Modified Acellularization for Successful Vascular Xenotransplantation

The purpose of this study was to estimate the possibilities of an acellular matrix using a modified acellularization protocol, which circumvents immunological, microbiological, and physiological barriers. We treated porcine subclavian arteries with various reagents to construct acellular grafts. Afterwards, these grafts were interposed in a mongrel dogs' abdominal aorta. Six dogs underwent interposition with fresh porcine grafts (control group), and seven had interposed acellular grafts (acellular group). The control and acellular group dogs were sacrificed at 1, 3, 5 (n=2 in each group) and 12 months (n=1 in acellular group) after the operation. Histopathological examinations were then performed, to assess the degree to which re-endothelialization, inflammation, thrombus formation, and calcification occurred. The entire acellular group, but none of the control group, exhibited re-endothelialization. The degrees to which inflammation, thrombosis, and calcification occurred were found to be lower in the acellular group. We also discovered many smooth muscle cells in the medial layer of the xenograft that had been implanted in the dog sacrificed 12 months after the operation. These results suggest that the construction of xenografts using our modified acellularization protocol may offer acceptable outcomes as a vascular xenograft.

Key Words : *Xenograft; Vascular Endothelial Cell; Graft Rejection*

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INTRODUCTION

Although a great deal of effort was put into the development of xenografts and homografts in Korea between 1970-1980 (1, 2), no successful experiments with these techniques has been reported. During this period, O'Brien and colleagues reported the standardization of a homograft cryopreservation approach (3). This approach was also used in clinical practice.

Homografting, though it had many benefits and resulted in superior outcomes compared to xenografting, also had many limitations, including early calcification, immunologic rejection, and generally poor durability. Due to these limitations, research into xenograft-related immunosuppression emerged as another target of research. The Toronto group reported a wide variety of outcomes from immunosuppression via the removal of the xenograft's antigens (4). Bader et al., as well as Teebken et al. also reported that the endothelium of the recipient could be repopulated into the acellularized xenograft (5, 6).

We hypothesized that xenografts in which the antigens had been removed (especially the endothelial variety), might exhibit less pronounced immunological rejection characteristics than other varieties of xenografts. In acellular xenografts, repopulation with the recipient's endothelium might also be conducted, showing possibilities of the production of a practically acceptable xenograft.

Although many studies have been conducted and reported good results under these hypotheses, we wanted to improve the acellularizing processes, especially in the process of nuclease treating and the removal of phospholipids to prevent the calcification of xenografts, to make more ideal xenografts.

MATERIALS AND METHODS

Materials

We used the left subclavian arteries of pigs (weighing between 200-250 kg, cadaveric donors) in the construction of xenografts. Mongrel dogs, weighing between 20-25 kg, were employed as recipients. This study was approved by the Institutional Animal Care and Use Committee of Korea University. The animal care conducted throughout this study was consistent with the guidelines and rules of *the Guide for the Care and Use of Laboratory Animals*.

All chemical reagents and buffers, unless stated otherwise, were obtained from the SIGMA Chemical Company (St. Louis, Mo, U.S.A.).

Acellular matrix (de-endothelialization) processing

The processes by which the de-endothelialization of the

porcine left subclavian artery were based on the methods developed in several previous reports (7). The construction of a viable biological matrix for the xenograft requires the depletion of cellular antigens, and the maintenance of the matrix for subsequent tissue repopulation.

