Assessment of the microcirculation in cardiovascular disease

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INTRODUCTION

Microcirculation is the collective name for the smallest components of the cardiovascular channels—the arterioles, capillaries and venules, each with their own characteristic structure and function. Together they are the site of control of tissue perfusion, blood–tissue exchange and tissue blood volume. The arterioles are resistance vessels since a major fraction of total pressure dissipation occurs in this segment of the vascular tree. A change in arteriolar structure or function affects local tissue perfusion, but also systemic arterial blood pressure control. The capillaries are the major exchange vessels; the nutrients required to sustain the cells in a tissue flow across their surface. Finally, venules are important capacitance vessels because most of the tissue blood volume is localized in these microvessels.

The high degree of control needed to ensure optimal microvascular function is met by a range of control mechanisms. These include very rapidly acting local metabolic and myogenic mechanisms, endothelium-dependent mechanisms of release of vasoactive substances, nervous and hormonal influences, but also slowly acting structural mechanisms. In fact, as the most embryonic part of the vascular tree, the microcirculation has an impressive ability to remodel the deployment of its vascular components, to change vessel length and number and to alter structural features of the vessel wall. These long-term structural changes are increasingly recognized as important features of cardiovascular disease.

The pathology of the microcirculation in (cardiovascular) disease reflects visible manifestations of acute mechanisms of diameter change as well as structural damage, including wall thickening, vessel tortuosity, growth (angiogenesis) and atrophy (rarefaction). The visible nature of the changes in pathology has for a long time determined the mainly descriptive approach in microcirculation research. In the past decades, major advances have been made in the quantitative study of microcirculatory structure and dynamics. The purpose of this article is to review some of these developments. We do not aim at detailed technical analyses, for which the reader is referred to specialized literature. Rather, we want to provide an overview of technical possibilities to study the microcirculation and their implications in understanding the pathophysiological role of this fascinating segment of the cardiovascular system.

QUANTIFICATION OF MICROVASCULAR STRUCTURE AND FUNCTION

Histomorphometry

One of the most remarkable early publications on the microcirculation is a monograph by R. Thoma, Professor of Pathology at the University of Dorpat, published over a 100 years ago [1]. It describes the ‘histogenesis’ and ‘histomechnics’ of the developing chick embryo microcirculation. Thoma used a combination of relatively simple microscopy and tissue fixation to describe the early development of capillaries, arterioles and venules. By a wonderful combination of experimental observation and deduction, he formulated three basic rules for vascular development which are still valid today: (i) the number of vessels that grow in a tissue is determined by genetic influences and metabolic needs of the tissue, (ii) the vessel diameter depends on the flow of blood through its lumen, and (iii) the vessel wall thickness is determined by the transmural tension. A particularly visionary aspect of Thoma’s observations is that he pointed out the strategic role of the endothelium as a mediator of mechanically induced vascular growth responses.

The early histological methods explored by Thoma and his 19th century contemporaries were refined during the 20th century. The combination of histology and (electron) microscopy revealed the basic microscopic anatomy of the microcirculation in many tissues in mammals [2]. Two techniques...
have been particularly helpful in the study of the three-dimensional anatomy of the microcirculation: light microscopy of India-ink injected tissue specimens and scanning electron microscopy (SEM) of corrosion casts. The use of India ink or other dyes has been especially helpful in the study of capillary networks and the quantification of capillary densities. A change in capillary density is a key phenomenon during normal development, inflammation, ischaemia, wound healing, and in tumour growth. Hudlicka's chapter in the Handbook of Physiology [3] contains an in-depth review of the application of India ink or other dyes in the study of capillary growth.

The combined use of SEM and corrosion casts to study the architecture of the microcirculation was introduced in the 1970s [4]. It involves a precasting procedure to remove the blood. Subsequently, casting media, such as methacylate mixtures or other plastic resins, are infused intra-arterially into an artery supplying the target tissue. It is generally accepted that perfusion pressure should be equal to the physiological pressure at the site of infusion. An essential next step is the hardening (polymerization) of the casting medium and the dissection of the cast samples. These are relatively complex and sensitive procedures. A well-prepared cast allows detailed measurements of vessel diameters and lengths. A recent book edited by Motta et al. [5] provides a comprehensive review of methods and applications of SEM/corrosion casts.

