Molecular and Cellular Biochemistry **264:** 45–49, 2004.

Review

Verification of coronary angiogenesis by quantitative morphology

Karel Rakusan

Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada

Abstract

The angiogenic response may be reliably evaluated only by the methods of quantitative morphology. These methods may appear deceivingly simple but they contain several possible pitfalls. This review presents major principles of proper methodology for determination of tissue vascularization using quantitative morphology. Description of appropriate preparation of the tissue is followed by a survey of methods available for visualization of the vascular structures, by the description of principles for proper sampling and measurements and, finally, by a section on how to present and interpret the results. (Mol Cell Biochem **264**: 45–49, 2004)

Key words: arterioles, capillaries, microvascular bed, morphometry, tissue processing

Introduction

A final check of the results for any angiogenic study should be the finding of an increased or decreased vascularization of the tissue. For practical reasons, it is more convenient to study various angiogenic processes *in vitro*. This approach, however, has several important limitations. First of all, most studies are based on cell cultures of endothelial cells derived from human umbilical veins, which are easy to work with and which are also readily available. These cells, however, are far from representative. First of all, they are of fetal origin and, in addition, endothelial cells from large vessels have several characteristics different from those, observed on endothelial cells from the microvascular bed. Moreover, isolated cells obviously lack the interaction with the remaining structures in the tissue, which are so important in the final angiogenic process: blood cells, pericytes, smooth muscle cells, connective tissue, mast cells and tissue macrophages. Therefore, it is imperative to verify any finding obtained from *in vitro* studies by analyzing the effect in the real tissue.

Terminal vessels are relatively stable structures. The turnover of endothelial cells in adult mammalian hearts is probably slightly faster than in most of the other tissues. Nevertheless, the half-life of these cells is estimated to be approximately 300 days [1]. In spite of this, formation of new vessels may be surprisingly fast. As reviewed by Hudlicka and Tyler [2], in regenerating tadpole tails, capillary sprouts meet and are connected within 2–4 h, while flow is established within 10 h. During wound healing in mammals, first indication of vascular growth is noticeable 24 h after the injury, the first capillaries are formed within a few days and vascularization is completed within 6–8 days.

An increased amount of vascular components in tissue, resulting in an increased vascular surface per volume of tissue may take either of two forms: as an increased size of the vessel or as the establishment of a new channel. While in both cases the result is an increased vascular surface density, the latter is more effective at increasing oxygen supply to the tissue [3]. Thus, both the amount of new material as well as its spatial arrangement is of functional importance.

Apart from the occasional attempts to evaluate vascular supply by the determination of the tissue blood content, most studies of tissue vascularization are based on quantitative morphology. The blood content may be determined using radioactive markers of plasma and/or red blood cells in whole tissue (total vascular volume) or tissue without any visible vessels (capacity of the terminal vascular bed) [4]. Red blood cell volume may also be estimated by measuring the hemoglobin content of the tissue [5].

Address for offprints: K. Rakusan, Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5 (E-mail: krakusan@uottawa.ca)

Morphometric methods appear to be deceptively simple. As a result, many investigators try to assess the tissue vascularization without any previous experience or preparations. The results are then unreliable. This may be illustrated by an unacceptable variation of the reported results. For instance, the numerical capillary density in the left ventricle of the adult male mouse was reported to be anywhere between 250 capillaries per mm^2 [6] and 3500 capillaries per mm^2 [7], which represents 14-fold difference. The range of recently reported values of numerical capillary density in the left ventricle of the adult male rat is even wider. The vast majority of reported values in this species are between 2000 and 4000 per mm^2. Nevertheless, we can encounter reports of values as low as 400 per mm^2 [8], 440 per mm^2 [9], and 900 per mm^2 [10] but also values of 5200 per mm^2 [11] and even 18,000 per mm^2 [12]. Thus, the highest reported value is 45 times higher than the lowest reported value in the same tissue and in the same species. Similar discrepancies may be detected also for capillary length densities and for the remaining morphometric parameters. This clearly demonstrates the lack of proper controls in performing morphometric operations as well as a lack of proper expertise of journal reviewers.

