Development of Fingertip Synchrotron Radiation Microangiography toward Clinical Prediction of Diabetic Microangiopathy

Toshiharu FUJII[*1, 2], Naoto FUKUYAMA[*1], Yoshimori IKEYA[*1], Chiharu TANAKA[*1], Yoshiro SHINOZAKI[*1], Kazuhito FUKUSHIMA[*3], Keiji UMETANI[*4], Yuji IKARI[*2] and Hidezo MORI[*1]

[*1] *Division of Physiology, Tokai University School of Medicine*
[*2] *Division of Cardiology, Tokai University School of Medicine*
[*3] *Department of Radiology, National Cardiovascular Center*
[*4] *Research & Utilization Division, Japan Synchrotron Radiation Research Institute*

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Objectives: The spatial resolution of conventional angiographic systems is not enough to predict diabetic microangiopathy in arterioles (20–200 μm).
Methods: To determine whether fingertip synchrotron (SR) radiation microangiography has enough spatial resolution to quantitate arteriolar diameter changes, and whether an arteriolar paradoxical vasoconstriction is a characteristic observation for diabetic microangiopathy, diameter reduction as arteriolar branching and difference of the diameter changes induced by acetylcholine between control (n = 5) and diabetic rats (n = 5) were analyzed.
Results: Fingertip SR microangiography visualized the arterioles with a diameter range of 30–300 μm and demonstrated vascular diameter reduction as branching with a fixed ratio (r = 0.93, P < 0.004 and r = 0.73, P < 0.001). A vasodilatory reaction was induced by acetylcholine in the control (142.4 ± 61.9 to 190.9 ± 73.5, P < 0.05, n = 25), in contrast, paradoxical vasoconstriction in diabetic rats (201.6 ± 83.0 to 16 0.4 ± 67.9, P < 0.05, n = 37). Histological angiopathy was noted only in the diabetic rats.
Conclusion: In conclusion, the fingertip SR microangiography is useful to predict diabetic micrangiopahty.

Key words: Synchrotron Radiation, Microangiography, Atheromatous Disease, Acetylcholine

INTRODUCTION

The numbers of those afflicted with diabetes mellitus (DM) have shown a rapid increase in recent years [1]. DM is a major risk factor for progression of arteriosclerosis, which causes fatal cerebro-cardiovascular events such as stroke and acute coronary syndrome [2]. Therefore, the development of diagnostic technology for the early stage of DM is a pressing social as well as clinical need.

DM induces microangiopathy including vascular endothelial dysfunction (VED). It have been reported that paradoxical vasoconstriction (PVC) in response to vasoactive medication such as acetylcholine (Ach) develops in an early stage of atheromatous disease (initial blood flow control abnormality) [3]. That is, if VED could be detected clinically at an early stage, and if VED without significant pathological vascular disease is a sign for the reversible phase of the DM microangiopathy, the possibility would be raised for preventing the progression of DM-induced lethal atheromatous disease. It is also known that arterioles with a diameter of 20–200 μm and maximum ratio of wall thickness to vascular diameter play central roles in local regulation of flow distribution [4–6]. Therefore, it can be speculated that arterioles also play a central role in VED at an early stage of DM microangiopathy. However, because the spatial resolution (SPR) of conventional angiography is around 100–200 μm, it is almost impossible to evaluate these arterioles quantitatively in clinical settings [7].

Synchrotron radiation (SR) is an established X-ray source that can be used in microangiography. SR is generated by the emission radiated from the huge synchrotron accelerator, and is an electromagnetic radiation characterized by white light with extremely strong luminance and remarkable directionality [8–10]. Up to now, in laboratory animals successful imaging of the coronary arterial microcirculation and ischemia of the hind limbs have been reported by microangiography using SR [11, 12].

