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Histological structure affects recellularization of decellularized arteries

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ABSTRACT

Decellularized arteries were prepared to evaluate the *in vivo* recellularization of biological material after implantation. Porcine aortas and radial arteries were decellularized using high-hydrostatic pressure to form materials with histologically-different structures. Successful removal of cells from decellularized arteries was evaluated by hematoxylin–eosin staining and measurement of residual DNA. Cell remnants were eliminated completely from the decellularized arteries, and histological structures were preserved. Cells adhered to all decellularized artery samples, but infiltration of cells was observed only from the adventitial side of the decellularized radial artery. Rats were implanted subcutaneously with a decellularized aorta or radial artery to evaluate *in vivo* performance. Decellularized aortic tissue prevented cell infiltration better than that of the decellularized radial artery, suggesting that the elastin lamina in decellularized tissues prevents cell infiltration and suppresses recellularization.

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1. Introduction

Many biological materials, such as protein-based materials and decellularized tissues, have been used in multiple clinical and research contexts [1–3]. These materials are expected to recellularize and remodel following *in vivo* implantation into recipients [4,5]. However, cell infiltration and remodeling are difficult to predict. Lu et al. reported that the extracellular matrix (ECM) of decellularized tissues affects cell infiltration and remodeling [6,7]. That study used decellularized porcine aortic tissue and enzyme digestion to evaluate remnant proteins in the tissues. However, the histological structure of the tissue influences cell infiltration and remodeling, and this structure is changed by ECM digestion [8].

Arteries predominately comprise ECM and cells, and the composition of arterial ECM depends on the location of the artery. Arteries localized in regions of high blood-pressure contain more elastin than do those localized in regions of low blood-pressure [9]. Therefore, decellularized arteries have a different ECM composition and histological structure. Our group has reported the preparation of decellularized tissues from structures including the cornea, aorta, bone marrow, and small diameter arteries by using a high-hydrostatic pressure (HHP) method [10–14]. Low-temperature washing effectively preserves the ECM structure of decellularized carotid and radial arteries when preparing a small-diameter vascular graft using HHP [14]. Furthermore, implantation of a decellularized artery into a rat carotid showed that recipient cells infiltrate the radial artery more quickly than they do the carotid artery, suggesting that the ECM composition of decellularized arteries affects both cell infiltration and remodeling after transplantation.

In this study we examined whether the histological structure of decellularized arteries could affect cell infiltration and recellularization. We selected the porcine aorta as an elastin-rich artery and the radial artery as a collagen-rich artery to compare the effects of histological structure on cell infiltration and remodeling. Arterial tissues were decellularized, and their ECM structure was evaluated by histological staining. Cells were seeded to evaluate cell adhesion and infiltration into the decellularized arteries. These tissues were also implanted subcutaneously into rats and carotid artery implantation models were used to evaluate the *in vivo* performance of the decellularized arteries.

2. Materials and methods

2.1. Decellularization of arteries by HHP

Porcine aortas and forefeet were purchased from a local slaughterhouse (Tokyo Shibaura Organ Co., Ltd., Tokyo, Japan), and the radial arteries were harvested from the forefeet. The aortas and radial arteries were trimmed of surrounding tissue and fat and washed in saline. The aortas were then cut into pieces of approximately 20 × 20 mm. All

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arteries were packed in plastic bags with saline and hermetically sealed. The decellularization process was performed according to our previous report [14]. Briefly, arteries were first subjected to a pressure of 980 MPa for 10 min at 30 °C using a cold isostatic pressurization machine (Dr. Chef; Kobelco, Takasago, Japan). The arteries were then washed with DNase in saline for 7 days at 4 °C, followed by changing the washing solution to 80% ethanol in saline for 3 days. After washing, the arteries were preserved at 4 °C until use.

2.2. Evaluation of decellularization and histological structure

Decellularized arteries were fixed in paraformaldehyde (Wako Pure Chemical Inc., Osaka, Japan), dehydrated gradually in ethanol, and paraffin sections were prepared according to standard procedures. Sections of the decellularized arteries were stained with hematoxylin–eosin (H–E) to evaluate cell removal and in Elastica van Gieson (EVG) stain to evaluate histological structure. The stained sections were observed under an optical microscope (Coolscope; Nikon Co., Ltd., Tokyo, Japan).

The residual DNA content of untreated and decellularized arteries was measured using phenol/chloroform extraction and ethanol precipitation. After these treatments, the quantity of residual DNA was measured by spectrophotometry at a wavelength of 260 nm.

2.3. Enzymatic degradation test

Degradation of decellularized arteries was measured using an enzymatic degradation test [15]. Decellularized arteries were immersed in enzyme-containing solutions at 37 °C for 24 h. Collagenase and pronase (both 100 U/mL, Wako Pure Chemical) in phosphate-buffered saline containing 50 mM $CaCl_2$ were used for the test. The samples were weighed at different times to calculate the residual weight percentage of the samples.

