MAIN TEXT

A tissue-engineered, decellularized, connective tissue membrane for allogeneic arterial patch implantation

Masashi Yamanami[1] (iD) | **Keiichi Kanda[1]** (iD) | **Kazuki Morimoto[1]** |
Tomoya Inoue[1] | **Taiji Watanabe[1,2]** | **Osamu Sakai[1,3]** | **Daisuke Kami[4]** |
Satoshi Gojo[4] | **Hitoshi Yaku[1]**

[1]Department of Cardiovascular Surgery, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

[2]Department of Cardiovascular Surgery, Japanese Red Cross Kyoto Daiichi Hospital, Kyoto, Japan

[3]Department of General and Cardiothoracic Surgery, Graduate School of Medicine, Gifu University, Gifu, Japan

[4]Department of Regenerative Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

Correspondence
Keiichi Kanda, Department of Cardiovascular Surgery, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan.
Email: kei@koto.kpu-m.ac.jp

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Abstract

Background: We have previously applied in vivo tissue-engineered vascular grafts constructed in patients' subcutaneous spaces. However, since the formation of these vascular grafts depends on host health, their application is challenging in patients with suppressed regenerative ability. Therefore, the allogeneic implantation of grafts from healthy donors needs to be evaluated. This study aimed to fabricate allogeneic cardiovascular grafts in animals.

Materials and methods: Silicone rod molds were implanted into subcutaneous pouches in dogs; the implants, along with surrounding connective tissues, were harvested after four weeks. Tubular connective tissues were decellularized and stored before they were cut open, trimmed to elliptical sheets, and implanted into the common carotid arteries of another dog as vascular patches ($n = 6$); these were resected and histologically evaluated at 1, 2, and 4 weeks after implantation.

Results: No aneurysmal changes were observed by echocardiography. Histologically, we observed neointima formation on the luminal graft surface and graft wall cell infiltration. At 2 and 4 weeks after implantation, α-SMA-positive cells were observed in the neointima and graft wall. At 4 weeks after implantation, the endothelial lining was observed at the grafts' luminal surfaces.

Conclusion: Our data suggest that decellularized connective tissue membranes can be prepared and stored for later use as allogeneic cardiovascular grafts.

KEYWORDS
allogeneic arterial patch implantation, connective tissue, decellularization, tissue engineering

1 | INTRODUCTION

Materials with high biocompatibility and growth potential are ideal for achieving revascularization in congenital heart disease.[1] However, neither artificial materials nor biomaterials of xenogeneic origins, which are currently widely applied, can completely satisfy these conditions.[2] In recent years, tissue engineering approaches have been used for the development of biocompatible grafts.[3–6] Several kinds of vascular grafts are being developed using in vivo tissue engineering, which utilizes the patient's body as a bioreactor.[7–10] Previously, we reported that in vivo tissue-engineered vascular tissues, easily constructed in the subcutaneous tissues of the tissue recipients, functioned as superior grafts in animal experiments.[8,9] We have applied this technology to pulmonary arterial patch augmentation in a 2-year-old patient and reported a good clinical course.[11] Now, more than five years later, the postoperative course remains excellent.[12]

Autologous vascular grafts can be fabricated using connective tissue membranes prepared using the encapsulation phenomenon that occurs when foreign materials are implanted under the skin. However, this process requires at least four weeks.[8,9] Moreover, since the formation of vascular grafts depends on the health of the graft recipient, who often is a high-risk or very young patient, it may be challenging to use these grafts in patients with severe disease in which regenerative activity is suppressed because the tissues may not be fully mature. Therefore, the possibility of allogeneic grafts from healthy donors to patients should be evaluated. The objective of this study was thus to fabricate such allogeneic grafts using animals.

2 | METHODS

2.1 | Preparation of tubular connective tissues

All experimental procedures and protocols followed ARRIVE guidelines.[13] We are committed to the 3Rs of laboratory animal research. All experimental procedures and protocols were approved by the Animal Experiment Ethics Committee of the Kyoto Prefectural University of Medicine (Protocol 29-16) and were performed in accordance with the US Animal Welfare Act.

A total of four adult female dogs (Toyo beagles, 2 years old, weight ~10 kg) were purchased from Oriental BioService, Inc (Kyoto, Japan). One dog was used for tubular connective tissue preparation, and the other three were used in the implantation experiments. Four dogs were randomly assigned. All dogs were kept in single cages with ad libitum water and standard food. Since dogs had been subjected to irreversible and extensive invasion at the end of the experiment, dogs were euthanized using an intravenous injection of a pentobarbital sodium overdose (Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan).

Five silicone rods (diameter 5 mm and length 10 cm; 3-2316-04, AS ONE, Osaka, Japan) were used as molds (Figure 1A). Dogs were anesthetized using an intramuscular injection of ketamine (20 mg/kg; Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and xylazine (0.1 mg/kg; Bayer Yakuhin, Ltd., Tokyo, Japan). Small incisions were made in the shaved dorsal skin, and five molds were placed in dorsal subcutaneous pouches. After four weeks, dogs were anesthetized, and the implants were harvested. After the silicone rod molds were removed, the tubular connective tissue tubes were harvested. Five tubular connective tissues were obtained, one of which was used for in vitro decellularization experiments and the other used as a control. The other three were decellularized and used in transplantation experiments.

