (+42%) in the extensor digitorum longus muscle (EDL) was observed than of those in the untreated contralateral EDL (14). Accordingly, the PC coverage of capillaries was higher in the post-exercise VL biopsies than in the pre-exercise ones (study-1: +17%; AR in study-2: +13%). The findings of the two studies suggest a pro-angiogenic impact of PCs on capillary ECs
in skeletal muscles of rats and humans after an increase in contractility. The interaction of the two cell types might be communicated by the high number of PC projections (‘PEGs’) inserting into EC pockets (‘sockets’) in skeletal muscles as shown here and similar to those in rats (13, 31).

Given this close spatial relationship between PCs and ECs, it seems reasonable that both cell types communicate intensively. The existence of mechanical contacts in form of gap junctions between ECs and PCs is controversially discussed (reviewed in (2)).

Pericytes have phagocytic activity and are involved in the control or the permeability of the blood–brain barrier and the regulation blood flow through capillaries (8). It was furthermore speculated that PC also contribute to the realization of EC-mediated angiogenesis (16). Actually, there was a significant reduction in the PC coverage during early phases of angiogenesis in skeletal muscles of rats subjected to chronic electrical stimulation what suggested an anti-angiogenic impact of PC in this experimental model (13). In contrast, long-term peripheral vasodilation (prazosin model) or prolonged stretch (extirpation model) was accompanied by an increase in PC coverage (14). Thus, the role of PCs in controlling physiological angiogenesis obviously depends on the nature of the initial stimulus (14), because sprouting (16) and splitting angiogenesis as well as subsequent capillary remodeling require a dynamic interaction of PCs with ECs. The details of the mutual interaction is widely unknown so far.

Recently, it was shown that the PC pool in the skeletal muscles of mice is composed of two distinct populations (5, 6). The first PC subtype (type-1), which does not express the intermediate filament nestin, may differentiate into fat cells, while the second PC subtype (type-2), which expresses nestin, are part of the stem cell pool for muscle fibers (8) and may regulate angiogenesis (6). However, the contribution of
the two PC subtypes to the various angiogenesis modes has to be characterized in more detail, especially in human skeletal muscle.

Taken together, only an inconsistent picture emerges from the comparison of the structural indicators of skeletal muscle capillaries derived from the animal studies and our analysis of human skeletal muscle biopsies derived from study participants undergoing endurance training: some findings are divergent (abluminal EC processes, EC mitosis, breakage of the BM), while others changes are aligned (PC coverage). Thus, the question whether exercise-induced angiogenesis in humans is implemented by sprouting cannot be unequivocally answered at this point.

Needle biopsies of human skeletal muscle are too small to facilitate the production of a number of randomized sections sufficient for an isotropic uniform random sampling strategy (32), which would be required for a formally correct stereological analysis. Nevertheless, the morphometric findings represent acceptable approximations. We furthermore would like to underline that the values for T of compartments presented in this investigation represent only quantitative structural descriptors of the capillary ultrastructure and do not provide any evidence of cause/effect relationships between the capillary phenotype and angiogenesis. For a functional interpretation of the data e.g. their possible relation to oxygen and substrate diffusion, other indicators, e.g. the harmonic mean-based thickness have to be determined.

In summary, the findings of our study on the ultrastructure of capillaries in human VL biopsies after training indicate that increased PC coverage of capillaries and BM thinning are related to exercise-induced angiogenesis, while EC thickening is induced by the training stimulus.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.
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Figure Legends

Figure 1: Transmission electron microscopy to demonstrate the ultrastructure of capillaries in VL biopsies. Shown are representative micrographs of capillaries from VL biopsies collected from a participant before (A) and after (B) the endurance exercise stimulus. C: Regularly in pre- and post-exercise biopsies, ECs send forth projections often extending in pairs (arrowheads) into the capillary lumen. D: In many instances, the ECs in capillaries of post-exercise biopsies appeared enlarged (asterisks). Occasionally, we have seen capillaries with short (E) or long (F) abluminal projections (arrowheads) that may represent sprouts outlining the capillary lumen (asterisk). Electron micrographs were derived from participants of: A: study 2-NR pre-exercise; B: study 2-AR post-exercise; C: study 1 pre-exercise; D: study 1 post-exercise; E: study 2-AR post-exercise F: study 2-AR post-exercise. TJ in A-F marks presumptive tight junctions.

Figure 2: Identification of a capillary sprout on serial ultrathin sections. Unilaterally, an EC hollow with clearly visible lumen (asterisks) grew out of the cross-sectioned capillary (A; see also higher magnification in B). On the following two serial sections (C,D), which were separated by 500 nm distance from each other, this abluminal EC sprout extended (arrowhead) to terminate as small EC profile (arrow) surrounded by a BM.

