Although artificial vessels are available for large diameter arteries, there are no artificial vessels for small diameter arteries of < 4 mm. We created a decellularized vascular scaffold (length, 10 mm; outer diameter, 1.5 mm; inner diameter, 1.3 mm) from rat abdominal arteries. We measured the biomechanical characteristics of the scaffolds, implanted them to defects made in rat carotid arteries, and evaluated their patency and the endothelial cell linings. Silastic grafts were implanted as controls. The decellularized scaffolds demonstrated similar mechanical characteristics to normal arteries. All of the control grafts were occluded. Fibroblast-like cells were discovered in the thrombus, and fibrous organization was apparent. In contrast, patency of the grafts in 10 of 12 animals was observed 4 weeks after implantation. The internal cavity of the patent scaffold was completely lined by endothelial-like cells. Thus, the possibility of small artery reconstruction using decellularized scaffolds was demonstrated.

Key words: Regeneration medicine, Reconstruction, decellularized scaffolds, Small diameter artery

Introduction

Artificial vessels for small diameter arteries (< 4 mm) have not yet yielded successful clinical outcomes. Many unresolved challenges remain, including early occlusions, neointimal hyperplasia, and long-term functions. Presently, clinicians use autograft vessels for the reconstruction of arteries in the thigh (from groin to knee) and coronary arteries with the unavoidable sacrifice of healthy tissues. The adoption of tissue engineering technology for the regeneration of arteries may provide a solution. Several studies reported the development of hybrid-type vascular grafts in which vessels made with bioabsorbable polymers underwent seeding with bone marrow-derived cells, myofibroblasts, or vascular endothelial cells. These tissue-engineered vascular grafts may decrease the incidence of occlusion; however, they require definite periods of cell proliferation and cell seeding before transplantation into patients. Therefore, they have serious limitations for injury cases for which rapid treatment is crucial.

Another technology for tissue reconstruction has recently emerged, in which decellularized tissue scaffolds are utilized. Biological tissues were harvested and subjected to a decellularization process; the residual extracellular matrix was used as the scaffold for tissue reconstruction. These scaffolds demonstrate biomechanical characteristics similar to natural tissues and afford prominent cell infiltration from surrounding tissues after implantation. The successful application of these scaffolds was reported in the reconstruction of small caliber arteries (3-4 mm) and cardiac valves. However, the reconstruction of
arteries of < 2 mm in diameter with these scaffolds has not yet been thoroughly investigated, although results obtained through tissue engineering approaches have already been reported.\textsuperscript{16,17}

Therefore, in this paper, we investigated the reconstruction of these small arteries with decellularized allografts. Namely, we constructed a decellularized vascular scaffold from rat abdominal arteries, measured their biomechanical characteristics, implanted them in defects made in rat carotid arteries, and evaluated their patency, biomechanical characteristics, and endothelial cell linings.

Materials and methods

Decellularized vascular scaffolds

Rat abdominal aortas were harvested and decellularized with an ultra-high pressure treatment according to the method of Fujisato and Funamoto.\textsuperscript{10,18} Abdominal aortas were harvested from Sprague-Dawley (SD) rats (male rats, 9 weeks of age, with body weights of 300–320 g). Immediate pretreatment was performed by immersing them in processing solution that contained DNase, RNase, EDTA/2Na, and TritonX-100 for 1 hour. They were then subjected to processing under ultra-high pressure (Dr. Chef; Kobelco, Japan) at 25°C. The processing times were 15 min for raising pressure, 10 min for holding specimens at 10,000 atm, and 15 min for reducing pressure. Finally, they were post-treated by immersion in rinse solution for 24 h. With this processing, the cells and microorganisms in the tissues were completely removed\textsuperscript{10,18} and the decellularized scaffolds (length, 10 mm; outer diameter, 1.5 mm; inner diameter, 1.3 mm) were prepared. Prepared grafts were kept in sterilized phosphate buffer solution (PBS) and used for further experiments within 1 week.

Control grafts made of Silastic tubes (length, 10 mm; outer diameter, 1.1 mm; inner diameter, 0.7 mm) were prepared. Silastic tubing (Imamura, Tokyo, Japan) was purchased and cut to pieces of 10 mm in length. They were then cleaned with acetone, ultrasonically washed in distilled water, serialized with ethylene oxide gas, and used for the animal experiments.