2.2 | Decellularization

Tubular connective tissues were connected to a perfusion pump (TP-10SA; AS ONE) and decellularized in a sterile beaker in a 37°C bath by perfusion with 1% sodium lauryl ether sulfate (SLES; Sanyo Kasei, Kyoto, Japan) for 3 h, deionized water for 15 min, 1% Triton X-100 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 30 min, and 500 U/ml DNase (Worthington Biochemical Corp., Lakewood, NJ, USA) in phosphate-buffered saline (Takara Bio Inc., Shiga, Japan) for 3 h. Each perfusion was followed by a rinse with deionized water. (Figure 1D) Perfusion rates were maintained at 2 ml/min. Decellularized tubular connective tissues were stored at −20°C until further analysis. This decellularization protocol was modified from the protocol reported by Kawasaki et al.[14]

2.3 | DNA quantification and histology

Decellularized and untreated tubular connective tissues were desiccated in a dry heat incubator for 12 h until the tissue fragment weight reached a plateau. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). The Quaut-iT PicoGreen kit (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used to quantify DNA at 485 nm using an Infinite F-200 fluorescence reader (Tecan Group, Männedorf, Switzerland), using a lambda DNA standard curve. Haematoxylin-eosin (H&E) and 4′,6-diamidino-2-phenylindole (DAPI) staining of serial

FIGURE 1 Preparation of tubular connective tissues. (A) Silicone rods (diameter 5 mm, length 10 cm) used as molds. (B) Molds embedded into dorsal subcutaneous pouches became completely encapsulated in connective tissue. (C) Decellularized tubular connective tissue. Scale bar = 10 mm. (D) Schematic of the decellularization process. PBS, phosphate-buffered saline; SLES, sodium lauryl ether sulfate; Tx-100, Triton X-100 [Color figure can be viewed at wileyonlinelibrary.com]
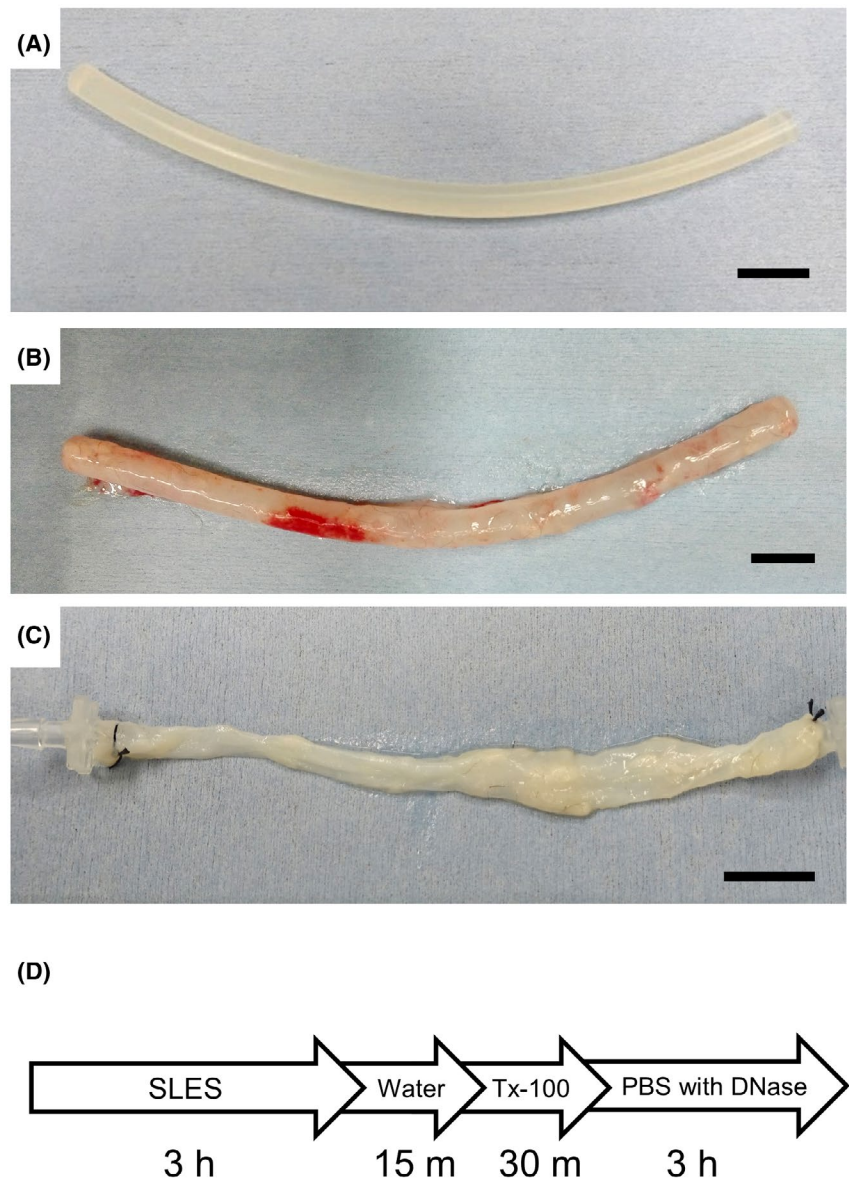
sections of the tubular connective tissues were performed to assess the uniform removal of the cellular component.