In the present study, we will deal with major principles of proper methodology in determination of tissue vascularization using quantitative morphology. We will start with tissue processing, followed by a survey of methods available for visualization of the vascular structures, principles for proper sampling of measurements and, finally with a description of morphological indices of microvascular supply.

Preparation of the tissue

Morphometric data are dependent on the size of the structure from which they are derived. In dealing with the heart which is an organ continuously changing its size and shape, it is prudent to start with taking the samples from hearts arrested at a well-defined stage of the cardiac cycle, for instance in diastole, which may be induced by either the injection of KCl solution or by an overdose of pentobarbital. Subsequently, we may use either frozen or fixed sections. Frozen sections are eminently suitable for certain investigations, especially those, based on immunochemical methods. Their disadvantage, however, is a certain degree of spreading of various structures and the need to cut relatively thick sections. More common is the use of fixed tissue. Fixation by perfusion under an appropriate pressure is the first choice, especially when vessel size measurement is also planned. It is usually recommended to use the perfusion pressure corresponding to the values of the mean arterial blood pressure recorded *in vivo.* According to our own experiments, however, the capacity of the terminal vascular bed is relatively insensitive to the perfusion pressure until a certain critical value is reached which is in the range of 160–180 mm Hg (unpublished results).

For an estimation of the numerical density of various vascular structures, simple immersion of the tissue into a fixative solution yields comparable results [13]. In our experience, fixation with an isoosmolar gluteraldehyde solution in phosphate buffer is the most satisfactory method of fixation.

Visualization of the vascular structures

There is no perfect method for the visualization of vascular structures. Their selection will depend on the major purpose of the study, additional aims of the project as well as on the expertise of the investigator and on his or her laboratory facilities. Sometimes, facility and convenience for fast sampling of a large amount of morphometric data may prevail over the search for elaborate obtaining of a "perfect" visualization of the vascular bed.

Initial attempts to visualize coronary vessels were based on the injection of a suspension of dye, usually mixture of Indian ink and gelatin [14, 15]. The suspension was kept warm (40 °C) in order to decrease its viscosity. Subsequently, perfusion of dye was replaced by a silicon elastomer, which solidified within the vessels and by subsequent maceration it was possible to obtain coronary casts. Alternatively, prolonged immersion in increasingly concentrated ethanol and methyl salicylate rendered the tissue translucent and the vasculature clearly visible [16]. The latter method yielded a good three-dimensional image of the vasculature, which was suitable for studies of spatial arrangements of the coronary vessels. Quantification of coronary casts, however, was less reliable. In addition, in both approaches, an increased viscosity as well as variations in the total volume injected may influence the results.

Presently, the vast majority of morphometric studies are based on evaluation of histological sections. Traditional staining for capillaries and arterioles in samples of cardiac tissue include the alkaline phosphatase method [17], PAS method [18], staining by methylene blue [19], toluidine blue [20] or silver staining [21]. A disadvantage of the first two methods is that they are not suitable for the use in the cardiac samples obtained from animals in the early stages of postnatal development. The method of alkaline phosphatase probably does not detect all the vascular structures present [22]. A more complete image on frozen sections may be obtained by a combination of staining for alkaline phosphatase and dipeptidase IV. The former stain detects proximal portions of the capillary bed, close to the feeding arteriole while the latter stains the distal portions [23]. We have established proficiency with the silver staining method applied to 1 μm thin sections of cardiac tissue embedded in historesin, see Fig. 1. Plastic embedding is far superior for quantitative tissue analysis to the use of