The purpose of this study was to determine whether finger-tip SR microangiography can predict arteriolar microangiopathy. Studies were designed to evaluate whether finger-tip SR microangiography can demonstrate VED at the arteriolar level in DM rats. The reasons for that SR microangiography was applied to the fingertip vascular beds are as follows. Monochromatic SR angiography for thick objects such as brain, heart or abdominal organs generally involves a huge X-ray dose beyond the safety limit for the human body. Moreover, the high resolution feature suffers because of various kinds of X-ray scattering. In contrast, fingertip angiography can be performed under an acceptable X-ray dose 0.14R (roentgen, 1.4 millisievert: mSv) per one frame at an exposure time of 2 msec

Hidezo MORI, Division of Physiology and Cardiology, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
Tel: 81-463-93-1121 (ext 2532) Fax: 81-463-93-6684 E-mail: coronary@is.icc.u-tokai.ac.jp

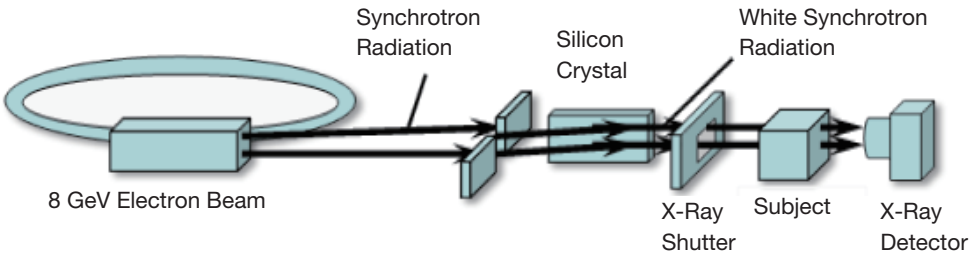


Fig. 1 SR Microangiographic Imaging System
Bending the accelerated electron beam in the 8 GeV accumulation ring of SPring 8 produces a white X-ray (synchrotron radiation). The white synchrotron radiation was monochromatized by a silicon crystal and radiated to the rats, while injecting iodine contrast materials. X-ray absorption images were detected by a saticon camera system with a super-high resolution.

and without compromising high-resolution. The X ray dose of 10 sec continuous exposure becomes (0.14 × R 50 frames/sec × 10 sec) 70R (700 mSv) and intermittent exposure of 5 frames/sec (2 msec per each frame) becomes 0.7R (7 mSv). Thus the fingertip SR microangiography would enable us to detect changes of arteriolar diameter induced by Ach administration even in clinical settings.

MATERIALS AND METHODS

SR Microangiographic Imaging System

SR microangiography on experimental animals was performed in a SR experiment facility (SPring 8, Hyogo Prefecture, Japan). SR is generated as a white SR by accelerating the electron beam generated from the electron gun by the synchrotron accelerator into the energy of 8 GeV. The resulting white SR is converted into monochromatic X-ray of 33.2 KeV (K absorption edge of the iodine) by a silicon crystal (Fig. 1). This monochromatic X-ray with an energy level just above the K absorption edge of iodine allows us to detect a small amount of iodine in the microvessels [13]. Monochromatic X ray that passed the objects (anesthetized rats) described below was detected by a saticon camera. A visual field was set to 9.5 mm × 9.5 mm in this SR microangiographic imaging system and SPR was 9.5 μm [10].

Animal preparation

All animal studies were conducted under protocols approved by Tokai University Animal Experimental Committee. Ten male rats (Japan SLC, Shizuoka, Japan) were divided into a control group (n = 5) and a DM group (n = 5). The five rats of the control group consisted of Fisher 344 rats (n = 3), and LETO rats (Otsuka Pharmaceutical, Tokushima, Japan, n = 2). The five rats of the DM group consisted of a type I DM model in Fisher 344 rats (n = 3), that had been treated with 100 mg/kg of streptozotocin (STZ; Sigma, St. Louis, Missouri, USA) 3 months before the microangiography, and a type II DM model of OLETF rats (n = 2) (Otsuka Pharmaceutical, Tokushima, Japan). The OLETF rat is a type II DM model that is characterized by impaired glucose tolerance, hyperinsulinemia, and hyperlipidemia with visceral steatosis type obesity. The excessive secretion of insulin detected by the glucose tolerance test manifests an early stage of DM, and the decrease of insulin secretion by the glucose stress induces overt diabetes with the progression of DM [14]. LETO rats served as controls for the OLETF DM rats. All of these rats were bred for three months in the same environment which is also approved by the Animal Experimental Committee.