In the past 10–15 years, the introduction of in-situ immunohistochemistry has revolutionized the histomorphometric study of the vascular system. Immunohistochemistry allows the recognition of a range of cellular and subcellular components of the microvascular wall [6]. Powerful new microscopy techniques (e.g. confocal microscopy, fluorescent imaging) have been developed to enhance visualization of these cellular and subcellular components. This approach has provided a wealth of data on the molecular control of endothelial, smooth muscle and connective tissue cell function in the vessel wall as well as the extracellular matrix and extrinsic (nervous, endocrine) influences on vessel wall structure and function. At the level of the microcirculation, such approaches now permit the study of the cellular and molecular mechanisms involved in vascular growth [7]. However, the application of immunohistochemistry has mainly focused on the larger segments of the vascular tree thus far. It is beyond the scope of this article to review these developments. Instead, the reader is referred to a recent review article discussing the application of immunohistochemistry to quantify the cellular and subcellular composition of the vessel wall [8].

**Intravital microscopy**

The histological methods described above have been particularly useful in the study of the architecture and molecular control of the microcirculation. However, tissue specimens have to be removed from the organism for analysis. This ex-vivo approach is not suitable for the study of microcirculatory dynamics. Intravital microscopy is the technique of choice for the measurement of microcirculatory pressures, flows, diameters, capillary exchange processes and cell–wall interactions.

The craftsmanship of Dutch lens polishers allowed Antoni van Leeuwenhoek (1632–1723) to make his pioneering microscopic observations on living objects. Italian scientists in the 17th century, in particular Malpighi, made the first systematic microscopic observations of microvessels connecting the arterial and venous system in frogs lungs. Microscope technology steadily improved throughout the centuries that followed. However, it was not until the late 1950s and 1960s that major advances were made in the ability to measure small vessel dynamics in a quantitative framework [9]. Although microscope technology was available, adequate methodologies for animal experimentation in vivo (anaesthesia, surgery, selection of proper tissues) had to be developed.

Since the early 1960s, a number of tissues have been systematically investigated with intravital microscopy (Table 1). The most widely used preparations are the mesentery [10] and the cremaster muscle [11, 12]. The former is usually studied in rabbit or rat; the latter mainly in the rat, but also in hamster or mouse. These tissues have to be exteriorized to allow transillumination. Striated muscles, such as the gracilis [13] and spinotrapezius muscles [14] of rat and the tenuissimus muscle of cat or rabbit [15, 16], are also widely used. They can be studied in situ by inserting a small light source under the muscle. Other well-known preparations are the hamster cheek pouch [17, 18] and the rat or rabbit pia mater [19]. Both can be studied acutely, but also chronically with transparent windows (vide infra). A number of other tissues summarized in Table 1 are less commonly used, mostly because of complicated surgical procedures and lack of reproducibility of the preparation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Species</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Striated muscle</td>
<td>rat, hamster</td>
<td>[11, 12]</td>
</tr>
<tr>
<td>Cremaster muscle</td>
<td>rabbit, cat</td>
<td>[15, 16]</td>
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<tr>
<td>Tenuissimus muscle</td>
<td>rat, dog</td>
<td>[13]</td>
</tr>
<tr>
<td>Gracilis muscle</td>
<td>rat</td>
<td>[14]</td>
</tr>
<tr>
<td>Spinotrapezius muscle</td>
<td>rat, cat, rabbit</td>
<td>[10]</td>
</tr>
<tr>
<td>Mesentery</td>
<td>hamster</td>
<td>[17, 18]</td>
</tr>
<tr>
<td>Cheek pouch</td>
<td>cat, rat, rabbit</td>
<td>[19]</td>
</tr>
<tr>
<td>Pia mater</td>
<td>rat</td>
<td>[12]</td>
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<tr>
<td>Kidney</td>
<td>rat</td>
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<tr>
<td>Intestine</td>
<td>rat</td>
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The most important architectural features of the microcirculation of these tissues have been reviewed
Microcirculation in cardiovascular disease

Fig. 1. Vascular branching pattern of the rat cremaster microcirculation. The alpha-numerical vessel classification refers to first- (A1), second- (A2), third- (A3) and fourth- (A4) order arterioles. Similar classifications are used for venules. Reproduced from [2].

