Multiscale analysis of coronary branching and collateral connectivity: coupling vascular structure and perfusion in 3D

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Chapter 4

Organization and collateralization of a subendocardial plexus in end-stage human heart failure

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Abstract

In the failing myocardium a subendocardial plexus can develop. Detection of the presence or function, however, of such a plexus does not form part of the present diagnostic spectrum for heart failure. This may now change as new methods for high-resolution imaging of myocardial perfusion distribution are being developed.

A severely hypertrophic heart was harvested during transplantation and analyzed for morphology of the intramural coronary arterial vasculature. The heart only had one coronary ostium, and the main branches of the coronary artery were cannulated. A fluorescent casting material was infused that was allowed to harden under physiological pressure. The entire heart was frozen and placed in a novel imaging cryomicrotome and sequentially cut in 25 \( \mu \text{m} \) slices. High-resolution images of each cutting plane were acquired, allowing a detailed three-dimensional reconstruction of the arterial vasculature.

The epicardial layer of the free wall demonstrated a normal vasculature with penetrating branching arteries. The endocardial layer and the septum revealed a highly interconnected vascular plexus with large vessels oriented parallel to the apicobasal axis. An extensive endocardial network with collaterals was detected, forming connections between the main epicardial branches. We conclude that an outward remodeling of transmural vessels did not prevent the generation and growth of subendocardial conduit arteries.

The orientation and vascular volume in the plexus provides an opportunity for detection by novel techniques of MRI contrast imaging currently developed. Knowledge of the effect on perfusion studies is required to prevent a misinterpretation of subendocardial perfusion images in heart failure.
Introduction

In the normal condition the organization of the coronary circulation is such that the large conduit arteries run across the epicardium and branch almost perpendicular into the ventricular wall. Subsequently, these transmural arteries branch repetitively into smaller branches until connecting to the capillary network. In normal cases the end branches of these arterioles are not strongly interconnected [1]. However, in some postmortem failing human hearts with long-lasting remodeling, a subendocardial plexus has been previously demonstrated as containing extensive interconnections between the end branches of the transmural vessels [2, 3]. However, these earlier studies do not report detailed structural information, probably since the visualization techniques applied were not well suited for such quantitative analysis [3, 4].

Recently, we developed an imaging cryomicrotome [5] that, for this study, was used to analyze the coronary circulation of an explanted human heart. The heart was diagnosed as suffering from hypertrophic cardiomyopathy. Before the heart was processed in the cryomicrotome, the coronary arteries were filled with a fluorescent polymer replica material. After each microtome cut, the images were recorded with proper optical filter settings such that a stack of high-resolution fluorescence images provides information on the three-dimensional (3-D) structure of the epicardial and intramural coronary arteries. In this way a well-developed subendocardial plexus was found that was not diagnosed before explantation.

Interestingly, current diagnostic procedures for heart failure do not include a test for the presence of a subendocardial plexus. This may be explained by the absence of a proper in vivo diagnostic technique. Consequently, the lack of clinical data results in a poor understanding of the role of the vascular plexus in the development of heart failure. A rapid development of especially MRI techniques for perfusion and/or blood volume distribution may offer future opportunities for an in vivo diagnosis of abnormal subendocardial vascularization [6].

The aim of the present study was to provide a more quantitative description of the subendocardial plexus in terms of vascular orientation and interconnectivity between the arteries of the plexus. Knowledge on these properties may reveal the mechanisms driving this pathophysiological remodeling and stimulate the development of techniques for in vivo diagnosis.

Materials and methods

Heart preparation

A 36-yr-old woman suffering from severe hypertrophic cardiomyopathy (New York Heart Association class IV), ejection fraction of 64% at rest, septal wall thickness of 15 mm estimated by echocardiography, and previous arrhythmias leading to ventricular fibrillation with total atrioventricular block presented at our hospital. The patient had a history of severe exercise intolerance with blood pressure drop and diastolic heart failure, necessitating
transplantation surgery. No signs of coronary disease were found. The patient received metoprolol tartrate medication until elective cardiac transplantation surgery at the University Medical Center at Utrecht, after which the heart was harvested. In compliance with local medical ethics guidelines and the declaration of Helsinki, informed consent was obtained for detailed investigation of the myocardium after explantation.