First, about 3 cm of the left subclavian artery of the pig was resected in a slaughter house, after which debridement and trimming were performed aseptically. Then, the tissue was taken to a laboratory in an aseptic bottle filled with a phosphate buffer solution (pH 7.0) (8). After trimming and cleansing, the graft was treated with hypotonic Tris buffer solution (TBS, pH 8.0). This facilitated the loosening of the original tissue cells. It is important, in this step, not to allow overly aggressive proteolytic degradation that may destroy the entire matrix, including the collagen that comprises much of its structural integrity. A protease inhibitor, phenylmethyl-sulfonyl fluoride (PMSF, 0.35 mL/L), was used to control protease activity (4, 7). Following this procedure, the damaged or destroyed cellular debris, nuclease, and other enzymes were washed and deactivated using Hanks' balanced salt solution (HBSS) (4, 7, 9). Then, the xenografts were treated with alkylphenoxypolyethoxyethanol (Triton-X 100; nonionic detergent) along with TBS and PMSF. Triton-X 100, which destroys the cellular membrane and removes floating materials (4, 7, 10), was applied. After 24 hr of HBSS treatment at 4°C, the tissue was treated with nuclease. Nuclease digests the DNA, RNA, and exonuclease in the native cells without collagen lysis. We selected DNAase I (1.0 μg/mL) and a RNAase A (1.0 μg/mL) as our experimental nucleases for this step (4, 7, 11). The nuclease reaction time was set for 2 hr, based on several of the authors' preliminary experiences with such procedures. After the nuclease treatment, the tissue was retreated with HBSS, TBS, and Triton-X 100 for the next 24 hr (4°C). One-percent sodium dodecyl sulfate (SDS), a detergent, was applied in order to effect the selective solution of the remaining cellular membrane, and to selectively remove phospho-

lipids from the tissue (12, 13). The removal of the phospholipids and cellular materials was important for the prevention of xenograft calcification (9, 14, 15). One-percent SDS was allowed to react in a 2-Hydroxyethypiperazine N-2-ethansulfonic acid (HEPES) buffer solution (pH 7.4) for at least 19 hr. This section of the procedure was also set up according to the results of several of the authors' preliminary experiences with this procedure.

The summarized protocol for acellular matrix processes is shown in Table 1.

Methods for the confirmation of the acellular matrix

We conducted histopathological examinations of the xenograft as an acellular matrix, using light microscopy and electron microscopy. Unfortunately we could not show the data of the mechanical properties, so we defined acellular matrix as an absolute absence of cellular materials (not collagenic matrix) in histopathological examination.

Operation methods

Anesthetic induction was conducted using ketamin (40 mg/kg, i.m) and propofol (1.5 mg/kg, i.v). After endotracheal intubation, 3% isofluorane was used to maintain anesthesia. Mechanical ventilation with 100% oxygen was then conducted, blood pressure was monitored through the femoral artery, and EKG tests were performed. Each recipient animal underwent a left abdominal horizontal incision along the spine, and the abdominal aorta was dissected via a retroperitoneal approach. Aortic clamping was conducted proximally and distally to the abdominal aorta at sites with no branches, resulting in an operation field of approximately 4-5 cm. The 3 cm length of the xenograft was interposed. The canines' average aortic diameter was 1.36 ± 0.16 cm, which was almost half of the xenograft's diameter, at 2.63 ± 1.50 cm. Therefore, it was necessary to reduce the diameter of the xenograft.

The de-cellularized xenografts were transplanted to the abdominal aorta (20-25 kg) of each of the 7 dogs in the acellular group, and fresh untreated xenografts were transplanted to another six dogs, which comprised the control group.

In order to minimize the interference of external factors in this study, we used no antibiotics in this procedure, except at the beginning of induction and the end of the operation. No anticoagulants or immunosuppressants were used throughout the study period. The donors (pig) and recipients (dog) were all raised in a general environment, rather than an aseptic environment.

Histopathological examinations after operation

Many authors reported the ingrowth of endothelial cells and smooth muscle actin positive cells into the de-endothelialized xenograft (repopulation) by 2 month to 11 months

Table 1. Protocol for acellular matrix processing

1) Donor subclavian artery harvesting & trimming
2) TBS (pH 8.0)+PMSF (0.35 mL/L, 4°C, 24 hr stirring)
3) Rinsing in HBSS (4°C 2 hr stirring)
4) TBS+Triton-x 100 (alkylphenoxypolyethoxyethanol)+PMSF (4°C 24 hr stirring)
5) Rinsing in HBSS (4°C 24 hr stirring)
6) Digestion with DNAase I (1.0 μg/mL) and RNAase A (1.0 μg/mL) (37°C, 2 hr)
7) Rinsing in HBSS (4°C 24 hr stirring)
8) TBS (pH 8.0)+Triton-x 100 (alkylphenoxyethoxyethanol) (4°C 24 hr stirring)
9) Rinsing in HBSS (4°C 24 hr stirring)
10) HEPES (pH 7.4)+1% SDS (19 hr)
11) Fixed solution