There are many common features, but also distinct differences. For instance, spontaneous contractile activity and intermittent flow are common to most vascular beds. On the other hand, only a limited number of beds receive input from one central artery (e.g. cremaster), whereas most others do not. There are also major differences in the branching patterns of the terminal part of the arteriolar tree. On the one hand, some tissues have a tree-like dichotomous branching pattern (e.g. cremaster), whereas in other tissues many arteriolar–arteriolar connections, or arcades (e.g. spinotrapezius), are found. The nature of this branching pattern is of great influence on the vascular pattern analysis. The most commonly used classification scheme designates the main arterioles entering into the microcirculation as first-order arterioles (A1; see Fig. 1). The subsequent branches are referred to as second-, third-, fourth- and (sometimes) fifth-order arterioles (A2–A5). A similar scheme is used for the venules. An alternative approach, of particular use in tissues with many arcades, is to refer to the arterioles entering into the microcirculation as the feeding arterioles and the arterioles terminating in capillaries as the terminal arterioles. In between are the arcading arterioles that form connections with other arterioles. These two different branching patterns may be the result of fundamentally different growth processes.

Table 2. Chronic window techniques to observe the microcirculation in conscious animals

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Species</th>
<th>References</th>
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<tbody>
<tr>
<td>Ear</td>
<td>rabbit</td>
<td>[20, 21]</td>
</tr>
<tr>
<td>Skin</td>
<td>rabbit</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>[23, 30]</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>[26, 27]</td>
</tr>
<tr>
<td></td>
<td>hamster</td>
<td>[24, 25]</td>
</tr>
<tr>
<td>Cheek pouch</td>
<td>hamster</td>
<td>[17]</td>
</tr>
<tr>
<td>Pia mater</td>
<td>rat</td>
<td>[31, 32]</td>
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<td></td>
<td>mouse</td>
<td>[33]</td>
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Chronic window techniques in conscious animals

The use of intravital microscopy to study microvascular dynamics as discussed above has one clear disadvantage: the need for anaesthesia and, sometimes extensive, surgery of the experimental animals. This limitation has inspired the development of chronic window techniques in conscious animals (Table 2). The first report on a transparent chamber dates from 1924 [20]. It concerned a rabbit ear chamber, which was modified and improved several times [21]. In 1934, Williams [22] adapted the chamber for use in the skin of the rabbit, whereas Algire [23] implanted the chamber into the dorsal skinfold of the mouse. Subsequently, transparent chambers were developed for the hamster cheek pouch [17], the dorsal skinfold of the hamster [24,
Dorsal microcirculatory chamber (DMC)

Most chambers described above contain regenerated tissue or preformed connective or skin tissue. The DMC contains intact striated muscle, the cutaneous maximus muscle. The chronic observation of a normal microcirculation in a striated muscle is a major advantage in pathophysiological and pharmacological studies. Striated muscle tissue comprises a large part of body mass and largely determines peripheral vascular resistance. This is an important consideration for studies of disease models involving a change in vascular resistance, such as hypertension or heart failure. Furthermore, the study of vasoactive drugs is best performed in tissues that contribute significantly to peripheral vascular resistance.

The technical details of the DMC in rats have been published elsewhere [28, 29, 36]. The DMC consists of two halves made of polycarbonate. Polycarbonate is an extremely lightweight material with very low heat conductivity. The two halves are connected by four stainless steel pins that maintain an interdistance of 500–650 μm between the cover slips. This allows enough space for one layer of cutaneous maximus muscle (200–300 μm).