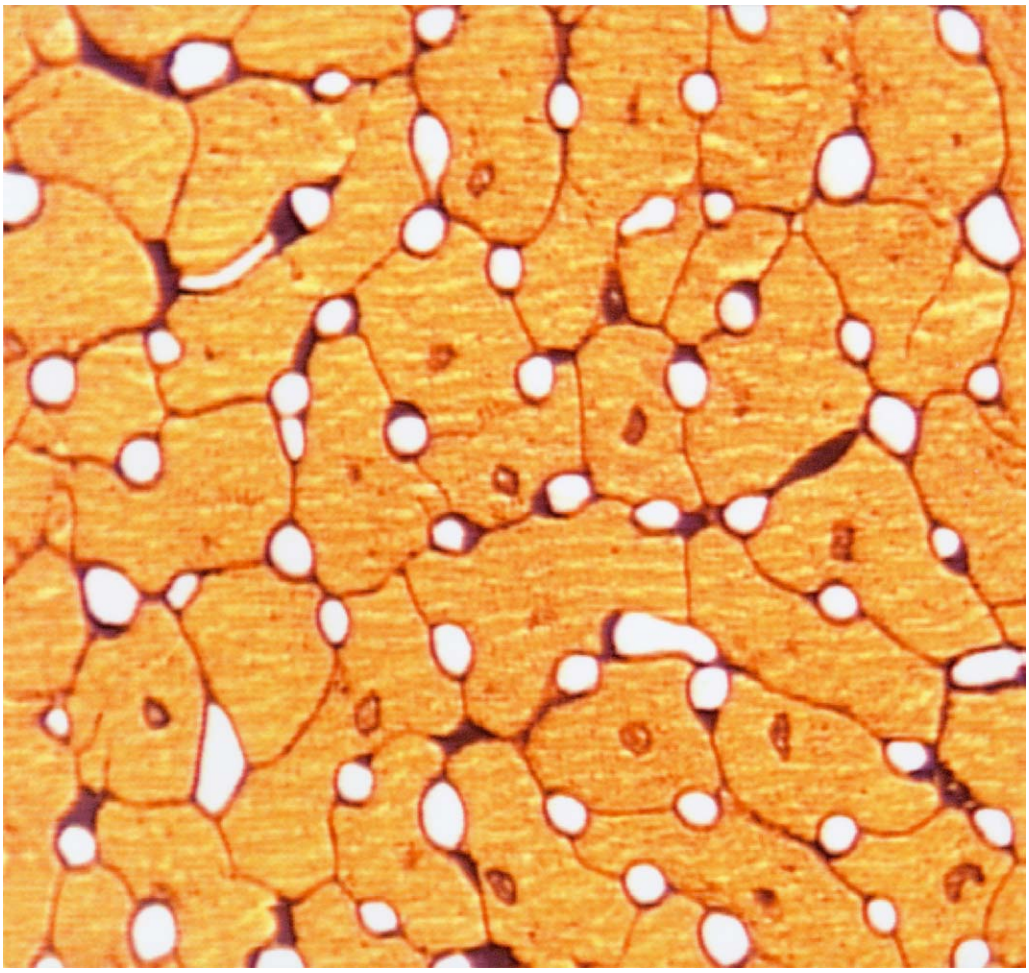

Fig. 1. Cross-section of rat myocardial tissue as used for quantitative evaluations. For methodology see [31].

paraffin embedded tissue (possibility of thinner sections, less spreading and distortion on cut sections).

Very suitable marker of endothelial cell surfaces is also GSI lectin [22]. Finally, many recent papers have described immunolabeling methods for visualization of coronary microvessels using antibodies for fibronectin and/or factor VIII (von Willebrand) [24], platelet endothelial adhesion molecule (PECAM-1) [25], anti-endothelial antibodies CD31 [9], CD34 [26] or 408 [27].

It is also possible to safely detect capillaries and arterioles in low-magnification EM-sections. The only problem in this case is difficulty in obtaining a sufficiently large tissue area for representative sampling [28].

