Quantitative Analysis of Vascular Structure in Decellularized Liver Using 3D Computed Tomography

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Abstract Recently, decellularized liver (DC-liver) has been developed as a scaffold for the generation of liver tissue because DC-liver has a vascular structure that is used for oxygen delivery. The structure has been evaluated by the appearance of the resin cast. In the present study, three-dimensional computed tomography was used to obtain images of the vascular structure. Imaging at the hepatic lobule level was achieved by coating the resin cast with a contrast dye or gold. We defined indices to evaluate blood vessel conformation and structure. The indices were measured and calculated from the images. The results suggest that DC-liver has a fine vascular structure quantitatively similar to that of native liver. This method is valuable for the development of whole liver engineering.

Keywords: animal cell culture, image analysis, tissue cell culture, whole liver engineering, decellularized scaffold.

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1. Introduction

Liver transplantation is the only effective treatment for patients with severe liver disease. However, the major problem with transplantation is the chronic shortage of donors [1]. Therefore, construction of liver tissues that can express liver function is desirable as an alternative to donor liver.

The mass of liver tissue necessary for human survival is considered to be $\geq 30\%$ of the original mass, because rats and humans can survive even when 70% of the liver is resected [2, 3]. The average weight of the liver in 8-week-old male Wistar rats is 8.2 g, and that in humans is 1.2 kg for 59 ± 11 kg body weight [2]. The required weight of alternative liver tissue is ≥ 2.5 g for rats and ≥ 360 g for humans. Although centimeter-scale tissue is needed for construction of > 1 g liver tissue, the maximum thickness of liver tissue constructed *in vivo* is ≤ 1 mm. Construction of blood vessel network is needed for the creation of such centimeter-scale liver tissue. Therefore, we aimed to construct a vascular network based on the internal vascular structure of decellularized liver (DC-liver). We then attempted to construct centimeter-scale liver tissue based on DC-liver with a vascular network [4, 5].

We have reported optimization of the decellularization method to obtain DC-liver, which retains its vascular structure even when cellular components are removed [4]. Many researchers who have studied decellularization of organs have used resin to observe the vascular structure in a decellularized organ [4–9].

They have previously discussed the residual vascular structure solely from the appearance of the resin cast. Although the aim is to retain the tree-like vascular structure, quantitative analysis based on resin cast has never been reported at organ level. However, quantitative analysis of the vascular structure is important for construction of vascular networks in decellularized organs. We report quantitative analysis of vascular structure by DC-liver and native liver using three-dimensional (3D) computed tomography (CT).

2. Materials and methods

2.1 Molding of vascular structure

All experiments were performed at room temperature unless stated otherwise. Six-week-old male Wistar rats underwent abdominal surgery under inhalation anesthesia. In some rats, blood in the whole liver was removed by perfusing the liver with calcium–magnesium-free phosphate-buffered saline via an 18-G cannula inserted into the portal vein (native whole liver). In other rats, after perfusing with the saline, the liver was partially resected, leaving only the right lobe (native right lobe). Triton X-100 solution (4%) was introduced into the right lobe via the portal vein to produce DC-liver. Resin was prepared using Batson's No. 17 Plastic Replica and Corrosion Kit (Polysciences, Eppelheim, Germany). Red resin was injected into the portal vein and blue resin into the hepatic vein. The volume of resin injected into the portal and hepatic veins was 0.8 ml in whole liver and 0.3–0.4 ml in the right lobe. After injection, the liver was left for at least 3 h to allow consolidation of the resin. DC-liver was dissected before decellularization of Triton X-100. The other livers were dissected after consolidation of resin. The resin-injected liver was soaked in 33% KOH solution, and incubated at 50°C for 3–6 h. Then, the surrounding liver tissue was removed with KOH solution. KOH treatment was repeated until the surrounding liver tissues were completely removed from the resin. The experimental protocol was reviewed and approved by the Ethics Committee on Animal Experiments of Kyushu University.

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Table 1 Compositions of contrast dye coating solutions.

Component	Final concentration [%]						
Oypalomin	10	1	1	1	1	1	1
Isodine	16	1	-	1	1	1	1
Triton X-100	5	-	1	1	1	1	1
Solvent	Water	Ethanol	Water	Ethanol	Ethanol	Water	Water
The times of repeated dip and dry	15	5	5	5	1	5	1
Abbreviation	LA	LB	LC	LD-5	LD-1	LE-5	LE-1

Fig. 1 Index for quantitative analysis of vascular structure.