The DMC should be implanted in relatively young rats. The most optimal period is when the animals are 5–6 weeks old. The surgery is done under anaesthesia and takes about 1 h for an experienced person. An aluminium cap is placed over the chamber and fixed with stainless steel wire to avoid post-surgery scratching and nibbling and to improve wound healing. During the first 1 or 2 weeks after surgery, wound edges are cleaned regularly using iodine and saline. Approximately 3 weeks after surgery, rats can be used for experiments for the first time. A preparation is used if it meets the following criteria: (i) the rat should show no sign of discomfort or disease; (ii) the preparation should show good optical clarity and no signs of microbleeding or inflammation, as indicated by leucocytes sticking to the venular wall, excessive venular growth or tissue disintegration; (iii) the presence of a normal microcirculation, as evidenced by vasomotion. A schematic representation of the experimental set-up is given in Fig. 2. In our laboratory we have used the rat DMC to study microcirculatory changes in experimental hypertension, diabetes and heart failure as well as in research on vasoactive drugs [29, 36–40]. Fig. 3 summarizes the results of the pharmacological studies. It shows a marked heterogeneity of the different microvascular segments in sensitivity to vasoactive drugs.

Microcirculatory dynamics

The major advantage of intravital microscopy is that it allows the assessment of microcirculatory dynamics. A particular segment of the vascular tree can be selected on the basis of the classification criteria described above. Next, the change in diameter, flow and pressure can be followed in time or after pathophysiological or pharmacological manipulations. In addition, phenomena like cell–wall interactions or vascular permeability can be observed as a function of time.

The method of choice for measuring diameter changes is video-image shearing as described originally by Intaglietta and Tompkins [25]. This consists of the optical shearing of the image of a blood vessel along a line that is perpendicular to the axis.
of a vessel, in order to align the opposite edges of the vessel. The spatial transfer to obtain this alignment is the direct measure of the diameter of the blood vessel. This is a measure of the internal diameter, not taking into account possible differences in vessel wall thickness. A special advantage of this method is that mean diameter can be calculated as the average of measurements at more than one site in the vessel and that it is insensitive to lateral displacement.

Microcirculatory blood flow measurements are based upon flow velocity measurements. The most accurate method is the so-called dual window method, developed originally by Wayland and Johnson [41] and refined by others [see 42 for a review]. With this method, the velocity of passing erythrocytes is assessed. Circulating erythrocytes have a certain distribution over the cross-sectional area of the microvessel and travel at different velocities. Microscopic images constitute place- and time-dependent light intensity signals. Sensors placed on the intermediate image collect time-dependent signals from these spots. The passing erythrocytes produce characteristic peaks in the upstream and downstream signals of the photodiodes. By processing these signals in an appropriate way, a measure of erythrocyte velocity is obtained. Specifically, cross-correlation techniques are used to determine the average delay between two spots. This delay is inversely proportional to erythrocyte velocity. The dual-slit method relies on changes in optical density. Thus, it is essential that changes in illumination spectrum and power are minimized. In the past few years, laser Doppler systems are used increasingly for the measurement of blood cell flux in the microcirculation [43].

Microcirculatory pressure measurements are based upon micropuncture and subsequent servo-nulling techniques [44]. In this procedure, the blood pressure cannula is a micro-pipette filled with a concentrated salt solution. These micro-pipettes have external diameters between 1 and 3 μm. They are inserted into the vessels with the aid of micromanipulators. The servonulling refers to the property that the measurement system maintains constantly, the electrical resistance measured in the low-resistance pipette fluid. It maintains this constancy by generating a pressure equal to the local microvascular pressure and pushing the blood back from the pipette.

Intravital microscopy is also applied to study rheological properties of erythrocytes and leucocytes and to assess the interactions of circulating cells with the vessel wall. Cell shape, cell deformation and viscoelastic properties of these cells can be analysed from microscopic video recordings [45]. Recent attention has shifted from rheological to cell–wall interactions [46]. Leucocyte rolling and sticking are believed to be of primary importance in the inflammatory process. These phenomena can be visualized with intravital microscopy.