2.4. Cell seeding on decellularized arteries

L929 mouse fibroblasts were seeded onto decellularized arteries to evaluate cell adhesion and infiltration. Decellularized arteries were cut longitudinally so the luminal and adventitial surfaces were exposed, and then placed in culture plates. Minimum Essential Medium with 10% fetal bovine serum was added to the plates, followed by incubation at 37 °C for 4 h. Sterilized stainless steel rings were placed on the decellularized arteries to prevent them from floating in the medium [16]. A suspension of L929 cells (1.0×10^4 cells/sample in Minimum Essential Medium) was added to the rings in the plates and incubated for 12 h. After the incubation, cell adhesion and infiltration were evaluated by H–E staining.

2.5. Subcutaneous implantation of decellularized arteries into rats

The animal study was performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1985) and the institutional guidelines for the care and use of experimental animals of Tokyo Medical and Dental University.

The decellularized arteries were dissected into 10×10-mm segments. Male Wistar rats (10 weeks-of-age) were anesthetized with 0.5 mL/kg pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan), and the segmented decellularized arteries were implanted subcutaneously into the back and sutured to the dermis ($n = 5$). Seven days after implantation, the rats were killed by pentobarbital overdose, and the decellularized aortas were harvested along with surrounding tissues. The harvested tissues were fixed in paraformaldehyde and subjected to H–E staining. The distance that cells had infiltrated into the decellularized arteries was calculated using Image J software (NIH, Bethesda, MD, USA), and the five cells that had infiltrated the furthest into each section were measured [17] ($n = 5$). The distance was measured as the length of a perpendicular line between the cell and the edge of an implanted artery. Average length was calculated using the upper five measured lengths in a section.

2.6. Implantation of decellularized arteries into the rat carotid

A rat carotid artery implantation model was used to evaluate cell infiltration into decellularized porcine aortas and radial arteries. The decellularized porcine aorta was not a suitable size for implantation into the rat carotid artery, so a reconstructed tubular graft was used (rolled graft). Vascular grafts of HHP-decellularized aortic sheets were constructed by rolling aortic sheets around a glass rod of 1-mm diameter with the epithelial side of the sheet facing the rod. The sheets were adhered together using fibrin glue (Bolheal®, Chemo-Sero Therapeutic Research Institute, Kumamoto, Japan) or a proline suture to bond the edges of the sheets. These tubular grafts were implanted into rats as small-diameter vascular grafts.

Male Wistar rats (10 weeks of age, $n = 3$/artery) were anesthetized with 0.5 mL/kg pentobarbital, and a decellularized artery (inner diameter: 1–1.5 mm) was implanted into the carotid using a cuff (inner diameter, 0.6 mm and outer diameter, 0.9 mm) and ligated with 5-0 silk sutures. The rats were killed with an overdose of pentobarbital after 7 days, and the decellularized arteries were extracted and evaluated by H–E staining.

3. Results and discussion

3.1. Decellularization of the aorta and radial artery

Cell removal was evaluated by H–E staining and measurement of residual DNA content. No nuclei were observed in the H–E-stained sections of any aortic or radial artery tissue (Fig. 1B, D). Residual DNA content of the decellularized tissues decreased to near the detectable limit (Fig. 2). EVG staining was used to evaluate histological structure of the decellularized arteries. Elastin stained deep blue and collagen stained pink. No difference was detected in the EVG-stained sections of the untreated and decellularized arteries (Fig. 1E–H). Decellularized aortas contained many more elastin fibers than did decellularized radial arteries, whereas decellularized radial arteries were richer in collagen than were decellularized aortas.

HHP uniformly affected all parts of the tissues; cells were destroyed, and the cell remnants were removed by washing. We previously reported that low-temperature washing effectively preserves the ECM structure [14]. We used the same low-temperature washing procedure here to prepare decellularized arteries. Few cell components remained in the decellularized arteries, as shown by H–E staining and residual DNA content values. EVG staining indicated that collagen and elastin were preserved in the arteries after decellularization. The decellularized aortas served as an elastin-rich artery model, while the decellularized radial arteries provided a collagen-rich artery model.

3.2. Enzymatic degradation test

Degradation of the decellularized arteries was evaluated using enzymes. The decellularized radial arteries were degraded by collagenase within 2 h, whereas the decellularized aortas were degraded more slowly by collagenase, and approximately 20% of the aorta remained after 24 h (Fig. 3A). Meanwhile, digestion with pronase yielded different results (Fig. 3B). No difference in degradation was observed between decellularized aortas and radial arteries after 6 h, and the decellularized radial arteries were intact until 12 h. The decellularized aortas were partially degraded by pronase after 4 h and approximately 10% remained after 24 h.

Degradation of the ECM is part of the *in vivo* remodeling process. Matrix metalloproteinase-1, -8, and -13, collectively called collagenase,

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