TBS, Hypotonic Tris buffer; PMSF, Phenylmethyl-sulfonyl fluoride; HBSS, Hanks' balanced salt solution; HEPES, 2-Hydroxyethypiperazine N-2-ethansulfonic acid; SDS, Sodium dodecyl sulfate.
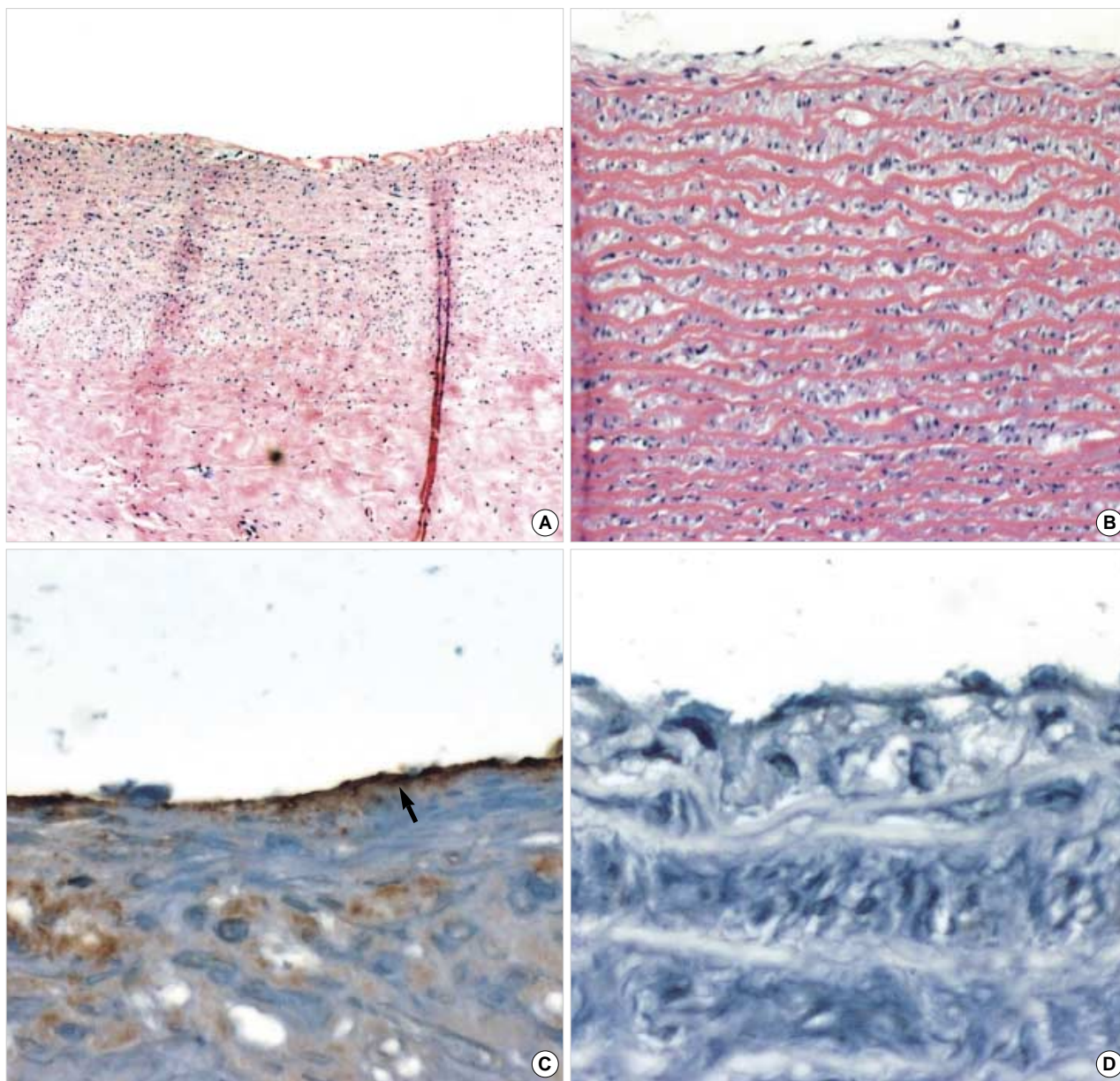
Fig. 1. Endothelial cells of cellular matrix. (A) Dog aorta (hematoxylin and eosin stain). (B) Pig graft (hematoxylin and eosin stain). (C) Immunohistochemical staining of dog aorta with rabbit anti-human Factor VIII antibody, which stained the endothelial cytoplasm positively (arrow). (D) Immunohistochemical staining of pig graft with rabbit anti-human Factor VIII antibody, which did not stain endothelial cytoplasm.