The final application of intravital microscopy to assess microvascular dynamics is the study of permeability characteristics. Classically, indicator-dilution techniques have greatly enhanced insight into transcapillary exchanges of small molecules. Other techniques to study exchange across the microcirculation include the osmotic transient technique and lymphatic protein flux analyses [47]. Intravital microscopic observations have been used to define the structural basis of capillary permeability as well as the dynamics of the transport processes involved. The visualization of transvascular transport requires the addition of fluorescent tracers.
CLINICAL MICROCIRCULATION TECHNOLOGY

Of similar importance to Thoma's early contribution to experimental microcirculation research is the work of O. Müller in Tübingen in the 1920s and 1930s on clinical microcirculation [48]. Müller used microscopy through the intact human skin to observe capillary structure and function. In the same period, Landis [49] was the first to measure conjunctiva capillary pressure. It was not until the 1960s and 1970s that more sophisticated techniques were developed for analysing dynamic phenomena in the human microcirculation. In particular, the introduction of fluorescence video microscopy, video densitometry, automated blood cell velocity measurement, laser Doppler and transcutaneous oxygen pressure measurements have been important technical improvements. A recent monograph edited by Bollinger and Fagrell [43] gives an excellent overview of these developments in clinical microcirculation research. Here we shall focus on two areas of application: (i) dynamic capillaroscopy and (ii) vascular network analyses.

Dynamic capillaroscopy

Capillaroscopy in humans is based on the possibility to directly observe capillaries located superficially in the body, e.g. in the skin, retina or conjunctiva. A relatively simple stereomicroscope with magnification from $\times 10-100$ is sufficient for morphological analyses of skin capillaries in the hand or foot nailfold. The bulbar conjunctiva can be observed with an eye microscope. Nailfold and conjunctiva are now the most widely used tissues for clinical capillaroscopy. Lip, gingiva and tongue are less often studied [43].

Dynamic capillaroscopy was initially performed by measuring capillary blood cell velocity with the naked eye. Important advances were made by Bollinger et al. [50] and Fagrell et al. [51] by the introduction of video photometric measurement of blood cell velocity. In the past few years, laser Doppler fluxmetry has further facilitated the capillary blood cells velocity measurement in humans. In combination with diameter measurements, this allows the calculation of capillary flows.

A further important technical advancement was the introduction of fluorescent dyes for use in humans [43]. The two most important dyes available for use in humans are sodium fluorescein and indocyanine green. Both were derived from use in ophthalmological angiography. The major advantages of using dyes are that they improve image contrast and allow the study of dynamic phenomena, such as transcapillary transport of molecules. Fluorescence video microscopy is now an essential technique for microvascular network analyses in humans and for the study of vascular permeability.

Microvascular network analysis in humans

A change in microvascular network geometry has been recognized as an important factor in the normal development of tissues as well as in the pathophysiology of certain (cardiovascular) diseases, such as hypertension, heart failure, diabetes and ischaemia. However, almost all evidence for this role is derived from histological and intravital microscopic studies in animal models. Therefore, there is a great need for clinical research to test the hypotheses derived from animal work. In the meantime, both native capillaroscopy and fluorescent angiography have been used to measure capillary densities and microvascular network geometry. Native capillaroscopy has been applied to determine capillary densities in nailfold [52, 53]. Prasad et al. [54] have recently used combined intravital capillaroscopy and fluorescein angiography to study capillary density in the forearm skin of patients.

A tissue that seems particularly suited for microvascular network analysis in patients is the retina. Qualitative analysis of retinal microvascular patterns is a diagnostic tool already used in clinical medicine for some 100 years [55]. Stanton et al. [56, 57] have recently introduced more refined quantitative methods for retinal microvascular network analyses. Their method is based on the projection of fundal photographs, allowing the determination of arteriolar and venular densities and diameters [56]. The administration of fluorescein and application of computerized morphometry allow even more detailed analyses of network properties, such as branching patterns and bifurcation angles [57].