Implantation

The experiment was conducted in compliance with the guidelines issued by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University, and the study protocol was approved by the committee (Approval No. 0070175). Experimental animals were anesthetized with ketamine. A segmental defect of 10 mm in length was created in the carotid artery. The decellularized graft was implanted to the arterial defect and anastomosed using 8-0 nylon sutures in 10 full-thickness-layer simple stitches. The artery and grafts were flushed with heparin-added saline several times during the surgery. Fibrin gel (6:1 ratio of 5% fibrinogen from bovine plasma to 1% thrombin from bovine plasma) was applied over the anastomosis sites for sealing. On the other hand, Silastic tube graft was anastomosed with putting over the both ends of artery over the tube and ligaturing them by nylon sutures, since the tube was so hard and needlework was proved very troublesome. No anticoagulant or antiplatelet agents were administered after surgery. The number of specimens was 12 for the decellularized scaffolds and 5 for the Silastic tube grafts.

Harvesting of samples

Four weeks after surgery, the experimental animals were anesthetized with ketamine and the implantation sites were revealed to examine the pulsatility of the grafts and the artery. The grafts were retrieved together with 3-mm lengths of proximal and distal parts of the artery. The inner lumen was gently rinsed with PBS, and the thrombus formation in the grafts was examined. Six of the retrieved scaffold samples were subjected to mechanical testing, and the other 6 were fixed with 4% paraformaldehyde and subjected to histological observation.

Mechanical testing

Mechanical tests were performed to determine the biomechanical characteristics of the decellularized vascular scaffolds and normal carotid arteries. Decellularized scaffolds (n = 6) harvested 4 weeks after implantation were examined in this mechanical test. Decellularized scaffolds (n = 5) immediately after processing were also measured. Normal abdominal aorta (n = 5) from 12-week-old SD rats were served as control.

In the test, the specimen was kept in a tissue bath filled with PBS at 37°C. Internal pressure was applied at a constant rate of 60 mmHg/min up to 140 mmHg. The change in diameter was monitored by a charge-coupled-device camera, and the relation between the internal pressure and the external diameter was measured. From the acquired relations between the pressure and the diameter, the curve within the region of 70–120 mmHg was fit to the equation

\[
\ln \left( \frac{p}{p_0} \right) = \beta \left( \frac{d}{d_s} - 1 \right)
\]
Reconstruction of small diameter arteries

and the stiffness parameter, $\beta$, was determined. In this equation, $p$ denotes pressure, $p_s$ represents reference pressure (set to 95 mmHg in this study), $d$ denotes diameter, and $d_s$ is the reference diameter at $p = p_s$. This parameter corresponds to the reciprocal of the compliance.$^{19, 20}$

Histological observation

Histological observation was performed on paraffin-embedded thin sections stained with hematoxylin and eosin (HE). Immunostaining was performed for endothelial cells with factor VIII-related antigen and for smooth muscle cells with alpha-smooth muscle actin. The sections were examined by light microscopy.

Statistical analysis

The difference in the stiffness parameter, $\beta$, between the decellularized scaffolds immediately after processing, decellularized scaffold grafts after 4 weeks implantation, and the carotid artery was examined using one-way ANOVA and t-test. The difference in patency between the decellularized scaffolds and the control grafts was examined with Fisher’s exact test. All statistical analyses were performed using the “R” software (version 2.13.0: http://www.r-project.org/).

Differences were considered statistically significant if $P < 0.05$.

Results

Macroscopic observation and patency

Figures 1 and 2 are typical views of the decellularized vascular scaffolds graft and Silastic tube graft respectively just after implantation and at 4 weeks after implantation. Table 1 presents the results of the animal experiments. At 4 weeks after implantation, decellularized vascular grafts demonstrated patency in 10 of 12 animals. In these samples, the pulsatility of the grafts was comparable to that of normal arteries. No inflammation was detected, and capillary vessels lined

<table>
<thead>
<tr>
<th>Grafts</th>
<th>Patent</th>
<th>Occluded</th>
<th>Graft patency</th>
</tr>
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<tbody>
<tr>
<td>Decellularized vascular scaffolds</td>
<td>10</td>
<td>2</td>
<td>83%</td>
</tr>
<tr>
<td>Silastic tube grafts</td>
<td>0</td>
<td>5</td>
<td>0%</td>
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Table 1. Patency of the decellularized vascular scaffold grafts and of the Silastic tube grafts at 4 weeks post-implantation. Significant difference ($P < 0.05$) was confirmed between their patencies.

Figure 1. Macroscopic observation of the decellularized vascular scaffold grafts immediately after implantation and at 4 weeks after implantation. Arrowheads indicate the grafts. (A) The decellularized vascular scaffold grafts immediately after implantation. (B) The decellularized vascular scaffold grafts at 4 weeks after implantation. (C) Cross-section from the central region of the graft in (B). No thrombus was found in the lumen.