Table 2 Conditions of gold coating.

Abbreviation	Thickness of coating Au [nm]	Current [mA]	Sputtering period [sec]
Au-20	20	40	20
Au-30	30	40	40
Au-35	35	40	45
Au-40	40	40	50
Au-50	50	40	70

2.2 Coating of resin cast

2.2.1 Coating with contrast dye solution

Oypalomin (Fuji Pharma, Tokyo, Japan) is a non-ionic contrast dye used in the urinary tract and blood vessels, and contains 300 mg/ml iodine. The dye was diluted with water or ethanol because of the high viscosity and high iodine content. The coating solution contained Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), which is a non-ionic detergent, and Isodine (Meiji, Tokyo, Japan), which contains 7 mg/ml iodine. The resin that was used to form the cast of the vascular structure in the liver was coated with coating solutions with different compositions as outlined in **Table 1**.

2.2.2 Gold deposition coating

A JFC-1600 ion coater (JEOL, Tokyo, Japan) was used for gold deposition. The gold target was set in the machine. The resin casts were then coated under conditions as shown in **Table 2**. Coating

was performed by turning the resin cast to allow coverage of the entire surface. The thickness of the gold coating was determined by the relationship between the current and sputtering time.

2.3 Scanning using 3D-CT and data analysis

A 3D-CT scanner (Somatom Sensation 64; Siemens AG, Munich, Germany) was used to scan the resin casts of the vascular network. Slice collimation in the 0.3 mm range was needed to obtain high-resolution 3D-CT images of small vessels. Using a detector collimation of 12×0.3 mm, a pitch of 0.9 and a rotation time of 1.0 s, approximately 30 s was required to cover this range using a 64-row CT detector. Another scanning protocol and reconfiguration conditions for 3D-CT imaging of DC-liver using a 64-row scanner were: 120 kV, 188 mA, 0.4 mm slice, 0.2 mm reconstruction increment (z-axis resolution), U90u ultra sharp kernel, and 100 mm field of view. The portal or hepatic veins were aligned along the Z dimension to reduce artifacts. The 3D scan data comprised many slices. The photographs of 10 slices were selected for subsequent analysis. The photographs at equally spaced intervals were selected, depending on the Z dimension of each sample. The data were saved in jpeg format, inverted white to black, and binarized. The cross-sectional areas of the rat liver and that of the vascular structure were calculated, as well as the number of vascular structures per liver tissue area. Vascular structure with an area from 0.076 mm^2 (6 pixels) to 2 mm^2 was counted as one vascular structure. Based on these data, the ratio of vascular structure area to tissue area, the number of vascular structures per tissue area, and the cross-sectional area per vascular structure were obtained. Based on the structure of the liver lobule, the cross-sec-

tional surface can be assumed to be the aggregate of equilateral triangles with the blood vessel as the vertex. Therefore, the distance between the centers of blood vessels was calculated by $\sqrt{[(2/\sqrt{3})/\text{number of vascular structures per liver tissue area}]}$. The average diameter of the vascular structure was calculated based on the cross-sectional area per vascular structure when the structure was assumed to have a precise circular shape. These indices are illustrated in **Fig. 1**.

2.4 Statistical analysis

The results are expressed as means $\pm$ SD. Statistical analyses were performed using multiple comparisons with Tukey's test or a two-tailed unpaired Student's *t*-test. $P < 0.01$ was considered to be statistically significant.

3. Results

3.1 Coating of resin

The minimum diameter of the molded vascular structures was $\geq 100 \mu\text{m}$. The gaps between the tips of the branches of the vascular structure were $< 1 \text{ mm}$. However, below a diameter of 1 mm , the vascular structure could not be detected by 3D-CT (**Fig. 2B, C**). Continuity of the vascular structure was not depicted and there were many artifacts (**Fig. 2A–C**). This implies that the resin did not have sufficient X-ray absorption for 3D-CT detection.