2.4 | Implantation of patch grafts

Three dogs were anesthetized using intramuscular injection of ketamine (20 mg/kg) and xylazine (0.1 mg/kg). The decellularized tubular connective tissues were stored at −20°C for one week before they were naturally thawed, cut open, and trimmed to an elliptical sheet of 15 × 8 mm. A neck midline incision was made to expose both common carotid arteries. After heparin sodium (100 U/kg; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) injection, the carotid arteries were clamped. The anterior walls of the carotid arteries were cut into elliptical sheets, which

were allo-transplanted to the carotid arteries as vascular patches using 7-0 polypropylene (Prolene; Ethicon, Inc., Somerville, NJ, USA) running sutures ($n = 6$). The wound was closed using 4-0 nylon (NB204; Kono Seisakusho Co., Ltd., Chiba, Japan) sutures. Thereafter, dogs had free access to standard food and water. After implantation, no antiplatelet, anticoagulant, or immunosuppressive agents were administered.

2.5 | Ultrasonography

The dogs were followed until 1, 2, and 4 weeks after implantation, and the status of their patch grafts was evaluated using Doppler ultrasonography (S6V; SonoScape Medical Corp., Shenzhen, China).

2.6 | Macroscopic observation of harvested grafts

Patch grafts were resected 1, 2, and 4 weeks after implantation (each group $n = 2$). Dogs were anesthetized using an intramuscular injection of ketamine (20 mg/kg) and xylazine (0.1 mg/kg). After heparin sodium (100 U/kg) injection, the common carotid arteries containing the vascular patch grafts were harvested. The harvested arteries were cut open longitudinally, and grafts were macroscopically scored.

2.7 | Histology and immunohistochemistry

Patch-graft specimens were fixed with 10% formalin; embedded in paraffin; sliced into short-axis cross-sections; and stained with H&E, Masson's trichrome (MT), Elastica van Gieson (EVG), or von Kossa stain. Immunohistochemistry was performed using monoclonal antibodies against α-smooth muscle actin (α-SMA; M0851; Dako Japan, Kyoto, Japan; 1:100 dilution) and von Willebrand factor (vWF; A0082; Dako Japan, Kyoto, Japan; 1:5000 dilution).

3 | RESULTS

3.1 | Preparation of tubular connective tissues

The assembled molds embedded in subcutaneous pouches of the beagles for four weeks showed complete connective tissue encapsulation. The implants were readily harvested because the developed connective tissue and the subcutaneous tissue were connected only by fragile, irregular, and redundant tissues, which could be dissected easily (Figure 1B). The silicone rods could be smoothly removed from the end of the implants because there was no adhesion between the substrates and the developed connective tissues.

3.2 | Decellularization

After seven hours of decellularization, the tubular connective tissues became translucent and white macroscopically (Figure 1C). Histological changes are shown in Figure 2. Before decellularization, the tubular connective tissues contained many nuclei (Figure 2A,B), whereas, after decellularization, all cell components were completely removed (Figure 2C,D). After decellularization, the total
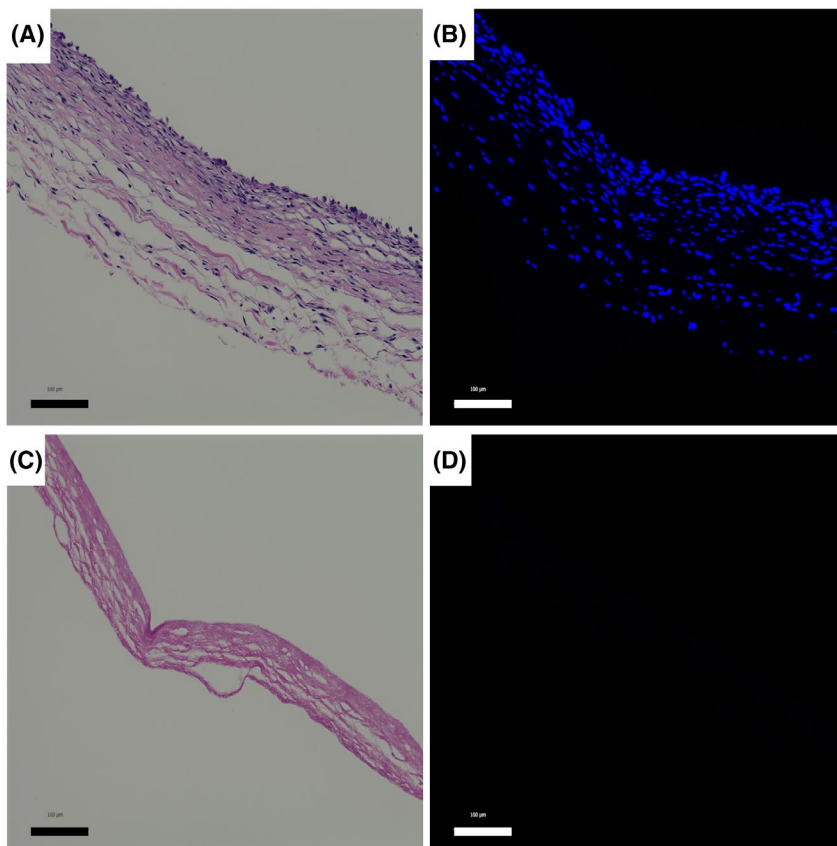