The heart weighted 613 g and had a coronary anomaly with a single right coronary ostium giving rise to the right coronary artery, left anterior descending artery (LAD), and left coronary artery (LCX). The LAD was positioned anterior and curved around the right ventricle, whereas the LCX was located at the posterior side of the heart and coursed in an almost straight line toward the apex. The LAD and LCX were cannulated separately and perfused with Tyrode solution as described earlier [7]. Subsequently, the heart was flushed with saline buffer containing $10^{-5}$ M adenosine at ~80 mmHg until the efflux remained clear of blood. Two batches of vascular casting replica material (Batson No. 17, Polysciences) containing either yellow or red fluorescent dye (Potomac yellow and Radiant red, Radiant Colour) were prepared [5]. Following perfusion, first the LAD and then the LCX were filled at 80 mmHg with yellow and red replica material, respectively, which subsequently was allowed to polymerize at ambient temperature. Equal pressure between the two cannulated arteries was maintained, thereby preventing large collateral flow between them. After polymerization, the entire heart was embedded in gel containing 5% carboxymethylcellulose sodium solution (Alfa Aesar, Karlsruhe, Germany) and 1% Indian ink (Royal Talens, Apeldoorn, The Netherlands) and was frozen for 48 h at -20°C. Subsequently, the frozen heart was placed in the imaging cryomicrotome and was serially cut from base to apex with a slice thickness of 25 µm. After each cut, the remaining bulk tissue was photographed with a 2,000x2,000 Kodak charged-coupled device (CCD) camera (Kodak Megaplus 4.2i), fitted with a Nikon 70–180-mm lens at the appropriate filter setting for yellow fluorescent (excitation 440/20 nm, and emission 505/30 nm) and red fluorescent (excitation 505/30 nm, and emission 635/30 nm; all filters by Chroma Technology), as well as with normal illumination suitable for outline imaging. Lens settings were adjusted to an in-plane resolution of 40x40 µm per pixel for each of the three imaging channels. The raw unprocessed image dataset was 30 Gb in lossless compressed png format.

**Image processing and analysis**

A maximal intensity projection (MIP) is formed by the maximal intensity of the images in a set of images at corresponding coordinates. To facilitate the image processing, the stack of 5,692 images was reduced to 2,846 by combining two sequential images into an MIP for each fluorescent color channel, thus increasing the voxel size to 40x40x50 µm$^3$. The resulting images were tested for bright pixels on the CCD camera chip, which may develop as a consequence of long-exposure times or heat development in the CCD camera. These unresponsive pixels were removed using an in-plane face neighbor averaging filter at these locations only. About 1% of images could not be used for analysis because of small-tissue remnants attaching to the cutting surface. These images were replaced with an MIP of the preceding and following image.
Removal of the glow of structure below the image surface was performed by image deconvolution with the estimated point spread function of the imaging cryomicrotome [8]. For this, a macro was used enabling piecewise the iterative deconvolve 3-D plugin of ImageJ (ImageJ Software, National Institutes of Health, Bethesda, MD).

Vascular tree geometry was obtained through the application of a topology-preserving peeling algorithm [9] after thresholding at 125 of the 255 grayscale level. This yielded ~9.6 million nodal points after peeling. By the enforcement of continuity between pixels and the requirement of a connection to a large artery in the basal plane, the tree size could be reduced to ~4.3 million nodal points. Pixels belonging to small spurious unconnected branches, as well as nodal points associated with the atria and autofluorescence of the aortic wall, were removed. The resulting tree was analyzed by our algorithm that was developed to seek vascular pathways via neighboring central nodal points, connecting the epicardial conduit arteries. Collateral paths were subsequently stored and numbered. Hence, the use of different fluorescent colors is no prerequisite for collateral identification, but vessels containing both colors certainly identify such a path. Vascular paths were plotted and verified against the original data by J. van den Wijngaard and P. van Horssen.

Image analysis and processing were performed on a high-end personal computer with an Intel quadcore Q6600 or Intel dualcore T8300 processor with the Microsoft Windows XP operating system and typically took several hours for image restoration, 120 h for image deconvolution, and 24 h for nodal peeling and analysis of nodal connectivity. All visualization of coronary collateral paths in three dimensions was performed in Amira (Amira 3.1, Visage Imaging, Fuerth, Germany). Because of imaging limitations, a 3-D reconstruction of the complete vasculature was downsampled twofold for viewing purposes only.

**Results**

Anterior and posterior views of the virtual cast are depicted in Fig. 1, A–C. The LAD (green) ran anterior and mainly perfused the septum of the heart but also curved around part of the right ventricular wall (Fig. 1A). The LCX (red) perfused the posterior portion of the heart with a small portion of the septum and left ventricular free wall. This anatomical configuration, however rare, is reported as one of the types that may be found in single coronary artery anomalies [10]. Fig. 1B clearly demonstrates penetrating arteries branching from the LCX, which then connect to a vascular system with mainly apicobasal- axis orientation.
Fig. 1: Reconstructed images of the coronary vascular bed from different projections. A: anterior heart view with perfusion of the left ventricular free wall (green) and descending anterior left coronary artery (LCX; red) partly visible. LAD, left anterior descending coronary artery. B: view with septal vasculature visible centrally. C: posterior heart view with LCX extending toward the apex. Mixing of fluorescent dyes is clearly noticeable especially in A.