Just before SR microangiography, rats were anesthetized by intraperitoneal injections of 50 mg/kg of sodium pentobarbital (Nembutal, Abbot Laboratories, North Chicago, IL, USA). The right common iliac artery was then exposed by skin incision, and a polyethylene catheter (Clay Adams, PE 50; I. D. 0.58 mm, O. D. 0.965 mm; INTRAMEDIC, USA) was inserted into the lower part of the descending aorta for contrast medium injection. Another catheter was inserted into the tail artery for vasoactive agent infusion and arteriolar blood pressure monitoring.

Experimental protocol

To determine whether there is sufficient SPR in fingertip SR microangiography and to determine whether the fingertip SR microangiography can detect functional angiopathy in DM rats, the angiographic and analytic procedures described in the next paragraph were done. In ten rats, SR microangiography was repeated two times in the following order; at baseline and during Ach administration with a 10 min intermission. Before the second microangiography, restoration of arterial blood pressure and pulse rate to the initial levels was confirmed. SR microangiography was performed by irradiating the left hind-limb fingertips of the anesthetized rats with the monochromatic X-ray while injecting iodine contrast medium (iopamiron 370, Bayer) into the descending aorta via the right common iliac artery. The contrast medium was injected at a rate of 2.4 ml/sec (Fisher rat: total 2 ml, LETO and OLETF: total 4 ml) from the inserted polyethylene catheter into the right common iliac artery. After a 10 min intermission and confirming stabilization of aortic pressure and pulse rate the second SR angiography was done in exactly the same way as at baseline while injecting Ach (Nacalai Tesque Tokyo Japan) at a rate of 3.28×10^{-11} mol/kg/min into the tail artery.

In order to verify whether the SPR of the SR system is sufficient to evaluate changes of arteriolar diameter

quantitatively, the analysis described below was done. The measurement of lumen diameter of microvessels was measured at three points for each vascular bifurcation: proximal portion tentatively named as "mother artery", and two distal sites after the bifurcation as the "1st (bigger) daughter artery", and the "2nd (smaller) daughter artery" (total of 38 data sets from 10 rats at baseline or during Ach administration). The measurement points were set at the just proximal and distal to the branching point. Linear interpolation between adjacent pixels was applied at an effective pixel side length of 5 μm. The operator clicked the position and direction of a vessels segment on Image-pro plus, the vessel diameter was computed as a distance between two half-density points from the apex to the bilateral background levels of a short axial density profile plot of the target segment. A copper wire of 100 μm in diameter, which was put just close by the imaging samples, was used as the calibration reference in quantitative angiographic analysis (Fig. 2, the left upper panel). To verify the accuracy of the measured microvascular diameter at baseline and during Ach administration, SPR of the fingertip SR microangiography was confirmed empirically. Vascular diameter was measured in the 38 sites of the visualized vascular bifurcations where vascular diameters were measured at all of the 3 points as described above. Arterial diameter, in general, is reduced as branching progressively [15]. Therefore vascular diameters of the 1st and 2nd daughter arteries were plotted against the proximal sites (38 and 38 data sets, respectively) and analyzed by linear correlation and regression analysis. Next, in order to show whether PVC develops only in DM rats, the changes of lumen diameters of microvessels from baseline condition to Ach administration were compared between normal and DM (n = 25, 5 normal rats, and n = 37, 5 DM rats). The quantitative angiographic analysis described above was performed on a personal computer (DELL Precision Workstations 620, DELL). The vessel diameter analysis was done with Image-Pro Plus ver. 4.0 software (Media Cybernetics, Silver Spring, USA). Image-Pro Plus is an image analysis, measurement, and processing software to automatic measurement of length or area indicative of shape or morphology.