FIGURE 2 Histological changes produced by decellularization. Haematoxylin and eosin (H&E; A,C) and 4′,6-diamidino-2-phenylindole (DAPI; B,D) staining of a short-axis cross-section of tubular connective tissue before (A,B) and after (C,D) decellularization. The upper side is the luminal surface. Scale bar = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]

DNA content of the decellularized tubular connective tissues was 0.83 ± 0.34 ng/mg dry weight (mean ± 1 standard deviation). The amount of DNA in the control tubular connective tissues was 492 ± 132 ng/mg dry weight.

3.3 | Implantation of decellularized connective tissues as patch grafts

Arterial patch transplantation of the decellularized connective tissue graft (Figure 3A) was easily performed using general vascular surgical procedures. After declamping, the implanted patch pulsated with little bleeding and without rupture or aneurysm formation (Figure 3B).

3.4 | Ultrasonographic evaluation

The luminal surfaces of the grafts were thickened one week after implantation, suggesting thrombus formation (Figure 4A). At two and four weeks after implantation, the luminal surfaces of the grafts became smooth, and no aneurysmal changes were observed (Figure 4B,C). Good blood flow at all times was confirmed using color Doppler (Figure 4D–F).

3.5 | Macroscopic observations

No abnormal overt inflammation or liquid pooling around the grafts was observed. Slight adhesions were observed around the patch grafts at both one and two weeks after implantation (Figure 5A,B). At four weeks after implantation, mild adhesion was observed around the patch grafts (Figure 5C); graft separation from the surrounding tissue was very easy. Before graft removal, good blood flow pulsation was observed over the entire length of the graft, and the thrill indicating local stenosis could not be palpated. Macroscopic observation revealed that the inner surfaces of the patch grafts one week after implantation were covered with fibrin (Figure 5D). At 2 weeks, inner surfaces of the
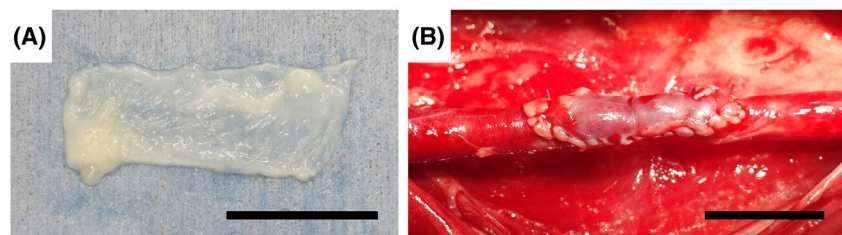

FIGURE 3 Implantation of patch grafts. Decellularized tubular connective tissues were cut longitudinally (A) and trimmed to an elliptical sheet of 15 × 8 mm. The resulting sheets were allo-transplanted as vascular patches (B). Scale bar = 10 mm [Color figure can be viewed at wileyonlinelibrary.com]
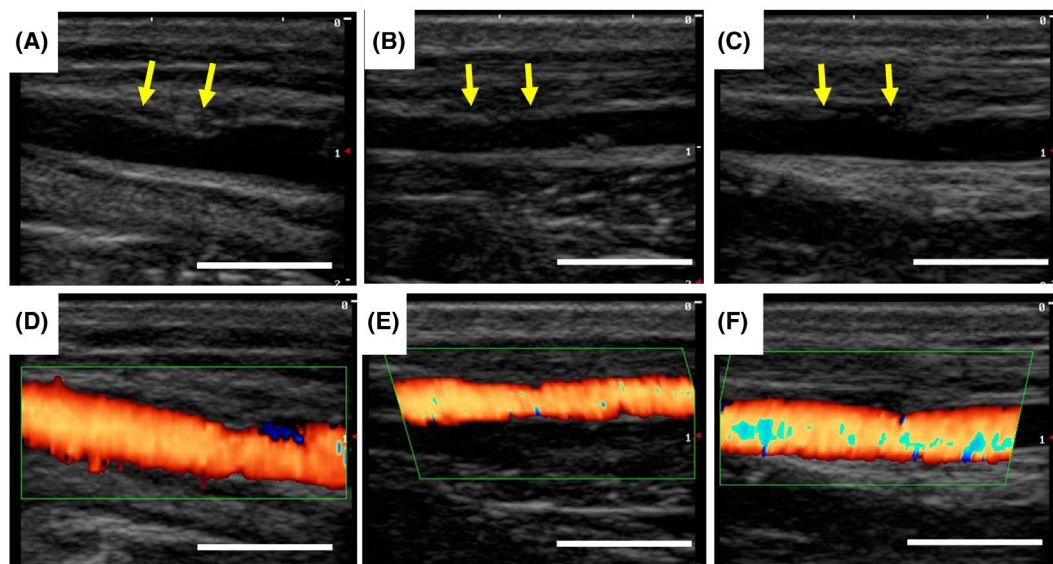