Collecting and reporting the data

Vascularization of cardiac tissue is commonly expressed as the numerical density of capillaries and arterioles, eventually in conjunction with numerical density of myocytes which provides opportunity for calculating the so-called myocyte (fiber) per capillary ratio. For estimation of capillary density, which is simply the number of capillaries per cross-section (usually expressed per square millimeter), the sampling should be done on real cross-sections which may be judged by the actual shape of myocytes. Regions located close to epicardium, endocardium or larger vessels should be omitted. Counting should be done with correction for edge effect, i.e. using only capillary profiles which are touching two of the four sides of the quadrangular counting field ("forbidden line"). Fields should be sampled from the well-defined part of the heart. In order to avoid a subjective bias, the counting should be performed in a blind fashion. The measurement error very often stems from insufficient sampling. There is always a conflict between aim for perfect picture and a cost or effort. General recommendation is "to do more less well" [29].

In some studies, a decreased myocyte to capillary ratio is taken as a proof of capillary angiogenesis. This is true only

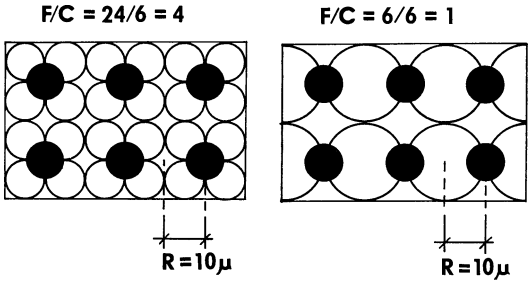

Fig. 2. Fiber (myocyte)/capillary ratio (F/C) decreases from 4 to 1 but the radius of Krogh tissue cylinder (*R*, i.e. half of the intercapillary distance) remains unchanged. Reprinted with permission from [30] courtesy of C.C. Thomas Publisher.

in situation, in which the total number of myocytes remains constant which is not the case in some experimental conditions. Moreover, a decrease in this ratio does not always mean an improved vascularization of the tissue, because it depends also on the size of myocytes. This may be demonstrated on Fig. 2, which schematically represents a situation, in which fiber (myocyte) to capillary ratio decreased from 4 to 1 but the distance between the two capillaries, which is functionally more meaningful, remains the same [30]. This is not necessarily a hypothetical situation: in mammalian hearts during early postnatal development we encounter a ratio of 4 to 1, while in adult hearts this ratio approaches the values of 1 to 1.

An ideal estimate for the formation of new vessels should be based on calculation of total length of capillaries and/or arterioles per unit of tissue volume or per organ. Such a calculation is possible from calculation of vascular length density (in mm/mm^3) which takes into an account a degree of anisotropy [31]. Close to ideal cross-sections are achieved by turning the blocks of tissue until the vast majority of myocytes attains circular shape. For a more accurate estimation of vascular length density it is necessary to obtain the degree of anisotropy. This may be achieved by various approaches. Mathieu and coworkers [31] proposed a relatively simple method based on obtaining the ratio of mean capillary profile counts originating from transverse and longitudinal sections of the muscle tissue and calculating the degree of anisotropy using a table or graph given in their paper. Even more accurate but also more exacting is a method of orthogonal triplet probes, which requires a device for generating sections with isotropic uniform random operation. This method was described by Mattfeldt and coworkers [32]. Finally, it is also possible to obtain a relatively accurate estimate of length density by determining first the angle at which the vessel is oriented within the plane of the section. This may be derived from the ratio of the long to short axis of the external diameter [33, 34]. This method is especially suitable for determination of arteriolar length density. Fortunately, orientation of the capillaries within the myocardial tissue is close to a perfect alignment along a preferred axis, i.e. anisotropy. The larger the deviation from the ideal orientation in space, the more capillary material is present in the tissue sample above the values estimated from the numerical capillary density on the tissue cross-sections. Correction factor for the normal rat hearts is in the range of 1.06 to 1.09 [35].

