Three-Dimensional Microstructural Abnormality of the Coronary Capillary Network After Myocardial Reperfusion — Comparison Between ‘Reflow’ and ‘No-Reflow’ —

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Background The 3-dimensional (D) structural abnormalities of the coronary capillary network and capillary volume changes after myocardial reperfusion were investigated using confocal laser scanning microscopy.

Methods and Results Using open-chest anesthetized rats' hearts, the left anterior descending artery (LAD) was occluded for 7 min followed by reperfusion. The hearts were divided into 2 groups: (1) reperfused area stained well by intravenous indocyanine green after reperfusion (Reflow), and (2) lack of staining in the reperfused area (No-reflow). The entire coronary microvasculature was filled with contrast medium using a Langendorff's perfusion system. Capillaries were observed 3-dimensionally in the control and reperfused areas in both the Reflow and No-reflow group and the capillary volume fraction was computed from the 3-D images. The reperfused area in both groups showed decreased capillary diameter with waving and shrinkage configuration. In the No-reflow group, marked interruption of capillary network was observed. In the Reflow group the capillary volume fraction was significantly reduced in the reperfused area compared with the control area ($14.8 \pm 4.1\%$ vs $22.2 \pm 5.6\%$, $p < 0.05$). Capillary volume fraction was further decreased in the No-reflow group compared with Reflow ($5.3 \pm 1.4\%$ vs $14.8 \pm 4.1\%$, $p < 0.01$).

Conclusion The morphological changes in coronary capillaries after reperfusion were characterized by waving and shrinkage, which resulted in a reduction in capillary volume. (Circ J 2004; 68: 868–872)

Key Words: Capillaries; Ischemia; Reperfusion

Previous clinical or experimental studies have suggested that the intramyocardial coronary microvascular network may be damaged during the no-reflow phenomenon after myocardial reperfusion.^{1–6} Although the structural changes in coronary capillaries after myocardial ischemia have been investigated by 2-dimensional (D) observation^{7–10} (optical and electron microscopic studies), precise assessment of the 3-D structural changes using the 2-D image techniques has not been done because the construction of most of the capillary network is enormously complex. Our newly developed unique contrast medium, which we have already reported, allowed us to investigate the 3-D microvascular structure using confocal laser scanning microscopy,^{11,12} and in the present study we intended to investigate 3-dimensionally the structural abnormalities of the coronary capillary network and changes in capillary volume fraction after myocardial reperfusion using the same technique.

Methods

Sample Preparation

Experimental procedures and protocols were conducted according to the institutional guidelines approved by the Animal Research Committee of Kawasaki Medical School. Adult male Wistar rats ($n=23$, 280–320 g) were deeply anesthetized by diethyl ether inhalation followed by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Additional doses were given as needed throughout the experiment. After endotracheal intubation, the rats were ventilated by a respirator pump (VS600®, Instrumental Development Corp). Arterial pH and blood gases were measured and kept in the physiologic range by adjusting the inspired oxygen concentration and minute ventilation. An arterial pressure catheter was inserted into the left femoral artery to monitor the aortic pressure. All rats were anticoagulated with heparin (500 U iv). After median sternotomy, the heart was exposed and the midportion of the left anterior descending artery (LAD) was occluded for 7 min with a 7-0 silk suture, followed by 3 min of reperfusion. A perfusion defect in the LAD region was confirmed by intravenous injection of indocyanine green (ICG) after the occlusion, and after reperfusion, ICG was injected again to divide the hearts into 2 groups (Reflow group: good perfusion of ICG in the reperfused area; No-reflow group: lack of ICG perfusion in the reperfused area). All the ICG staining was recorded on digital videocassette

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Fig 1. Langendorff perfusion system (see text for details).

for off-line judgment by 2 investigators.