Pathological study

In order to elucidate whether the arteriolar PVC can predict pathological changes in DM, hematoxylin-eosine staining was performed with conventional methods in the kidney, aorta, brain and heart. Histological analysis of the finger-tip arterioles was not done in this study.

Statistical analysis

The results were summarized as mean values ± SD. Statistical analysis was performed using EXCEL 2007 (Microsoft, USA). Linear correlation and regression analysis were applied to the two sets of data (the 1st and 2nd daughter segments versus the mother segments). Comparison of vascular diameter changes during Ach administration between DM and control rats was performed by the paired t-test. P value < 0.05 was considered to be statistically significant.

RESULTS

To determine whether fingertip SR microangiography can detect functional microangiopathy and predict pathological changes in DM rats, the SPR of the SR system was confirmed empirically and proceeded to evaluate whether PVC induced by Ach administration develops only in DM rats.

As shown in panel a-1 and magnified panel a-1 of Fig. 2 (the arrows in these figures), SR microangiography visualized fingertip arterioles. The minimum diameter of the measured fingertip arterioles was 30 μm (Fig. 3, right panel, the arrowhead).

To verify the accuracy of the measured microvascular diameter at baseline and during Ach administration, SPR of the fingertip SR microangiography was confirmed empirically. We measured the diameters of the 3 sites for each bifurcation site (yellow arrowheads in Fig. 2, panel a-2): the mother (the proximal site before bifurcation), the 1st daughter and the 2nd daughter arteries, and analyzed whether the measurements show a progressive diameter reduction as branching with a constant ratio [10].

In the 38 bifurcation sites from the 10 rats, all of the 3 arteries were visualized clearly enough to measure the diameters. The relations of the microvascular diameters of the 1st and the 2nd daughter arteries against the mother arteries were analyzed by correlation analysis and linear regression analysis as shown in the left and right panels of Fig. 3. Both plots revealed significant linear correlations ($r = 0.93$, $P = 0.004$, $r = 0.73$, $P < 0.001$, respectively). Regression analysis revealed regression equations of $y = 0.807x + 0.003$, and $y = 0.392x + 23.31$, respectively. These results indicated that the SR microangiography could reveal a diameter reduction as branching with fixed ratios of approximately 0.8 and 0.4. In other words, SR microangiography had enough SPR to evaluate the diameter changes of arterioles induced Ach administration for all of the measured sites.

To evaluate quantitatively a difference in the impact of vasoactive agents on microvessels between normal and DM rats, the changes of lumen diameters of microvessels during Ach administration were compared between those with and without Ach stress in all ten rats. Ach treatment induces PVC; diameter reduction during Ach, in DM rats (Fig. 2, the panel b-1 and b-2 and Fig. 4, the right panel) as contrasted with vasodilatory reaction; diameter increase during Ach in normal rats (Fig. 2, panel a-1 and a-2 and Fig. 4, the left panel). The change in the microvascular diameter caused by Ach was significant in both normal and DM groups ($P < 0.02$, and $P < 0.03$, respectively, paired t-test).

To confirm whether the vascular functional response reflects diabetic pathological findings, the histopathologies of the kidney, aorta, brain and heart of control and DM rats were compared. In all of the DM rats diabetic pathological changes such as deformed glomerulus and infiltrate of mononuclear cells in aortic wall (shown in the right upper and middle panels of Fig. 5) and diabetic vascular changes in heart and brain (data not shown) were noted in all of DM rats, but not in any of control rats. We used type I and II DM rats. Each number of the rats was too small to