When the resin cast was coated with composition LA (**Table 1**), the structure was depicted in greater detail by 3D-CT (**Fig. 2E, F**) compared to the uncoated resin cast. However, the vascular structure appeared to have uniform diameter at all points (**Fig. 2F**). Under physiological conditions, the diameter of blood vessel decreases with decrease in blood flow volume from the inlet to the peripheral vessels at the edge of the liver. This phenomenon was observed in the resin cast of the vascular structure, but was not observed in the 3D-CT images when the resin cast was coated with composition LA. The difference in appearance was expected because X-ray absorption increased too strongly by coating with the contrast dye. Therefore, we attempted to find the composition that gives sufficient contrast and allows detection of the fine structures of the resin-molded vessels.

We studied various concentrations of contrast dye and deter-

Fig. 2 Appearance of resin casts of vascular structure of native whole liver before (A) and after (D) coating with contrast dye. B and E are 3D-CT images. C and F are magnifications of the dotted square regions in B and E, respectively. A–C were uncoated and D–F were coated with composition LA (**Table 1**). The arrows show the z-axis direction.

gent, and different times for repeated dipping and drying. The compositions are shown in **Table 1**. Then, 3D-CT images were obtained using each composition (**Fig. 3**).

For compositions LB and LC, the terminal portions appeared thicker and diameters were more uniform than for the uncoated resin cast. In contrast, the terminal portions were detected in patches for compositions LD-5, LD-1 and LE-5. LE-1 was the most suitable composition for 3D-CT detection. In the image obtained using composition LE-1, continuity of the vascular structure was observed and the diameter decreased progressively from the inlet to the tip of the vascular structure.

However, during drying of the resin-molded vascular structure after soaking in the contrast dye solution, draining of the solution was slow in the high-density field of the tubular structures. Blotting of excess solution with filter paper was required to reduce the bias of coating. This procedure could lead to damage of the resin cast. Therefore, we explored other method to coat the molded resin without blotting.

The resin-molded vascular structure was coated with gold under the conditions described in **Table 2**, and 3D-CT images were obtained. At 50-nm thickness, the terminal portions appeared thicker and more uniform in diameter compared with the

Fig. 3 Appearance of resin casts of the vascular structure of native liver, coated with coating solution (left column) and 3D-CT images (middle column). The dotted square regions are shown at a higher magnification in the right column. The arrows show the z-axis direction.

Fig. 4 Resin casts of the vascular structure of native liver coated with gold (20–50 nm thick). The left column shows the appearance of resin casts before coating with gold and the second column shows the appearance after coating. The third column shows the 3D-CT images of the gold-coated resin casts. The dotted-square regions are shown at a higher magnification in the right column. The arrows show the z-axis direction.

uncoated resin casts. In contrast, at 20-nm thickness, the terminal portions appeared in patches (**Fig. 4**). The 3D-CT images of the resin-molded vascular structures coated with 30 and 40 nm gold were as good as those taken using composition LE-1.

Based on these results, LE-1 was the best coating composition for contrast dye solution and Au-35 was the best condition for coating by gold deposition. However, when coating with the contrast dye solution, we were concerned that the resin would be damaged by contact with the filter paper in the drying process. Therefore, we chose the Au-35 condition for coating in quantitative vascular analysis.

Resin casts of the portal vein system in native whole liver (WN-A), the portal and hepatic vein systems in native whole liver (WN-AV), the portal vein system in native right lobe (RN-A), the portal and hepatic vein systems in the right lobe (RN-AV), and the portal and hepatic vein systems in decellularized right lobe (RD-AV) were coated with 35-nm-thick gold, and 3D-CT images were obtained (**Fig. 5**).

3.2 Analysis of 3D-CT images

We compared the data obtained from 3D-CT images of the portal vein system, and portal and hepatic vein systems in native whole liver and native right lobe to evaluate the adequacy of this method. The ratio of vascular area to tissue area in the portal and hepatic vein systems was twice that of the portal vein system, both in the whole liver and in the right lobe (**Fig. 6A**). The number of vascular structures per tissue area in the portal and hepatic vein systems was higher than that in the portal vein system, both in the whole liver and in the right lobe (**Fig. 6B**). The distance between the centers of blood vessels in the portal vein system was greater than that in the portal and hepatic vein systems (**Fig. 6C**). The average diameter of the blood vessels in the portal vein system

Fig. 5 Samples coated with 35-nm-thick gold. The left column shows the appearance of resin casts before coating with gold. The middle column shows the appearance of the resin casts after coating. The right column shows the 3D-CT images of gold-coated resin casts. The arrows show the z-axis direction.