(16-18) after implantation. Elkins and colleagues, especially, reported as much as 80% repopulation of donor cells into decellularized xenograft by 11 months after implantation (18). We, therefore, conducted histopathological studies at 1, 3, 5 (n=2 in control and acellular groups) and 12 months (n=1 in acellular group) after the initial operation.

During the histopathological studies, we compared the degrees to which re-endothelialization, inflammation, thrombus formation, and calcification occurred in each group, via hematoxylin and eosin staining.

The re-endothelialization of the recipient's endothelium

to the decellularized xenograft was identified via immunohistochemical staining with rabbit anti-human factor VIII antibody (DAKO, Inc, Japan). We found that Rabbit Anti-human Factor VIII Antibody reacted with canine endothelium, but not porcine endothelium, which was consistent with the authors' preliminary experiences (Fig. 1).

The degrees of inflammation was measured according to the depth of invasion of the inflammatory cells from the adventia of the xenograft with a 100 scale (=1 cm) microscopic ruler. The degrees of the calcification and thrombus formation were also graded according to a calcified graft length/whole graft
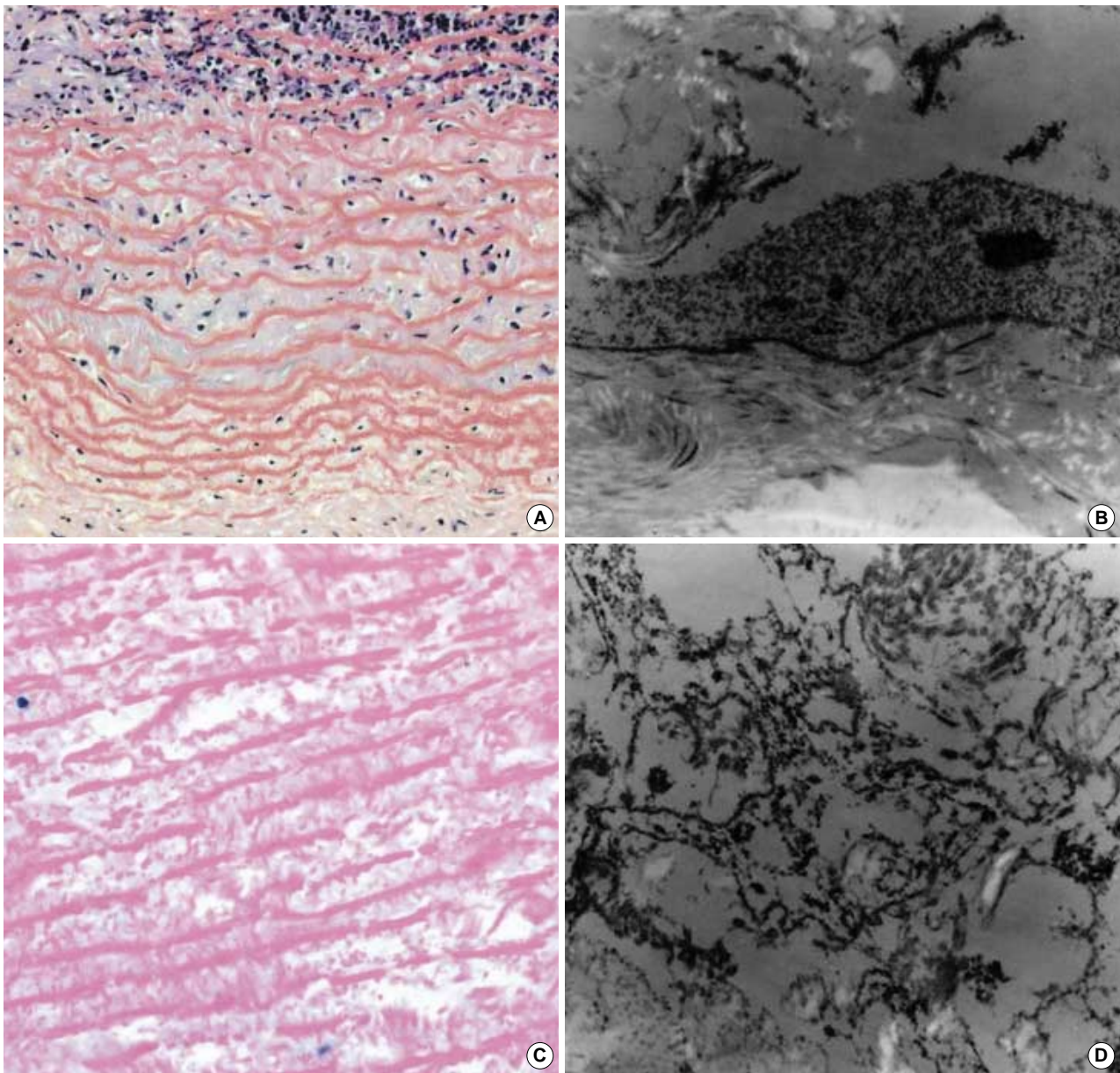
Fig. 2. Cellular matrix (Control, **A**, **B**) and acellular matrix (**C**, **D**); Cellular matrix (**A**, **B**) showed many cellular materials and stained nuclei, but acellular matrix (**C**, **D**) did not contain any cellular materials and showed collagen fibers only. (**A**) Cellular matrix (control group, hematoxylin and eosin stain, ×100). (**B**) Cellular matrix (control group , EM, ×10,000). (**C**) Acellular matrix (acellular group, hematoxylin and eosin stain, ×200). (**D**) Acellular matrix (acellular group, EM, ×8,000).