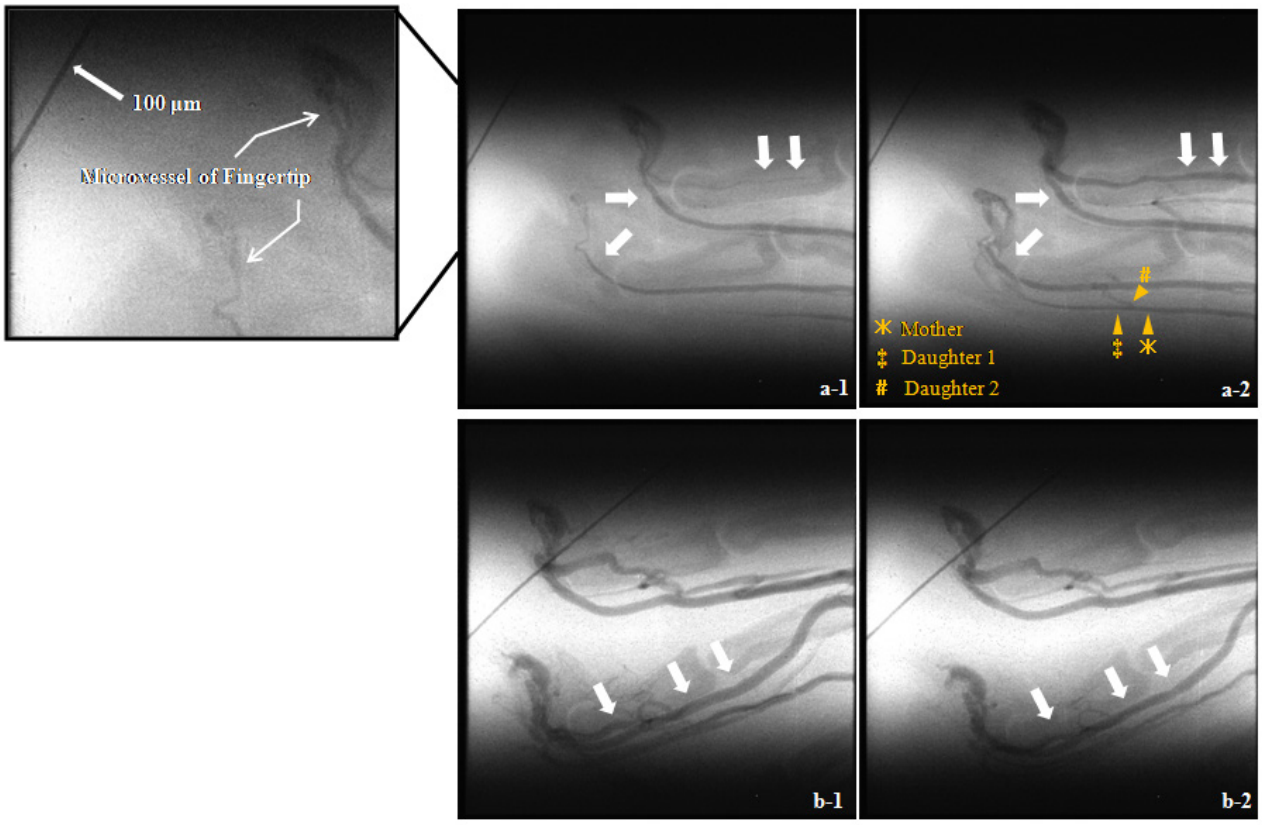


Fig. 2 Fingertip Microangiograms of the Rats with and without Ach Stress
The three upper panels are representative SR microangiograms of hind limb in a control rat; center: at baseline (a-1), left: magnified view of the baseline and right: during Ach administration (a-2). The two lower panels are those in a DM rat; center: baseline (b-1) and right: during Ach administration (b-2). The copper wire with a known diameter (100 μm, the magnified view of a-1) was used as a calibration for measurements of vascular diameter. The arrows indicate Ach induced vasodilation (the upper panels) and the PVC (the lower panels). The arrowheads (✱ ‡#) indicate an example of diameter measurement of the mother, 1st and 2nd daughter arteries.