More advanced stereological methods are also available for determination of vascular surface density (mm^2/mm^3) and volume density (mm^3/mm^3). These indices, however, are very dependent on the vascular diameter which is rather variable *in vivo* and influenced by tissue processing as well.

Conclusion

The major purpose of this brief review was to emphasize a surprising lack of proper standards in evaluating the angiogenesis by quantitative morphology. Possible reasons for the unacceptable variability of the reported results of the reported data on cardiac vascularization may be found in the lack of attention to methodology. Our review summarizes major methodological approaches reported in the current literature in terms of preparation of tissue, visualization of the vessels and proper sampling and reporting the results.

References

1. Hudlicka O: What makes blood vessels grow? J Physiol (London) 444: 1–24, 1991
2. Hudlicka O, Tyler KR: Angiogenesis. Academic Press, London, 1986, pp. 1–5
3. Rakusan K, Turek Z: A new look into the microscope: Proliferation and regression of myocardial capillaries. Can J Cardiol 2: 94–97, 1986
4. Rakusan K, Rajhathy J: Distribution of cardiac output and organ blood content in anemic and polycythemic rats. Can J Physiol Pharmacol 50: 703–710, 1972
5. Becker LE: Myocardial capillaries in rats with Co induced polycythemia. Am J Physiol 197: 981–989, 1959
6. Yang Y, Min J-Y, Rana JS, Ke Q, Cai J, Chen Y, Morgan JP, Xiao Y-F: VEGF enhances functional improvement of postinfarcted hearts by transplantation of ESC-differentiated cells. J Appl Physiol 93: 1140–1151, 2002
7. Rakusan K, Nagai J: Morphometry of arterioles and capillaries in the hearts of senescent mice. Cardiovasc Res 28: 969–972, 1994
8. Shyu K-G, Wang M-T, Wang B-W, Chang C-C, Leu J-G, Kuan P, Chang H: Intramyocardial injection of naked DNA encoding HIF-1α/VP16 hybrid to enhance angiogenesis in an acute myocardial infarction model in the rat. Cardiovasc Res 54: 576–583, 2002
9. Cosmas AC, Kernan K, Buck E, Fernhall B, Manfredi TG: Exercise and dietary cholesterol alter myocardial ultrastructure. Eur J Appl Physiol 75: 62–67, 1997
10. Liu Y-H, Yang X-P, Sharov VG, Nass O, Sabbah HN, Peterson E, Carretero OA: Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. J Clin Invest 99: 1926–1935, 1997
11. Anversa P, Li P, Sonnenblick EH, Olivetti G: Effects of aging on quantitative structural properties of coronary vasculature and microvasculature in rats. Am J Physiol (Heart Circ Physiol) 267: H1062–H1073, 1994