MICROCIRCULATION IN PATHOPHYSIOLOGY

The impressive technological developments in intravital microscopy have created possibilities to assess the role of the microcirculation in various disease states. We will conclude with a brief survey of the major pathologies in which the microcirculation has been implied.

Hypertension

Two central concepts in hypertension research point to the microcirculation as an important pathogenetic site: (i) microvascular rarefaction and (ii) increased arteriolar wall-to-lumen ratio. Both concepts are based on the generally observed increased peripheral vascular resistances as the major haemodynamic cause of arterial pressure elevation in hypertension. A number of reviews [38, 58-62] extensively discuss the experimental evidence for both concepts. In brief, small arteriolar and capillary rarefaction are observed in many tissues in models of experimental hypertension. In humans, capillary rarefaction was found in conjunctiva and nailfold of young patients with borderline hypertension [53, 63]. In more established essential hyper
tension, capillary and arteriolar rarefaction were found in a broad range of tissues [38, 52, 59]. Rarefaction of small arterioles and capillaries can contribute significantly to resistance and pressure elevation in hypertension [38]. It is not yet clear whether a reversal of microvascular rarefaction leads to a chronic fall in arterial pressure.

Increased wall-to-lumen ratio in hypertension is a phenomenon usually found in resistance arterioles slightly larger than those in the microcirculation [61, 62]. However, from the point of view of resistance control, the arteriolar tree should be viewed as a continuous structure with pressure dissipation—and therefore resistance control—all along the arterial tree. Both absolute wall hypertrophy and structural narrowing with the same wall mass ('remodelling') have been implied as causes of increased wall-to-lumen ratio in arterioles from hypertensive species [61, 62].

The key question in present microvascular hypertension research is what causes rarefaction and increased wall-to-lumen ratio? To a certain degree, these structural vascular changes may represent adaptations of the vascular system to the increased arterial pressure and related wall tension in hypertension. On the other hand, both rarefaction and remodelling may be part of attenuated growth of the vascular tree in subjects predisposed by genetic or intra-uterine factors to develop hypertension [64]. Longitudinal microvascular studies should be designed to answer this question.

Heart failure

Disturbance of microvascular function is now recognized as a potential feature of heart failure. Chronic heart failure leads to a range of neuroendocrine responses, including activation of the sympathetic nervous system, the renin–angiotensin system, and elevation of endothelin, atrial natriuretic peptide and vasopressin plasma levels. These neuroendocrine mechanisms profoundly influence resting tone and contractility of various microvascular segments, although the chronic outcome of this influence is still largely unknown. Preliminary data suggest an impaired response to both contractile and dilator influences on resistance-sized arterioles [69]. On the structural side, increased capillary basement membrane thickness and arteriolar hyalinosis have been reported in human heart failure [70]. It is still too early to draw conclusions on the exact contributions of these mechanisms in the pathophysiology of heart failure, but they seem to be a central feature of the reduced skeletal muscle function in chronic stages of the disease [71].

Other diseases

The microcirculation is a target of research in the study of various arterial occlusive diseases, causing tissue ischaemia. Other, more rarely investigated diseases with a potential microcirculation involvement include chronic venous incompetence, vascular disorders related to changes in collagen metabolism, lymphoedema and sympathetic dysfunctions. Bollinger and Fagrell [43] provide an overview of clinical investigations in these areas.

CONCLUSION

The early history of microcirculation research was characterized by a mainly descriptive approach. The three-dimensional architecture of a number of vascular beds was revealed and the basic concepts of the physiology of the microcirculation were developed on that basis. This period was followed by an explosive development in technology in the second half of the 20th century. This technology created possibilities at the molecular, cellular, whole-animal and clinical level to assess microvascular structure and function. Moreover, advances in video and computer technology permitted sophisticated image analyses and simulations of complex vascular networks. In parallel, research focused more on quantitative analyses of microvascular dynamics and mechanisms of microcirculatory control. Fundamental questions on the development of microvascular networks are being investigated. The pharmacology of microvascular reactivity is gradually revealed. Finally, and most importantly, the pathophysiological role of the microcirculation in disease is being defined.
REFERENCES