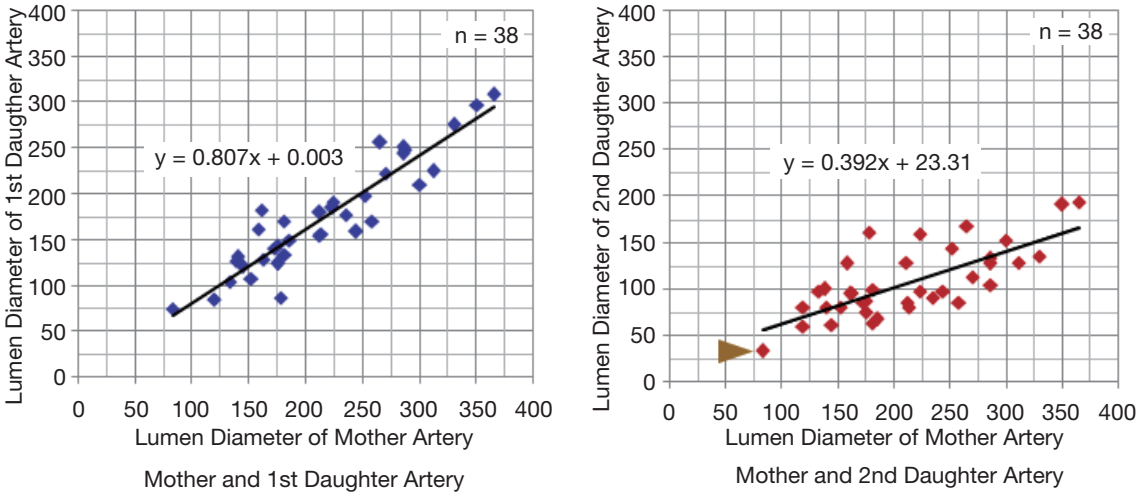


Fig. 3 Regression Analysis Regarding Microvascular Diameter Reduction as Branching
Vascular diameters of a mother artery were plotted to a horizontal axis, and vascular diameters of 1st (the left panel) or 2nd daughter artery (the right panel) were plotted to a vertical axis of 38 bifurcation sites. The slopes of the regression lines: 0.807, and 0.392, indicated the reduction rate of the microvascular diameter as branching. The arrowhead in the right panel indicates 30 μm that is the minimum diameter of the measured 2nd daughter artery.

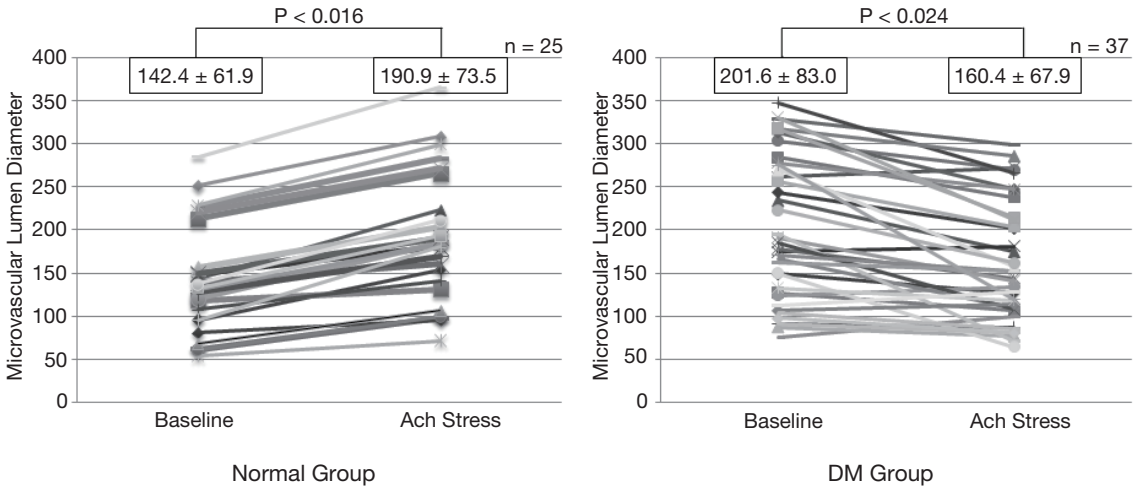


Fig. 4 The Changes of the Microvascular Diameter Induced by Ach Stress
The left panel: the diameter changes from baseline to during Ach administration in normal rats, and the right panel: those in the DM rats. Ach induced vasodilator reaction in normal rats, in contrast, paradoxical vasoconstrictive reaction (PVC) in DM group.

discuss the difference between the types.