length and the thickness of thrombus with the same ruler.

RESULTS

The histopathological findings with regard to the xenograft, which was processed as described above in the de-endothelialization method, indicated the construction of an acceptable acellular matrix, as shown in Fig. 2.

All of the experimental animals showed normal growth patterns during each scheduled period after operation, without antibiotics (except at the beginning of induction and at the end of operation), anticoagulants, or immunosuppressants.

We compared the degrees of re-endothelialization, inflammation, thrombus formation, and calcification via histopathologic examinations after operation.

Re-endothelialization

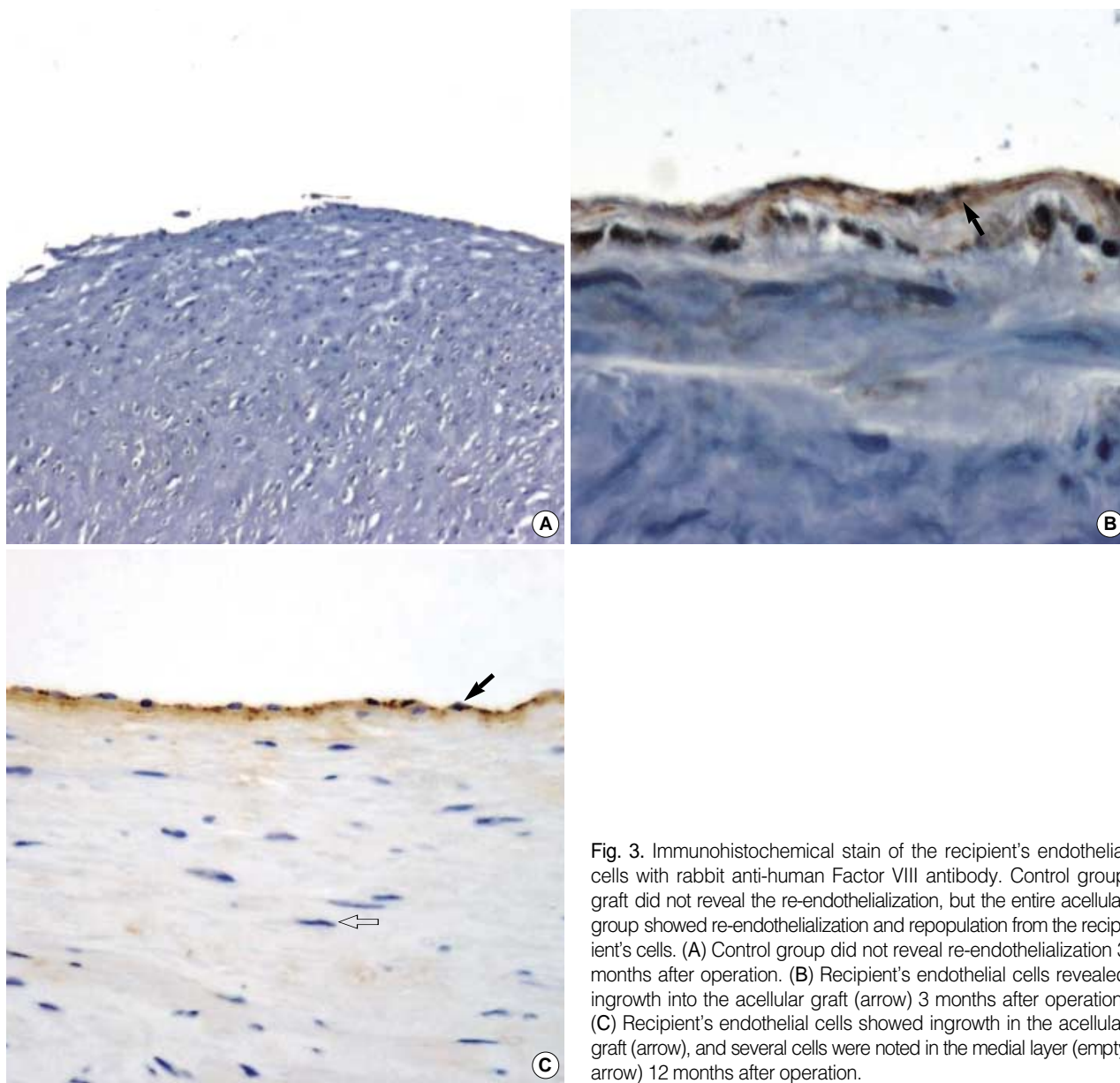All of the cases in the acellular group exhibited re-endothe-

Fig. 3. Immunohistochemical stain of the recipient's endothelial cells with rabbit anti-human Factor VIII antibody. Control group graft did not reveal the re-endothelialization, but the entire acellular group showed re-endothelialization and repopulation from the recipient's cells. (A) Control group did not reveal re-endothelialization 3 months after operation. (B) Recipient's endothelial cells revealed ingrowth into the acellular graft (arrow) 3 months after operation. (C) Recipient's endothelial cells showed ingrowth in the acellular graft (arrow), and several cells were noted in the medial layer (empty arrow) 12 months after operation.

lialization, but none of the control group did. The degrees to which re-endothelialization occurred also positively correlated with the postoperative growth. Although many authors have reported some differences, they uniformly state that the recipient's endothelium can exhibit ingrowth into any layer of the graft (8, 11). In our study, the dog that had been sacrificed at 12 months after operation exhibited several cells that were penetrating into the medial layer of the xenograft (Fig. 3C). The nuclei of the cells in the medial layer were shaped like spindles or polygons, and proved to be positive for smooth muscle actin immunostaining (Fig. 4). We identified these infiltrating cells to be smooth muscle cells, which had migrated from the recipient's aorta. It is also possible that some of these cells maight have been fibroblasts.

Inflammation

All of the experimental animals exhibited inflammatory reactions. The inflammatory cells were determined to have infiltrated to more than 50% of the entire thickness of the graft from the adventitia in the control group, and infiltrated to approximately 5-20% of the entire thickness from the adventitia in the acellular groups (Table 2).