FIGURE 4 Postoperative echocardiography at 1 (A,D), 2 (B,E), and 4 (C,F) weeks after implantation (A–C, B-mode imaging; D–F, Doppler echocardiography) showing graft thickening at 1 week after implantation. At 2 and 4 weeks after implantation, the patch grafts became smooth, and no aneurysmal changes were observed. Yellow arrows indicate the patch-graft regions. The left side is the proximal side. Scale bar = 10 mm [Color figure can be viewed at wileyonlinelibrary.com]
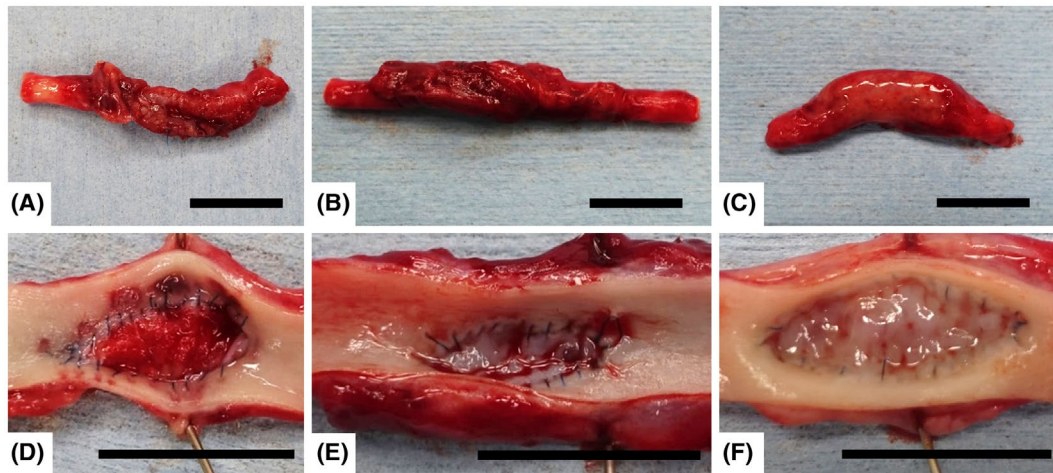
FIGURE 5 Macroscopic observation of harvested grafts. Vascular patch grafts resected at 1 (A,D), 2 (B,E), and 4 (C,F) weeks after implantation. Macroscopic appearance of the grafts (A–C). Inner surfaces of the patch grafts at 1 week were covered with fibrin (D). At 2 weeks, the inner surfaces of the grafts were partially covered with neointima (white-colored) near the anastomotic site; however, fibrin was still present, especially at the center of the patches (E). At 4 weeks, the inner surfaces of the graft were almost completely covered with neointima (F). The left side is the proximal side. Scale bar = 10 mm [Color figure can be viewed at wileyonlinelibrary.com]

grafts were partially covered with neointima near the anastomotic site; however, fibrin was still present, especially at the center of the patches (Figure 5E). At four weeks after implantation, the inner surfaces of the grafts were almost completely covered with neointima, with no intimal hyperplasia of the anastomosis site observed (Figure 5F).

3.6 | Histological observations

The boundaries of the patch grafts became obscure over time (Figures 6 and 7A,G,M). Infiltration of the vascular wall with cells derived from the host into decellularized allogeneic connective tissue progressed with time. MT staining revealed that the grafts at one week after implantation were composed of collagen-rich tissue, while at four weeks after implantation, collagen tissue became obscure (Figure 7B,H,N). EVG staining revealed an elastic fiber network in the neointima at four weeks after implantation (Figure 7C,I,O). Von Kossa staining revealed low-level calcification in all the grafts (Figure 7D,J,P,). Staining for α-smooth muscle actin (α-SMA) revealed α-SMA-positive cells in the neointima and graft wall at two and four weeks after implantation (Figure 7E,K,Q). Staining with von Willebrand factor (vWF) revealed endothelial lining of the luminal surfaces of the grafts at four weeks after implantation (Figure 7F,L,R).