Figure 2. Macroscopic observation of the Silastic tube grafts immediately after implantation and at 4 weeks after implantation. Arrowheads indicate the grafts. (A) The Silastic tube grafts immediately after implantation. (B) The Silastic tube grafts at 4 weeks after implantation. (C) Cross-section from the central region of the graft in (B). The graft was completely occluded.
the surface of the grafts. No blood clots were detected in the lumen of the grafts. In the remaining animal, the graft was occluded. A blood clot was discovered around the region of anastomosis between the graft and artery. The cause of the occlusion was believed to be a procedural complication of anastomosis during surgery. All of the Silastic tubes were occluded in control animals. No pulsatility was detected at the time of retrieval and the entire lumen was filled with thrombi, and no intima was observed. Thus, the patency of the decellularized scaffolds and the control grafts were 83% and 0%, respectively. Fisher’s exact test confirmed the significance of this difference (P < 0.05).

Mechanical Test

Figure 3 shows the mechanical response of the decellularized vascular scaffolds just after preparation and 4 weeks after implantation with patency, as well as that of carotid artery. The mean values are plotted with their SD. The horizontal axis shows the internal pressure, and the vertical axis represents the external diameter normalized by the diameter in the pressure-free state. As shown in the figure, the decellularized scaffolds demonstrated an elastic property under small internal pressure whereas they exhibited stiffness under higher pressure. Thus they had similar mechanical characteristics as that of normal arteries.

Figure 4 shows the measured stiffness parameters (β) for the 3 sample types. Although the decellularized scaffolds had a smaller mean value of β than normal abdominal aorta (8.62 vs. 10.02), the value of β increased to 12.50 at 4 weeks after implantation. However, no statistically significant differences were detected (P > 0.05).

Histological findings

Figure 5 shows the HE stained and immunostained histological sections for the typical patent case of the decellularized scaffold. The internal lumen of the scaffold was completely lined by endothelial-like (factor VIII positive) cells. Smooth muscle-like (alpha-smooth muscle actin positive) cells migrated into the wall of the decellularized scaffold and bridged the scaffolds from end to end. Although the neointima formation and thickening was evident, the cell population was observed to be relatively poor in the main substance of scaffolds. Figure 6 shows an HE-stained section of the Silastic tube graft. The Silastic tubes were completely occluded, and well-developed thrombi were observed. Fibroblast-like cells were found in the thrombi, and fibrous organization was apparent.
We performed reconstruction of an artery of < 2.0 mm in diameter and observed good patency with the use of decellularized vascular allograft scaffolds. In addition to such advantages of these grafts that they are free from serious infectious risks involved in xenografts and that greater number of grafts from heart-dead might be expected than the living grafts from brain-dead, good patency for such a small artery demonstrates the particular merits of the decellularized scaffold.

One of the most prominent characteristic is their excellent biocompatibility. For scaffolds derived from even different species, most of the antigens are removed in the decellularization treatment such that prepared scaffolds would not evoke immunological response inflammation. Since the scaffolds in this study were allografts, favorable biocompatibility was expected. Furthermore, the matrix of the decellularized scaffold itself may enhance cell invasion. For porous vascular grafts, myofibroblasts and endothelial cells from capillary vessels are observed to migrate through.
the porosity. In the present study, although the detailed pathway of cell migration was not examined, the matrix of the grafts was believed to be beneficial for cell migration.

Another important characteristic of arterial grafts is compliance. Occlusion is believed to be related to compliance mismatch. Most clinically utilized artificial vessels made of nonabsorbable synthetic polymers such as expanded polytetrafluoroethylene (ePTFE) or Dacron, lack extensibility and therefore have lower compliance than natural arteries. Thus, compliance mismatch arises in these grafts at sites of anastomosis and creates cross-sectional changes under pulsation that induce a disturbance in blood flow and stagnation. These hemodynamics are believed to lead to neointimal hyperplasia followed by occlusion. Further, it may decrease wall shear stress and induce intimal thickening. Several attempts have been made to develop grafts with similar compliance to natural arteries; however, the presently available synthetic materials of low elastic modulus have insufficient strength and durability. Grafts with compliance that is comparable to natural arteries could not be achieved solely with these materials. An attempt to achieve ideal mechanical characteristics by adopting a coaxial double-tubular graft was also proposed, but the graft may have been too complicated for use in clinics.