Figure 3: Characterization of the endothelial cell (EC)-pericyte (PC) interaction. On all electron micrographs of capillaries, the abluminal side of ECs was consistently surrounded by a BM, in which frequently PC profiles were embedded. Regularly, projections of the PCs (‘PEGs’) were seen that entered the ECs at their abluminal
surface (‘sockets’) to a different extent (A-F). Note the intracellular EC holes in D-F (arrowheads), which presumably represent pockets for invading PC projections.

Figure 4: Morphometry of the capillary ultrastructure from pre- and post-exercise biopsies of study-1. With the outcome of the point and intersection counting performed on micrographs of capillaries from the 10 pre and 10 post-exercise biopsies of study-1, morphometric indicators were computed. Means ± standard deviations are represented. * significantly different between pre and post-exercise biopsies. EC= endothelial cell; PC= pericyte
References


Table 1: The capillary supply in the VL biopsies collected before and after endurance exercise from the participants of the two training studies. Shown are the means ± standard deviations as determined applying morphometry after light microscopy of semithin sections. * different between pre and post-exercise. MCSFA = mean cross-sectional fiber area, AR = angiogenesis responders; NR = non-angiogenesis responders.

<table>
<thead>
<tr>
<th></th>
<th>Study-1</th>
<th>Study-2</th>
<th></th>
<th>Study-1</th>
<th>Study-2</th>
<th></th>
<th>Study-1</th>
<th>Study-2</th>
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<tbody>
<tr>
<td></td>
<td>n=10</td>
<td>AR (n=6)</td>
<td>NR (n=6)</td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
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<tr>
<td>C/F-ratio</td>
<td></td>
<td></td>
<td></td>
<td>1.76 ±</td>
<td>0.22</td>
<td>2.18 ±</td>
<td>0.23 *</td>
<td>1.42 ±</td>
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<td>Capillary density</td>
<td></td>
<td></td>
<td></td>
<td>464 ±  47</td>
<td>534 ±  60*</td>
<td>435 ±  95</td>
<td>520 ±  99*</td>
<td>482 ±  60</td>
<td>460 ±  52</td>
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<td>(mm^-2)</td>
<td></td>
<td></td>
<td></td>
<td>3874 ± 669</td>
<td>4120 ± 484</td>
<td>3314 ± 824</td>
<td>3458 ± 605</td>
<td>3385 ± 546</td>
<td>3428 ± 804</td>
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Table 2: Summary of the quantitative stereological analysis of the capillary ultrastructure of angiogenesis responders (AR) and non-angiogenesis responders (NR) of study-2. Transmission electron micrographs of transversally sectioned 960 capillaries in 12 VL biopsies of study-2 participants that were collected before and after the training sessions were subjected to a morphometric analysis of the listed structural indicators by point and intersection counting. Means ± standard deviations are represented. Abbreviations: A= area; Vv= volume density; T= mean arithmetic thickness; B= mean arithmetic circumference; EC= endothelial cells; PC= pericytes; BM= basement membrane; *: \( P \leq 0.05 \) between pre- and post exercise biopsies.

<table>
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<th>indicator</th>
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<th>NR (n=6)</th>
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<tr>
<td>A (cap) with BM and PC</td>
<td>23.4 ± 2.2</td>
<td>23.6 ± 2.6</td>
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<td>Vv (lumen; cap)</td>
<td>0.39 ± 0.03</td>
<td>0.36 ± 0.04</td>
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<tr>
<td>Vv (EC; cap)</td>
<td>0.28 ± 0.02</td>
<td>0.32 ± 0.03 *</td>
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<tr>
<td>Vv (PC; cap)</td>
<td>0.09 ± 0.02</td>
<td>0.11 ± 0.02 *</td>
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<tr>
<td>Vv (BM; cap)</td>
<td>0.24 ± 0.04</td>
<td>0.21 ± 0.03 *</td>
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<tr>
<td>Intraluminal projection proportion</td>
<td>0.11 ± 0.04</td>
<td>0.12 ± 0.03</td>
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<td>PC coverage</td>
<td>0.24 ± 0.03</td>
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<tr>
<td>EC nucleus density</td>
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<tr>
<td>T (lumen)</td>
<td>741 ± 32</td>
<td>680 ± 63</td>
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<tr>
<td>T (EC)</td>
<td>422 ± 20</td>
<td>496 ± 54 *</td>
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<td>T (PC)</td>
<td>151 ± 35</td>
<td>184 ± 28 *</td>
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<td>T (BM)</td>
<td>388 ± 62</td>
<td>339 ± 41 *</td>
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<tr>
<td>B (cap)</td>
<td>18.3 ± 0.9</td>
<td>18.7 ± 1.4</td>
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Fig. 4