4 | DISCUSSION

Aiming to fabricate an ideal vascular graft, multiple tissue-engineered vascular grafts (TEVGs) have been developed using in vitro and/or in vivo tissue engineering technology, some of which have been clinically applied.[3,5,6,15]

In vivo tissue engineering uses the patient's body as a bioreactor. Several groups have reported the development of a vascular graft that exploits the encapsulation that occurs when foreign materials are implanted under the skin.[7,10] We have also reported good results from multiple animal transplantation experiments of grafts made using in vivo tissue engineering techniques.[8,9] We have clinically applied a TEVG as a pulmonary artery dilatation patch for congenital heart disease with good results.[11] However, in pediatric cases, not only is the subcutaneous area in which the base material is embedded limited but good capsule formation may also not be achieved due to poor nutrition related to the underlying disease. Therefore, we reasoned that allogeneic transplantation from healthy adult donors should be considered for these cases.

In the case of allograft use, it is necessary to attenuate the antigenicity of the graft by, for example, crosslinking using glutaraldehyde and decellularization. However, glutaraldehyde treatment not only produces cytotoxicity in the tissues surrounding the graft if not washed out completely but can also make grafts more thrombogenic and susceptible to calcification.[16,17] Therefore, we hypothesized that decellularization would be a more suitable method for attenuating antigenicity. Decellularization is achieved using high-pressure treatment[18] or, more commonly, a detergent.[19]

Sodium dodecyl sulfate (SDS) is a detergent widely used for decellularization, but it has been reported to cause substantial ECM damage to acellular scaffolds.[19] Therefore, we developed a decellularization protocol

FIGURE 6 Histological examination of grafts. Low-power micrographs of H&E-stained short-axis cross-sections of the grafts at 1 (A), 2 (B), and 4 (C) weeks after implantation. The boundaries of the grafts became obscure over time. Infiltration of the vascular wall cells derived from the host into decellularized allogeneic connective tissue progressed with time. Black arrows indicate anastomoses. Black boxes indicate the regions enlarged in Figure 7. The upper side is the luminal surface. Scale bar = 500 μm [Color figure can be viewed at wileyonlinelibrary.com]
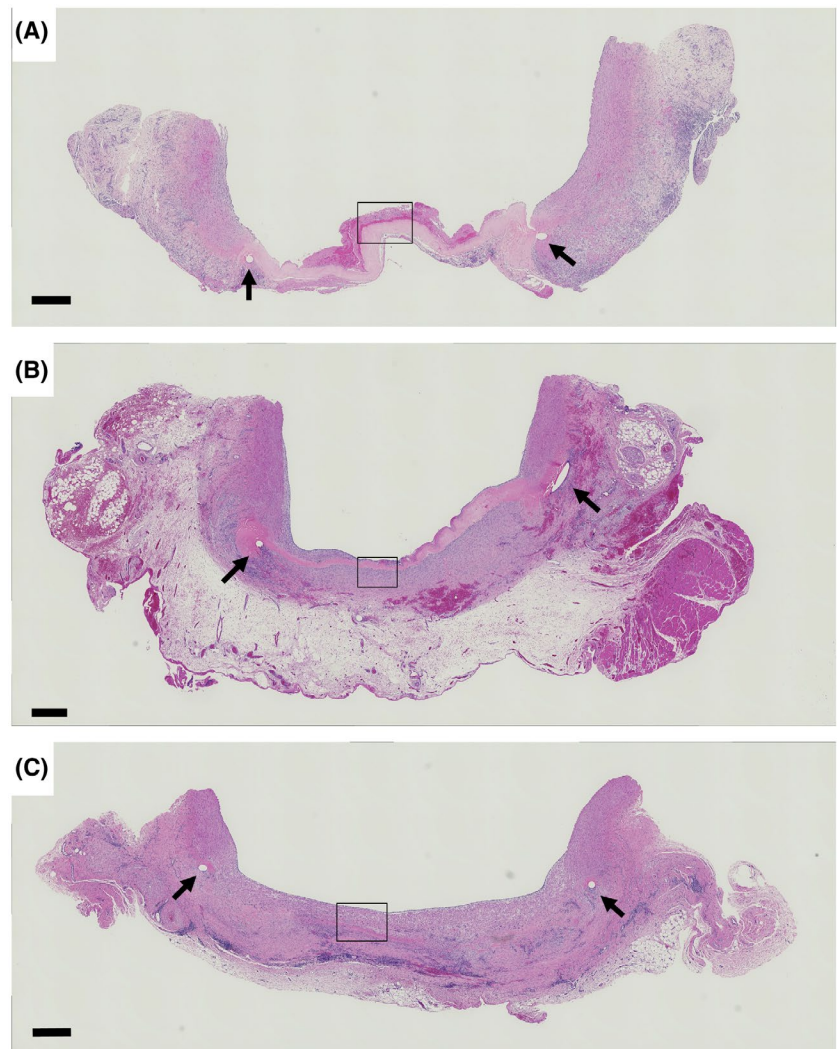
using SLES, a novel surfactant reported to decrease ECM damage.[14,20] However, as our perfusion decellularization protocol was designed for parenchymal organs such as the heart, it would be time-consuming to use.[14] Since, unlike parenchymal organs, the tubular connective tissues used in this study were extremely thin and had a simple structure, we hypothesized that the duration of the decellularization treatment could be shortened. Thus, we established a protocol that decreased perfusion time with SLES from 12 to 3 h and the perfusion time with DNase-added phosphate-buffered saline (PBS) from 24 to 3 h. After decellularization, the total DNA content was less than 50 ng/mg dry tissue weight, indicative of complete removal of the genetic material, based on a previous report.[21] The tubular connective tissues were reliably decellularized using this shortened protocol. As a result, the total process time for decellularization was reduced from approximately 37 h to approximately 7 h. Therefore, we transplanted vascular patches into beagles using decellularized grafts prepared using this protocol.