DISCUSSION

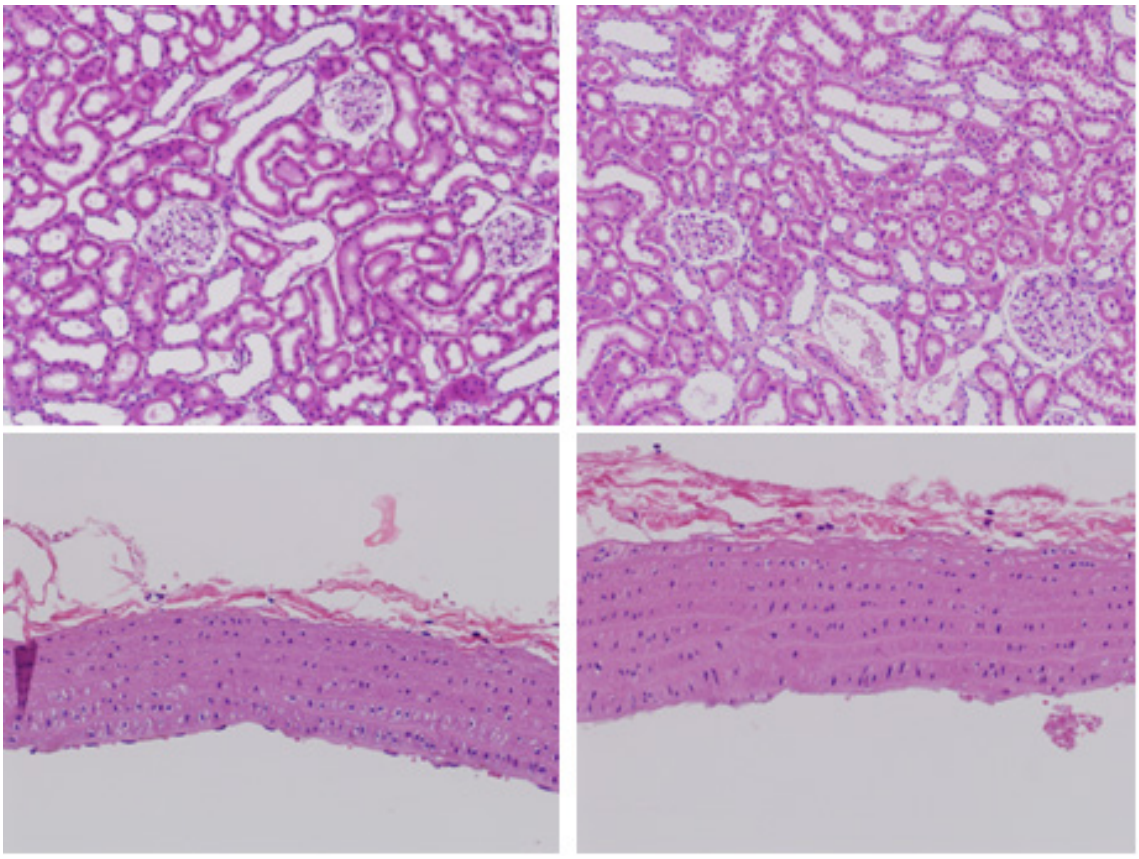
This study showed that fingertip SR microangiography has enough SPR to evaluate quantitatively arteriolar diameter changes and can detect functional microangiopathy predicting pathological changes in DM rats.

DM angiopathy is increasing with the increasing DM. Though early stage of DM angiopathy starts in VED, it progresses to an organic change after a while. DM angiopathy which is characterized by the accumulation of matrix protein, bridge formation of collagen protein from advanced glycation end product, thickening of intima-media complex, and calcification of vessel wall damages to key organs by stenosis or occlusion of systemic vessels [16, 17].