Fig. 2: Block of myocardial vasculature showing lower part of the LCX vasculature corresponding to the visible vascular structure as in Fig. 1B. The red dye is changed into yellow for clarity reasons. Transmural arteries penetrate the myocardial wall perpendicular to the ventricle wall surface until joining with the vascular plexus, which contains large arteries (1–3 mm diameter) oriented along the apicobasal heart axis (indicated by white arrows). The asterisk in the top right corner corresponds to the level of Fig. 3.

Fig. 2 demonstrates an enlargement of the bottom half of the LCX vasculature, and only the dye injected into the LCX is shown in the color yellow for clarity reasons. Clearly, the epicardial course of the LCX is demonstrated with the penetrating arteries branching into the ventricular wall at an almost perpendicular angle. These penetrating branches then connect with the subendocardial apicobasal-oriented vascular plexus where several large conduit arteries have been indicated by white arrows (diameter of conduit arteries, 1–3 mm).
Fig. 3: Combined image of reconstructed vasculature from a selected LCX perfusion region and outline images illustrating myocardial fiber orientation. The vessels penetrate the planes perpendicular in the subepicardium but are oriented parallel to the fibers in the plane at the subendocardium.

Fig. 3 depicts the orientation of both the penetrating and plexus arteries with respect to the fiber orientation as can be derived from the texture in epi-illumination images shown in the three parallel planes sagittal to the apicobasal heart axis. In the epicardial layer, the penetrating vessels are more or less perpendicular to the epicardium and run in a radial direction. However, in the subendocardium, the vessels run in the plane parallel to the apicobasal axis of the heart.

The interconnectivity of the two perfusion regions at their boundary is demonstrated in more detail in Fig. 4. Fig. 4, left, demonstrates the mixing of the two colors down to the level of single vessels. Fig. 4, right, has the same orientation but with decreased vessel opacity. Note that the boundary between the two colors is not necessarily the boundary between the perfusion areas. This is well illustrated by the one penetrating vessel coming from the LCX but colored red by plastic coming from the LAD.

By our using conservative settings for image processing, 113 distinct collateral connections were found between the epicardial conduit arteries, about 75% of them within the subendocardial vascular plexus. For clarity reasons, not all of the identified pathways are shown and, hence, a representative subset of 30 of these collaterals are shown in Fig. 4, right, each represented by a different color. The tortuous courses of these collateral pathways can be noted. From the online movie (note: supplemental movie may be found posted with the online version of this article), a clear impression of the 3-D nature of the vasculature can be appreciated.
Fig. 4: Left: reconstructed block of myocardial vasculature with 30 collateral paths identified at the level of the vascular plexus and shown in different colors. Right: with reduced opacity. Please note that line thickness of collateral paths does not represent actual collateral diameter.

Discussion

The 3-D intramyocardial vasculature of an explanted human heart with hypertrophic cardiomyopathy and severe end-stage heart failure was reconstructed at high-spatial resolution using a novel imaging cryomicrotome and analyzed employing dedicated software. We found a dense parallel network of conduit vessels with multiple interconnecting branches extending over the left ventricular subendocardium. The preferential direction of the intramyocardial vasculature ranged from perpendicular to the fiber sheets at the epicardial layer to parallel with respect to the apicobasal axis at the subendocardial layer. A large number of collateral pathways connecting the major branches of the epicardial arteries were identified.

Mechanisms in vascular plexus formation

The heart is a complex organ whose function critically depends on the concerted action of mechanical and electrical events that are made possible by proper tissue perfusion, which in turn depends on the mechanical events. Clearly, heart failure may develop starting from any malfunction in this chain of events. The anomalous origin of the coronary arteries in the heart presented here may have induced the abnormal vascular architecture we found at the microvascular level. However, the major coronary arteries were normally formed without evidence of atherosclerosis, and collaterals were only observed at the subendocardium, which argues against this theory. Furthermore, the single- coronary artery condition is not associated with hypertrophic cardiomyopathy per se, but a link to sudden cardiac death has been reported [10]. Therefore, it is more likely that chronic cardiomyopathy in the case described here has caused the adaptive formation of the collateral circulation at the subendocardium.
Subendocardial plexus in end-stage human heart failure

Prolonged episodes of ischemia in the heart studied may have led to hypervascularization and the development of a vascular plexus. Although the presence of collateral arteries in patients with assumed normal arteries was suggested previously from casting [1], angiography [3], or measurements of coronary wedge pressure after balloon occlusion [11], the presence of a vascular plexus has been described only in a few cases previously [2, 3]. In the case of wedge pressure measurement, it should be noted, however, that because of the nonlinear relationship between the coronary pressure and flow, high-zero flow pressures may be found, suggesting collateral flow, but actually represents extravascular compression of intramural vessels [12]. In the present heart, vascular development in the epicardial layer appeared normal, with penetrating vessels >1mm in diameter, exhibiting a dominant radial direction. In contrast, the main direction of similar-sized vessels in the subendocardial layer was parallel to the apicobasal heart axis. One would expect that with adaptive outward remodeling, these penetrating radial vessels should be able to supply the subendocardium with sufficient blood flow. From the present reconstruction, however, we cannot identify marked outward remodeling of these vessels. The basoapical orientation of conduit size vessels in the subendocardium suggests a lower resistance to flow in that direction than in the transmural arteries. Given the diameter and course of these vessels (see Figs. 1 and 2), we identify these vessels as conduit vessels.