In this study, the decellularized grafts just after preparation had comparable or slightly more compliant mechanical characteristics to natural arteries. This result coincides with reports on decellularized grafts from porcine carotid arteries and human umbilical veins. Damages to collagen fibers and removal of proteoglycans are considered to be responsible for such slight decrease in the stiffness. Compliance matching to the host artery was achieved in these grafts, and this matching was considered to be beneficial to the patency of the graft. On the other hand, the grafts showed greater stiffness after 4 weeks implantation. The invasion of cells into the grafts and the synthesis of extracellular matrix in the grafts by these cells may be responsible for such increase in stiffness. However, although they were within the range reported for normal arteries by Kawasaki, in which β ranged from 9.41-15.31 for brachial and femoral arteries, the statistically significant difference was not detected due to large individual variations. If we could evaluate the stiffness of the same specimen before and after the implantation, the paired t-test might detect the significant difference. However, we had to abandon such experiments since the mechanical test could not be carried out under aseptic condition and the serialization processes had strong impact on the mechanical properties of the specimen. Contrarily, the stiffness of the Silastic tube graft was estimated to be very large. If we notice the well known pressure-diameter relation for thin wall cylinders as

\[
\Delta d = \frac{d^2}{2tE} \cdot \Delta p
\]

in which \( t \) is the tube thickness and \( E \) the elastic modulus of the tube, and utilizing the reported material's mechanical properties of the Silastic, the stiffness parameter \( \beta \) of the control graft turned to be ca. 143. The fact that this value was more than ten times greater than that of the decellularized grafts might be one of the possible reasons why all of the Silastic tube grafts were occluded.

Although Silastic tubes were utilized as controls in this paper, allograft vessels without decellularization processing might be adopted as controls. Actually, in the pilot experiments of this study, we prepared such allografts from abdominal arteries that were cryopreserved without use of cryoprotectant solution and performed implantation to rat carotid arteries. Only one allograft from six was patent at four weeks after surgery. Mismatched histocompatibility and inferior cell viability were considered to yield such a high incidence of occlusion. Thus, allografts with inferior cell viability would bring us the same conclusion as Silastic tube controls. However, noticing the fact that the patency of fresh allografts was significantly better than that of the cryopreserved allografts, the patency of allograft was strongly dependent on their cell viability. Thereby allografts were not considered adequate as controls. Furthermore, we were speculating that the mechanical compatibility and biocompatibility had crucial importance for the success of vascular grafts. Allografts were known to have superior mechanical compatibility whereas have less biocompatibility due to mismatched histocompatibility. Hence, we adopted Silastic tubes as controls, which had good biocompatibility with inferior mechanical compatibility.

The decellularized scaffolds were anastomosed with conventional suture technique whereas the control Silastic tubes were anastomosed with the method based on the cuff technique. Such difference might have impact on the experimental results. Nevertheless, the cuff technique was commonly used in many animal experiments involving the minute handling of small vessels and was proved to yield successful anastomosis at least in relatively short period of four weeks after surgery. Further, the diameter of the Silastic tubes was matched to that of arteries.
at implanted site. Since hemodynamic factors play an important role in the thrombus formation, the matched size of graft would reduce the occurrence of hemodynamic disturbance and decrease the risk of occlusion. Thus we consider that the difference in the anastomosis technique did not have significant effect in the experimental results.

Lining of the internal lumen with endothelial cells is also important to ensure patency of the graft. In this paper, we confirmed complete bridging of endothelial-like cells over the graft as early as 4 weeks after implantation. Such rapid endothelialization would be attributed to the advantage of the decellularized scaffolds, since enhanced cellular infiltration was demonstrated for large diameter arteries and heart valves. Recently Negishi et al. reported the good endothelialization of the decellularized xenograft scaffolds utilizing the cuff method for anastomosis to realize the direct contact between the arteries and the grafts. Our results were consistent with them although the anastomosis method utilized was the standard suture technique, which was considered less favorable for endothelial cell immigration across the anastomosis sites since the ends of the arteries was only approximated to the grafts. The fact that the rapid endothelialization was proved under the situation similar to the clinical applications was meaningful from the practical utilization viewpoint.

Furthermore, the mechanical characteristic of the scaffolds similar to natural tissues possibly provided another advantage. Zhang et al. observed rapid endothelialization for elastic microporous polyurethane grafts (10 mm in length) implanted into the rat abdominal aorta whereas not for rigid ePTFE grafts. Noting that the grafts received repeated stretching by blood pressure and that endothelial cells and smooth muscle cells recognize substantial stretching and increase their proliferation, the low compliance of the scaffolds is considered beneficial for early endothelialization.

Although good patency of the decellularized scaffolds was demonstrated in this study, the length of the grafts was 10 mm and the experimental period was only 4 weeks. Risk of aneurysm formation cannot be denied since the matrix of the grafts was damaged in the process of decellularization. Longer grafts should be examined over a longer period of implantation to develop these arterial grafts for clinical use.

Conclusions

The possibility of the reconstruction of small arteries (< 2 mm) using decellularized scaffolds was demonstrated. Good patency of the scaffolds at 4 weeks after implantation was observed. The internal cavity of the scaffold was completely lined by endothelial-like cells. A match in compliance and the favorable material for cell migration were believed to contribute to the increased patency.

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