Complete filling of the coronary microvasculature was accomplished as follows. The heart was immediately isolated and connected to the Langendorff perfusion system (Fig 1). A polyethylene tube (15G) was inserted into the left ventricle (LV) via the mitral valve to remove the LV load. Adenosine (1 mg/min) was administered throughout each experiment, and the perfusion pressure and mean coronary flow rate were continuously monitored (CAMINO, V420-9 Laboratories Inc and MFV 3200, Nihon Khoden Inc, respectively). The heart was arrested in diastole through retrograde perfusion of oxygenated St Thomas Cardioplegic solution (25°C). Our specially prepared contrast medium (42°C)^{11,12} was administered by retrograde injection via the ascending aorta, from the nearest port of the circuit. The perfusion pressure was initially set at 120 mmHg for 10 s and then lowered to 85 mmHg throughout the remainder of the perfusion period (3 min). The whole heart was quickly immersed in a cold saline-circulation bath (0°C) for 3 min, so that the contrast medium solidified under the perfusion pressure. The time required to complete these procedures was less than 10 min. Finally, the heart was removed and immersed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde (4°C) overnight.

The fixed heart was cut along the long axis through the reperfused region, and the achievement of complete filling of the microvasculature by the contrast medium and the micro-perfusion pattern were examined using a stereoscopic microscope. For the confocal laser scanning microscopic study, the sample was embedded in 12% gelatin block, fixed once again overnight and then sliced into 200 µm thick samples using a vibrating microtome (VT 1000S, Leica Inc).

Confocal Laser Scanning Microscopic Observation

Capillaries were observed 3-dimensionally using confocal laser scanning microscopy (TCS-NT, Leica Inc, spatial resolution <1 µm). Observation was performed in control and reperfused areas in both the Reflow and No-reflow group. The microvasculature was clearly visualized by laser reflection mode as positive signals from the contrast

medium. Microscopic images were obtained at ×40 magnification and scanned for 50–60 µm in thickness divided by 1 µm for each slice.

Three-Dimensional Visualization and Capillary Volumetric Analysis

The confocal laser scanning microscopy images were automatically rendered (TCS-NT, Leica Inc) and displayed as 3-D images (Paint Shop Pro 4.12). For the capillary volumetric analysis, each slice image was digitized and quantified to either zero or one after they were converted into NIH stack files (NIH image 1.62). The region of interest (ROI) was manually focused on the capillary, excluding larger pre- and post-capillary microvessels, and confirmed by a stack files-scan. The percent vascular area out of the ROI was analyzed for each slice. By scanning this process along the depth direction, the capillary volume fraction (%) was analyzed as the voxel size ratio of the capillaries out of the total mass (MATLAB 5.2); (capillary volume fraction = capillary vol./[myocardial vol. + capillary vol.]).

Statistical Analysis

All data are expressed as mean ± SD. Comparison of the capillary volume fraction among controls, Reflow and No-reflow groups was performed by one-way ANOVA followed by Fisher's pairwise least-significant difference test for multiple comparisons using Stat View 5.0 PPC. The statistical criterion was defined as $p < 0.05$. To determine interobserver variability for the judgment of 'Reflow' and 'No-reflow', each ICG staining was also judged separately by the investigators who had participated in the consensus judgment. The interval between the consensus judgment and the reading to determine interobserver variability was >4 months. One investigator then repeated the judgment to determine intraobserver variability. The interval between the 2 judgments was 3 weeks. Kappa statistic¹³ defined as $\text{Kappa} = (\text{observer agreement} - \text{chance agreement}) / (1 - \text{chance agreement})$, was used to assess intraobserver and interobserver variability for the judgment of 'Reflow' and 'No-reflow'.

Fig2. Stereoscopic microscopic image of the No-reflow sample. Lack of capillary perfusion is seen in the reperfused area (arrow: ligation point).

Fig3. Confocal laser scanning microscopic images in the control region.

Fig4. Confocal laser scanning microscopic images of the reperfused region. Sample from (A) Reflow group, (B) No-reflow group.

Results

The Reflow group comprised 11 (48%) of 23 rats and 12 (52%) comprised the No-reflow group. By stereoscopic microscopy there was no capillary perfusion in the reperfused area in the No-reflow group (Fig2). The coronary microvasculature was observed 3-dimensionally (Fig3) by confocal laser scanning microscopy. Compared with the control region, the reperfused area in both groups showed decreased capillary diameter and density, with waving and shrinkage (Fig4AB). Despite these abnormal changes, the capillary network was apparent uninterrupted in the Reflow region, whereas in the No-reflow region it was markedly interrupted and there was partial leakage of contrast from capillaries in some specimens (Fig5).