By analyzing vascular diameter reduction as branching with linear correlation and regression analysis, we confirmed a constant ratio of diameter reduction from mother to daughter arteries in the vascular beds with a diameter of 30–300 μm (Fig. 3). This observation indicates that SPR of the present SR microagiographic system is more precise than the ability to detect the small vessels filled with small amount of iodine contrast materials (concentration resolution). Tanaka et al demonstrated a branching pattern with a fixed diameter reduction ratio continuous from the epicardial-to-intramyocardial branching in the dog coronary artery [15]. They analyzed branching patterns of coronary vessels determined by microangiography using SR as done in the present study, and showed constant ratios of the daughter vascular diameter to the mother vascular diameter were 0.84 in the epicardial coronary artery and 0.74 in the intramural coronary artery. If all of the vascular diameter measurements were done above the limit of SPR of the SR microangiography (9.5 μm), the degree of reduction should be constant. A marked deviation of the reduction ratio of the vascular diameter from the regression line means that the measurement was done beyond the limit of SPR of the angiography. We confirmed in this study the reduction ratio of vascular diameter was kept constant throughout the measurement in the range of 30–300 μm. The reduction ratios of the microvascular diameters as branching were 0.81 and 0.39, and the reduction ratio was constant throughout the range of the measurement. The smallest measurable microvascular diameter was 30 μm. Because this measurement is plotted on a straight-line approximation as shown in Fig. 3, it can be regarded that this SR system had enough SPR to quantitate the vessels with a diameter of 30 μm (theoretical resolution of 9.5 μm). Thus, we concluded SPR of the fingertip SR microangiography was high enough to verify the quantitative data for arteriolar PVC. The limit of SPR of SR microangiography depends on the resolution of detector its self, because the directionality of SR is nearly that of laser.

By performing fingertip SR microangiography with and without Ach stress, we succeeded in visualizing arteriolar PVC only in DM rats (Fig. 2, the panel b-1 and b-2 and Fig. 4, the right panel). In contrast, vasodilatory reaction was noted in control rats (Fig. 2, panel a-1 and a-2 and Fig. 4, the left panel). Histological study confirmed the pathological background for the arteriolar PVC demonstrated by the fingertip microangiography (Fig. 5).

By applying the fingertip SR microangiography into clinical settings, early detection of DM microangiopathy following early intervention would be possible. Retinal microangiopathy caused by DM is the most frequent cause for adult blindness. Renal microangiopathy causes lethal renal failure and DM microangiopathy frequently leads to lethal cerebral stroke and heart attack. Therefore, the early prediction of DM microangiopathy would clearly be clinically significant.



	Diabetic Pathological Changes	
	Control Group	DM Group
Renal Glomerulus	0/5 (0 %)	5/5 (100 %)
Aorta	0/5 (0 %)	5/5 (100 %)

Fig. 5 The Pathological Features of DM Rat
The upper panel: The right panel shows representative diabetic pathological changes of the renal glomerulus (denucleated convoluted tubule cell and deformed glomerular structure), in contrast to the left control kidney. The middle panel: The right panel shows diabetic pathological changes of the aorta (infiltration of mononuclear cells and thickened intimal layer), in contrast to the left control aorta. Summary of incidence of vascular and renal lesions is shown in the lower panel.

Obviously however, further experiments will be needed before moving on to a clinical study.

There are several limitations in present study. First, different types of rats were used as both normal rats and DM rats. However, vascular reaction for Ach loading showed as predicted without difference in both groups. Therefore, it is considered to have no impact to change the results. Second, contrast medium has a vasodilating effect, and the volume loading by mechanical infusion of contrast medium might dilate the vessels. Nevertheless, since VED was clearly demonstrated, these effects were regarded as little.

This study showed that the fingertip SR microangiography has enough SPR to quantitatively evaluate arteriolar diameter changes and can detect functional microangiopathy in predicting pathological changes in DM rats.

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