Thrombosis

All control group animals, upon gross examination, showed thrombus formation. Microscopic examination showed that the control group animals revealed thrombi of about 2,000-
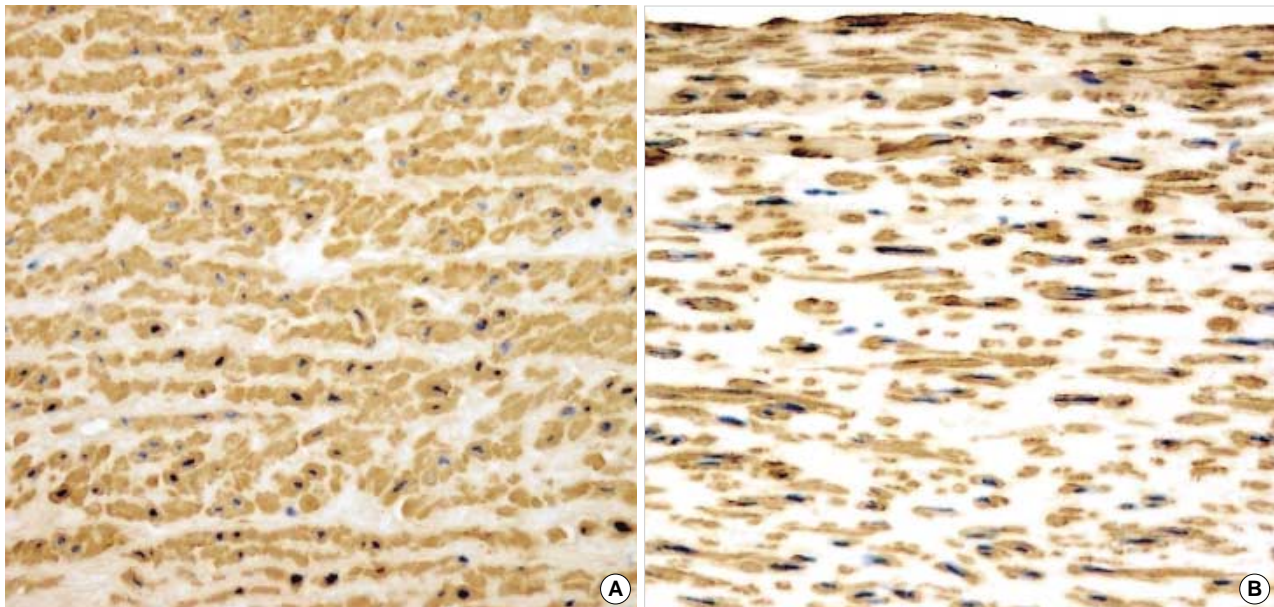
Fig. 4. Immunohistochemical stain of medial layer with smooth muscle actin (SMA). (A) Recipient's (dog) normal abdominal aorta. It showed a regular arrangement of the normal smooth muscle cells. (B) Donor's (pig) xenograft. The en-growing cells in the medial layer proved to be positive for SMA immunostaining but arranged irregularly, which had migrated from the recipient's aorta.

Table 2. Results of the acellular matrix vascular xenograft

Sacrifice*	No.	Matrix group	Re-endo-theliali-zation	Adven-titial inflam-mation†	Calcifi-cation‡	Thro-mbus§
1 month	1	Control	No	>50%	100%	3,000 μm
	2	Control	No	>50%	100%	2,000 μm
	3	Acellular	Yes	15%	Rare	None
	4	Acellular	Yes	10%	90%‖	None
3 months	5	Control	No	>50%	100%	2,500 μm
	6	Control	No	>50%	100%	3,000 μm
	7	Acellular	Yes	20%	None	None
	8	Acellular	Yes	5%	30%	None
5 months	9	Control	No	>50%	100%	3,000 μm
	10	Control	No	>50%	100%	2,000 μm
	11	Acellular	Yes	5%	Rare	100 μm
	12	Acellular	Yes	20%	70%‖	None
12 months	13	Acellular	Yes	10%	None	350 μm

*, Sacrificed months after operation; †, Involved depth from adventitia; ‡, % of the whole graft length; §, Thickness of thrombus; ‖ Severe calcification shown in graft and recipient's autologous vessel.
- Measurement conducted by a 100 scale (=1 cm) microscopic ruler.

3,000 μm thickness, and only one of the decellularized group animals showed about 1,000 μm thickness thrombus formation. Thrombus was rare in the remaining animals (Table 2). The decellularized group animal that exhibited a 1,000 μm-thick thrombus also showed the largest size discrepancy between the vessel diameters of the recipient and xenograft tissues.