All grafts performed well in the arterial pressure environment without any occurrence of aneurysm or rupture for 4 weeks after implantation. Hence, we hypothesized that as these decellularized grafts could withstand high-pressure environments, they could also be safely applied to pulmonary arteries.

Three elements, scaffolds, (stem) cells, and growth factors, are necessary components in tissue engineering.[22] To generate living and functional vascular grafts, most TEVGs have been incorporated with autologous cells at the beginning of their development.[3,5,23] L'Heureux et al reported the clinical application of TEVG in an arteriovenous (AV) shunt-based on allogeneic cell-sheet technology.[24] However, these grafts had been dehydrated, and subsequent cell seeding had not been performed. Another group reported the results of a phase-II trial of an AV shunt for end-stage renal disease patients using another TEVG, Humacyte.[6] These grafts were manufactured by seeding a 6-mm polyglycolic acid (PGA) scaffold with smooth muscle cells (SMCs) derived from organs and tissues of deceased allogeneic donors, which were decellularized
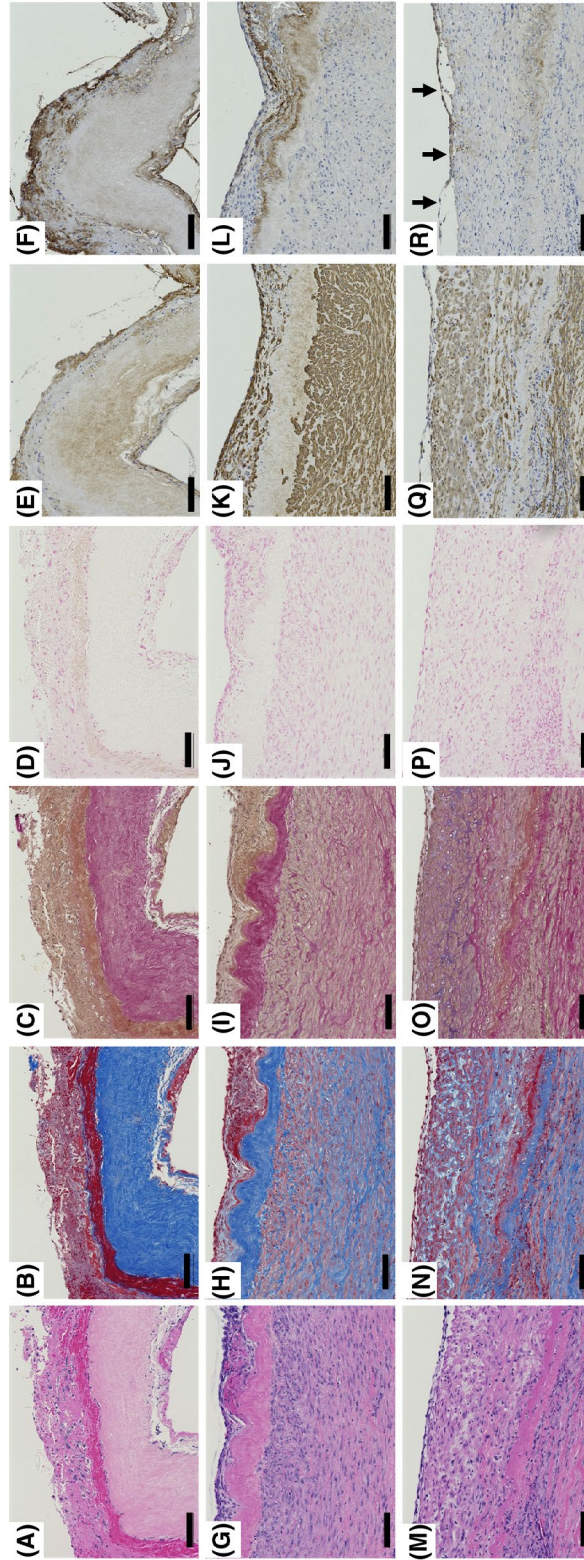
FIGURE 7 Histological examination of grafts stained with hematoxylin and eosin, Masson's trichrome, Elastica van Gieson, and von Kossa stains. Short-axis cross-sections of the grafts were obtained at 1 (A–F), 2 (G–L), and 4 (M–R) weeks after implantation. Staining with hematoxylin and eosin (A,G,M), Masson's trichrome (B,H,N), Elastica van Gieson (C,I,O), and von Kossa (D,J,P) stains. At 1 week, the graft was composed of collagen-rich tissue (blue) (B), while at 4 weeks, collagen tissue became obscure (N). Elasticavan Giesson staining revealed an elastic fiber network in the neointima (purple) at 4 weeks (O). Von Kossa staining revealed little calcification (black) in all grafts (D,J,P). Immunohistological staining for α-smooth muscle actin (α-SMA) (E,K,Q) revealed α-SMA-positive cells (brown) in the neointima and graft wall at 2 (K) and 4 (Q) weeks. Staining with von Willebrand factor (F,L,R) revealed endothelial lining at the luminal surfaces of the grafts at 4 weeks (R, black arrows). In all images, the upper side is the luminal surface. Scale bar = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]