Given the presence of only one coronary artery and the absence of signs of coronary artery disease, the pressure distribution across the epicardium likely was without major pressure differences. When we assume a normal arterial perfusion, this implies that shear stress between the plexus arteries, albeit with normal physiological variation [13], was low and was remodeling a shear independent process. This is further supported by a computer model study suggesting that when shear stress is the dominant factor for remodeling, parallel pathways are not to be expected [14]. It should be noted, however, that apart from a coronary stenosis [15], the transmural blood flow may well be impeded by changes in shear stress between tissue layers [16-18]. Shear forces and shearing angles between fiber sheets in the contracting myocardium may be strongly altered in hypertrophy and heart failure [19].

Methodological considerations

No fluorescent dye was infused into the right coronary artery, which, however, filled retrograde from the LCX. The vascular reconstructions show that the vessels in the anterior part of the left ventricular free wall were only partly filled because of scar formation and wall thinning.

Collateral connections in the subendocardial plexus were evidenced by two independent methods: 1) the admixture of both fluorescent dyes and 2) the contiguity of pathways between larger epicardial branches. The second method resulted in a quantification of the number of collateral pathways. We counted 113 collaterals but used conservative parameter settings in our algorithm to avoid an overestimation of the number of collateral pathways. Therefore, the real number of collateral pathways will be higher since especially thinner collaterals may have been missed. No collaterals were found in the subepicardium, underlining the preferential formation of a continuous subendocardial plexus, with connections to different epicardial arterial branches.
Previous visualization methods were limited since only two-dimensional images of the subendocardial plexus at a much lower resolution could be obtained [2, 3]. Some casting methods require tissue clearing, resulting in limited visibility of some of the deeper laying intramural vessels [20]. Other methods require a cutting of the ventricular wall causing a distortion of the ventricular wall and embedded vasculature. The present method of visualization not only significantly improves the spatial resolution of the intramyocardial vessels and preserves the 3-D geometry of the heart but also allows detailed quantitative observations at selected locations from selected angles within the entire vascular bed.

An alternative approach closest to ours is the ultramille system of Gerneke et al. [21] and its modifications [22, 23]. This method, however, required time-consuming staining of the cutting plane and does not image the entire heart. However, fiber direction could also be measured and the relative orientation of the vessels within the tissue sheets could be detected. The fiber orientation visible in our outline images (see Fig. 3) may be improved by a more optimal choice of illumination.

Although the fluorescent replica material was previously shown to penetrate to vessels of ~15 µm [5], replica material vessel kinking, vessel embolization, or minute air bubbles in some cases might prevent a full penetration of the entire vasculature, thereby reducing the quality of the vascular tree reconstruction.

**Clinical implications**

A better definition of the subendocardial plexus anatomy may be important for the interpretation of perfusion studies in cardiac imaging. The increased subendocardial vascular volume and directional orientation of the plexus vessel segments determine the distribution of contrast agents both in standard angiography and in other modalities such as MRI or ultrasound contrast imaging. Hence, these modalities provide the opportunity to detect the effect of a subendocardial plexus on perfusion measurements. An absence of awareness of the effect of such a plexus on subendocardial perfusion imaging may result in misdiagnosis of subendocardial ischemia.

Changes in fiber orientation may affect the electrical propagation and contraction patterns of the heart [24, 25]. A 3-D representation of the coronary arterial tree combined with models predicting geometrical changes as well as intramural compressive forces related to heart contraction may significantly advance our understanding and interpretation of these diagnostic techniques. The relationship between the vascular structure and the myocardial fiber direction in the present study underlines the importance of the contraction pattern of the myocardium. Contraction abnormalities importantly contribute to the resistance of the transport of blood from the epicardial vessels toward the subendocardium and papillary muscles.

**Conclusion**

Upon the development of hypertrophic cardiomyopathy to a severe form of heart failure in the described case, the myocardial vasculature in the inner half of the ventricular wall
achieved distinct different characteristics compared with the outer layer and a subendocardial plexus developed. Within the plexus, major vessels were found running in the long-axis direction, whereas the course of the epicardial vascular arrangement remained unaltered. Moreover, the number of collateral connections between perfusion territories became abundant and was concentrated in the subendocardium.

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