Fig6 shows the capillary volume fraction in the control area, the reperfused area in the Reflow group, and in the reperfused area in the No-reflow group. It was significantly reduced in the reperfused area in the Reflow group compared with the control area ($22.2 \pm 5.6\%$ vs $14.8 \pm 4.1\%$, $p < 0.05$), and was further decreased in the reperfused area in the No-reflow group compared with the Reflow group ($14.8 \pm 4.1\%$ vs $5.3 \pm 1.4\%$, $p < 0.01$).

There was excellent agreement in both the interobserver and intraobserver variabilities for the judgment of Reflow and No-Reflow ($\kappa = 0.82$, $\kappa = 0.91$, respectively).

Discussion

Previous investigations by optical or electron micro-

Fig 5. Contrast leakage from capillaries in the No-reflow sample.

scopy have reported ischemic morphological changes in coronary capillaries, such as bleb formation in endothelial cells, swollen endothelium, and plugging of platelets and leukocytes, and it has been speculated that these changes result from the no-reflow phenomenon after coronary reperfusion.^{9,10,14–16} However, those previous studies using 2-D images were limited in their observation of the 3-D capillary structure, which is enormously complex. Our newly developed method using confocal laser scanning microscopy with our unique contrast medium made it possible not only to observe capillary structure 3-dimensionally (without interruption), but also to analyze capillary volume fraction.¹² In the present study, we evaluated the 3-D capillary structure and the changes in capillary volume fraction after myocardial reperfusion.

Reperfusion Condition

In order to observe the morphological changes of the capillaries early after reperfusion, we determined the time of ischemia as 7 min according to previous studies in rats and dogs^{8,9} that reported early ischemic changes in rat capillaries occurring within the first 5 min.⁹ The onset of severe morphologic ultrastructural damage occurred much earlier in the rat than in the dog, maybe because of the animal's smaller size and higher metabolic rate, and the well developed and preexisting collateral circulation in the dog.⁸

Capillary Morphological Abnormalities and Capillary Volume Fraction

Our method made it possible to observe the complex structure of the capillary networks and to quantify the volume of the capillary bed. In the control area, the fine, well-regulated structure of the capillary network is demonstrated in high-resolution 3-D images. The capillaries were connected in patterns identified as Y, T, H, or hairpin and anastomosed through capillary cross-connections, as Kassab et al have described.¹⁷ The capillary volume fraction in the control area was 22.2%, which is comparable with

Fig 6. Capillary volume fraction in the control area and the reperfused areas in the Reflow and No-reflow groups.

our previous report!¹² Camilleri et al qualitatively assessed the microvascular structural changes following early myocardial reperfusion using carbon perfusion in a 'no-reflow' model and found that carbon black was present in arteriolar tufts, but little had reached the capillary level.¹⁸ Our images clearly show marked interruption of the capillary network in the No-Reflow area, resulting in reduction of the capillary volume fraction, and these results support the hypothesis that damage or interruption to the coronary microvascular network exists in clinical or experimental cases of 'no-reflow'.^{1–5} Furthermore, the leakage of contrast from capillaries may be related to myocardial hemorrhage after ischemia.¹⁹ Interestingly, morphological changes in the capillaries, such as 'coiling', 'waving' and 'shrinkage', were seen in the Reflow specimen and although the capillary network itself was virtually uninterrupted, the capillary volume fraction was reduced because of these structural changes. This particular finding suggests there is some degree of capillary damage after temporary coronary occlusion, even in cases of successful reperfusion (Reflow).

Study Limitations

Although the present study produced striking 3-D images of capillary construction and the morphological changes after coronary reperfusion, the mechanisms of these changes could not be demonstrated. However, the reduction in the capillary volume after myocardial reperfusion contributes to the further investigation of microvascular damage after myocardial ischemia, and to the understanding of the 'no-reflow' phenomenon in both clinical and experimental studies.

We observed capillary changes under only one condition of ischemia–reperfusion, whereas a time course of the 'no-reflow' phenomenon has been reported and therefore

further investigation, such as changing the duration of ischemia and reperfusion, is needed.

Conclusions

The 3-D coronary capillary structure after myocardial reperfusion is characterized by a waving and shrinkage configuration. The capillary volume fraction in the reperfused area of the Reflow group was significantly decreased compared with the control region, and further decreased in the No-reflow group.

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