following ECM production in an incubator equipped with a pulsatile pump. Both grafts were constructed using allogeneic vascular wall cells; however, before implantation, the incorporated cells were deactivated or removed. Our in vivo tissue-engineered autologous vascular graft was treated with 70% ethanol before clinical application for a pediatric pulmonary artery patch plasty to improve surgical handling.[11] In this TEVG, the incorporated cells were dead. Although none of these grafts were incorporated with functioning living cells, they actively stimulated tissue regeneration, providing excellent patency and vascular wall healing. The need for cell seeding is controversial. These clinical reports suggest that the incorporation of living cells in TEVGs is not always necessary if the TEVGs are composed of excellent extracellular matrices that induce rapid tissue regeneration.

In contrast to autologous grafts, the allogeneic decellularized TEVGs used in this study were depleted of cellular components. Although the amounts of glycosaminoglycans and growth factors that remained in the grafts are unknown, tissue regeneration, similar to that observed with the use of autologous grafts, was induced. Slight cell infiltration into the graft was observed one week after implantation, good cell infiltration was observed at two weeks, and almost all layers were infiltrated at four weeks. We consider the cells infiltrating into the graft wall to be derived from the native blood vessels in contact with the surrounding tissues and the graft. Endothelialization of the luminal graft surface is a result of invasion by native blood vessels. Cell infiltration into the graft was observed early after transplantation, and vascular wall reconstruction by vascular wall constituent cells was observed. Therefore, the biocompatibility of the vascular graft prepared using this technique is excellent.

Since graft preparation using the in vivo tissue engineering technology that we have developed takes place in the body, grafts can be fabricated at a very low-cost relative to those fabricated with the above-mentioned human-derived TEVGs in vitro. Although the donor for the recipient child needs to be a healthy adult, our technique is much less invasive than living-donor organ transplantation because the graft is prepared subcutaneously. This study shows the possibility of application of allogeneic in vivo TEVGs, targeting transplantation from healthy donors to children.

This study also had some limitations. First, to gain more insight, it is necessary to compare and contrast the transplantation of the allogeneic decellularized connective tissue membrane with vascular patch transplantation using an autologous connective tissue membrane as a control. Second, since the inflammation in the graft after transplantation was not evaluated, it is necessary to evaluate immunoreactivity in frozen sections. Third,

it is necessary to study the long-term behavior of the graft over the years to evaluate the degree of graft inflammation and the presence or absence of calcification. Fourth, as the decellularization procedure may affect the mechanical properties of the TEVGs, it is necessary to perform an in vitro mechanical assessment (burst pressure, tensile strength, suture retention tests, etc) of connective tissue before and after decellularization to optimize the procedures. Finally, it will be informative to examine the degree of degradation of the TEVG components after transplantation. The rate of decomposition of a TEVG composed of a biodegradable polymer can be estimated by measuring the residual molecular weight of the polymer after transplantation. However, the graft we produced primarily consists of collagen fibers derived from a living body, which are indistinguishable from host fibers that may slowly replace after transplantation. Therefore, further studies are required to quantitatively analyze the allogeneic ECM components such as distribution and change of collagen types at specific time points after transplantation.

Overall, we have demonstrated that decellularized allogeneic connective tissue membranes can be prepared, stored beforehand, and used as functional vascular tissues. They could, therefore, be an ideal cardiovascular graft option.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
Concept/design: Masashi Yamanami, Keiichi Kanda, and Satoshi Gojo; Data collection: Masashi Yamanami, Kazuki Morimoto, Tomoya Inoue, Taiji Watanabe, Osamu Sakai, and Daisuke Kami; Data analysis/interpretation: Masashi Yamanami, Keiichi Kanda, Daisuke Kami, and Satoshi Gojo; Drafting article: Yamanami Masashi; Critical revision of article: Keiichi Kanda, Satoshi Gojo, and Hitoshi Yaku.

DATA AVAILABILITY STATEMENT
The datasets generated during and/or analyzed during the current study are not publicly available due to also being part of an ongoing study but are available from the corresponding author on reasonable request.

ORCID

Masashi Yamanami https://orcid.org/0000-0001-6862-8046

Keiichi Kanda https://orcid.org/0000-0001-5966-4416

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