12. Odek-Ogunde M: Myocardial capillary density in hypertensive rats. Lab Invest 46: 54–60, 1982
13. Rakusan K, Flanagan MF, Geva T, Southern J, Van Praagh R: Morphometry of human coronary capillaries during normal growth and the effect of age in the left ventricular pressure-overload hypertrophy. Circulation 86: 38–46, 1992
14. Robert JT, Wearn JT: Quantitative changes in the capillary-muscle fiber relationship in human hearts during normal growth and hypertrophy. Am Heart J 21: 617–633, 1941
15. Rakusan K, Poupa O: Changes in the diffusion distance in the rat heart during development. Physiol Bohemoslov 12: 220–227, 1963
16. Bassingthwaighte JB, Yipintsoi T, Harvey RB: Microvasculature of the dog left ventricular myocardium. Microvasc Res 7: 229–249, 1974
17. Brown MD, Hudlicka O: Protective effects of long-term bradycardial pacing against catecholamine-induced myocardial damage in rabbit hearts. Circ Res 62: 965–974, 1988
18. Rakusan K, Turek Z, Kreuzer F: Myocardial capillaries in guinea pigs native to high altitude (Junin, Peru, 4105). Pflugers Arch 391: 21–24
19. Mall G, Mattfeldt T, Mobius H-J, Leonhard R: Stereological study on the rat heart in chronic alimentary thiamine deficiency—absence of myocardial changes despite of starvation. J Mol Cell Cardiol 18: 635–643, 1986
20. Capasso JM, Puntillo E, Halpryn B, Olivetti G, Li P, Anversa P: Amelioration of effects of hypertension and diabetes on myocardium by cardiac glycoside. Am J Physiol (Heart Circ Physiol) 262: 734–742, 1992
21. Rakusan K, Cicutti N, Kazda S, Turek Z: Effect of nifedipine on coronary capillary geometry in normotensive and hypertensive rats. Hypertension 24: 205–211, 1994
22. Hansen-Smith FM, Watson L, Lu DY, Goldstein I: *Griffonia simplicifolia* I: Fluorescent tracer for microcirculatory vessels in nonperfused thin muscles and sectioned muscle. Microvasc Res 36: 199–215, 1988
23. Batra A, Rakusan K: Geometry of capillary networks in volume overloaded rat heart. Microvasc Res 41: 39–50, 1991
24. Sabri A, Samuel J-L, Marotte F, Poitevin P, Rappaport L, Levy BI: Microvasculature in angiotensin II—dependent cardiac hypertrophy in the rat. Hypertension 32: 371–375, 1998
25. Rongish BJ, Hinchman G, Doty MK, Baldwin HS, Tomanek RJ: Relationship of the extracellular matrix to coronary neovascularization during development. J Mol Cell Cardiol 28: 2203–2215, 1996
26. Krams R, Kofflard MJM, Duncker DJ, Von Birgelen C, Carlier S, Kliffen M, ten Cate FJ, Serruys PW: Decreased coronary flow reserve in hypertrophic cardiomyopathy is related to remodeling of the coronary microcirculation. Circulation 97: 230–233, 1998
27. Osthoek PW, Moorman AFM, Sauer U, Gittenberger-de Groot AC: Capillary distribution in the ventricles of hearts with pulmonary atresia and intact ventricular septum. Circulation 91: 1790–1798, 1995
28. Anversa P, Loud AV, Giacomelli F, Wiener J: Absolute morphometric study of myocardial hypertrophy in experimental hypertension. II. Ultrastructure of myocytes and interstitium. Lab Invest 38: 597–609, 1978.
29. Gundersen HJG, Osterby R: Optimizing sampling efficiency of stereological studies in biology: Or "Do more less well". J Microsc 121: 65–73, 1981
30. Rakusan K: Oxygen in the Heart Muscle. C.C.Thomas, Springfield IL, 1971, p. 28
31. Mathieu O, Cruz-Orive L-M, Hoppeler H, Weibel ER: Estimating length density and quantifying anisotropy in skeletal muscle capillaries. J Microsc 131: 131–146, 1983
32. Mattfeldt T, Mobius H-J, Mall G: Orthogonal triplet probes: An efficient method for unbiased estimation of length and surface of objects with unknown orientation in space. J Microsc 139: 279–289, 1985
33. Anversa P, Capasso JM: Loss of intermediate-sized coronary arteries and capillary proliferation after left ventricular failure in rats. Am J Physiol (Heart Circ Physiol) 260: H1552–H1560, 1991
34. Chen Y, Torry RJ, Baumbach GL, Tomanek RJ: Proportional arteriolar growth accompanies cardiac hypertrophy induced by volume overload. Am J Physiol (Heart Circ Physiol) 267: H2132–H2137, 1994.
35. Rakusan K, Wicker P, Abdul-Samad M, Tarazi RC, Healy B: Failure of swimming exercise to improve the capillarization in cardiac hypertrophy of renal hypertensive rats. Circulation Res 61: 641–648, 1987