was similar to that in the portal and hepatic vein systems (**Fig. 6D**). Based on mass balance, it was supposed that the inlet and outlet volumes were equal, and twice the inlet volume was almost equal to the inlet plus outlet volumes. These results agree with the supposed phenomenon and the analysis method can be used for the

Fig. 6 Ratio of area of blood vessels to total tissue (A), number of blood vessels per total area (B), distance between centers of blood vessels (C), and diameter of blood vessels (D). Each sample was calculated based on the photographs of 10 slices. (E) Interval between blood vessels was calculated from the distance between the centers of blood vessels (F) and the diameter of the blood vessels (D). ** $p < 0.01$.

quantitative analysis of vascular structure.

The data for the right lobe in native liver were compared with those for the right lobe in DC-liver. The native liver and DC-liver had almost the same values for all the parameters (Fig. 6A–D).

The interval between blood vessels was estimated based on the average distance between the centers of blood vessels and the average diameter of blood vessels (Fig. 6E). The interval between blood vessels in RN-AV and RD-AV were estimated to be < 2 mm.

4. Discussion

Vascular structure in the liver can be analyzed by assessing the appearance of resin casts [4–9]. In the present study, we devel-

oped a method for obtaining clear 3D-CT images of vascular structures in native liver and DC-liver by coating resin casts with contrast dye or gold. Too much coating resulted in images depicting thicker vascular structures than they actually were. In contrast, the terminal portions appeared in patches in areas that did not receive enough coating. In our study, LE-1 and Au-35 were the best coating conditions for the resin casts. We chose the gold deposition for fine vascular analysis, because of the concern that the contrast dye coating would be damaged by the filter paper during the drying process. Coating with contrast dye solution can be scaled up if fine detection is not necessary. In contrast, if fine detection of vascular structure is desired, the gold deposition method should be used, even under scaled up condition. However, this requires large apparatus.

We defined five parameters for quantitative analysis. The results showed that these parameters are suitable for quantitative analysis because they conform to the theory of mass balance. Then, the vascular structures in native liver and DC-liver were compared using these parameters. No differences between the values of these parameters between native liver and DC-liver were observed. Quantitative analysis of DC-liver was achieved, which showed that the vascular structure in DC-liver was similar to that in native liver.

The calculated distance between vascular structures was approximately 1.5 mm. However, the distance between the tip of the resin-molded structures was < 1 mm. The difference between the calculated and actual distances was because of the measurement limitations of the Somatom Sensation 64 scanner. With this device, one pixel is 0.6×0.6 –1.0 mm and whole images are reconstructed with a resolution of ≥ 0.4 mm. Our data showed that the native liver and DC-liver had almost the same vascular structure. To obtain more precise data with higher resolution, the use of higher specification equipment such as a synchrotron X-ray machine is required. For example, if the synchrotron X-ray beam has light energy of 5–35 keV, energy resolution ($\Delta E/E$) is 10^{-4} . Although under $7.5 \times 7.5 \mu\text{m}$ per pixel is obtained, the analysis range is only 36×24 mm. This is too small for human use and has limitations in mice and rats. Moreover, compared with 3D-CT, synchrotron X-ray machine is expensive and analysis is time consuming.

We used 3D-CT to obtain images of the vascular structure at the hepatic lobule level. A recent ultrasound imaging study in rats only examined a part of the liver and comparison between native liver and DC-liver was not performed [10]. To the best of our knowledge, quantitative analysis of tubular networks has not been reported previously. Using this technique, optimization of the decellularization method can be achieved in rats and humans. This method is useful for construction of other organs using decellularized organs.

The DC-liver was obtained as a scaffold, which has a tubular structure for construction of a vascular network. The fineness of the structure was proven quantitatively. In the near future, based on 3D-CT images of tubular structures, it may be possible to simulate the oxygen supply through the vascular network, and the viability of seeded liver cells between vascular networks. This will be helpful for development of liver tissue engineering, such as optimization of recellularization or culture conditions.

5. Conclusion

Imaging of fine vascular structure in the liver was achieved by coating resin cast with contrast dye or gold. Images were obtained at the hepatic lobule level. The images suggested that the vascular structure of DC-liver was as fine as that of native liver.

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Conflict of Interest

We have no conflicts of interest relationship with any companies or commercial organizations based on the definition of Japanese Society of Medical and Biological Engineering.

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