Calcification

The degree to which calcification occurred was less pronounced in the acellular group animals than in the control group animals (Table 2). Two of the experimental group animal revealed moderate to severe calcifications, but these animals also revealed multiple calcifications in their non-operated autologous vessels. Therefore, it was impossible to confidently conclude that calcification could be caused by vascular xenotransplantation. In addition, the dog that was allowed to grow for the longest period (12 months after operation) did not show any calcification.

DISCUSSION

Xenotransplantation is an important procedure, due to the ease associated with graft supply. Xenotransplantation, however, has classically suffered from three primary problems.

The first problem involves immunological barriers. This is the most important and the most difficult problem to resolve. This problem occurs due to three distinct phenomena: hyperacute rejection (HAR), acute vascular rejection (AVR or delayed rejection), and T cell-mediated rejection. HAR is induced by preformed antibodies in the recipient, which bind the galactose alpha 1-3 galactose (alpha-Gal) epitopes in the pig's vascular endothelial cells. These binding antibodies then activate the complement, inducing graft failure (19). Although the exact cause of AVR or delayed rejection remains elusive, the existence of preformed antibody or

the return of removed antibodies is known as the cause of this form of xenograft rejection (20). T cell-mediated rejection can also cause problems during xenotransplantation, especially during the first week after the transplantation. The degree to which rejection occurs when a cadaveric xenograft is used (21), however, cannot be precisely defined.

In order to prevent immunologic problems, many authors have proposed the inactivation of the primary complement factors, control of the expression of the complement activation regulator, the removal of anti-xenograft antibodies, multiple drugs that decrease the amount of anti-Gal antibody secreting cells, immunosuppressants, and so on (19, 20, 22-25).

Microbiological issues comprise the second group of problems with regard to xenotransplantation. Complete eradication of exogenous viral infection and removal of endogenous retrovirus, however, has been reported (26).

The final problem associated with xenotransplantation is the physiological barrier. This involves the consequences of the incompatibility between porcine and human coagulation factors, and results in a characteristic 'intragraft thromboses' or 'disseminated intravascular coagulation (DIC)'. In order to resolve or avoid these problems, many authors have reported on the inactivation of the endothelium of the xenograft (27, 28) as well as anticoagulation therapy.

In our study, we planned to develop an ideal xenograft, which circumvents many of the barriers related to xenotransplantation. Our method for constructing this ideal xenograft involves de-endothelialization and the removal of much of the cellular material inherent to the xenograft.

The de-endothelialization processing in our study was based on the methods referenced by previous articles, but also had several unique aspects. We did not use glutaraldehyde fixation to prevent the calcification of the graft (29, 30). We treated the xenograft with 1% SDS, which remains a controversial technique. We chose a 2 hr duration time for the nuclease treatment. The above three aspects constitute the principal differences between our method, and those in the previous articles (7). The optimal treatment duration and temperature selected for our study were based on several preliminary experiences by the authors.

The comparison factors we selected as our foci for the microscopic study after operation included re-endothelium, the inflammation reaction (which reflects immunological rejection), thrombus formation, and calcification. Because vessels are not composed of unique cells, we were unable to note any specific findings regarding immunological rejections in the vessels. Therefore, we regarded inflammatory reactions as markers for immunological rejections, as stated in a previous article (31). Re-endothelialization was selected as one of our focal factors, as we had hypothesized that the ingrowth of the recipient's endothelium into the de-endothelialized xenograft would result in a more immunologically durable xenograft. Several cell types in the recipient exhibited repopulation. We clearly confirmed the ingrowth of smooth mus-

cle cells from the recipient's aorta. Thrombus formation and calcification are other immunological rejection factors, and can elicit graft failure (32). In all of our cases, it was necessary to reduce the diameter of the xenografts, which may have influenced postoperative thrombus formation.

The results of our study revealed that the re-endothelialization of the recipient's endothelium to the xenograft occurred in all of the experimental group animals, but not in all of the control group animals. Other factors occurred less frequently in the experimental group animals than in the control group animals.

Therefore, we conclude that the construction of xenografts using our modified acellularization protocol may offer acceptable outcomes as a vascular xenograft.

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