Acute Regeneration and Chronic Acellular Transformation of Rabbit Cryopreserved Aortic Allografts

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Received December 12, 2011; accepted March 14, 2012; published online May 15, 2012

An analysis of rabbit cryopreserved aortic allografts excised on postoperative days (POD) 2, 5, 11, 60, 210, 360, and 720, as well as controls that were untransplanted native aortas and cryopreserved aortas, was performed. On POD2, the number of medial smooth muscle cells in the allografts was reduced to approximately 50%. Ki-67 analysis revealed that medial smooth muscle cells in the allografts proliferated from the 2nd day. By the 11th day, their proliferation ceased and the number of medial smooth muscle cells was restored to almost the same level as in the controls. Polymorphic microsatellite DNA marker analysis disclosed that the restored medial smooth muscle cells were of donor origin. From 7 months through 2 years, the media of cryopreserved aortic allografts were transformed into acellular structures, in which the elastic fibers were preserved. On the other hand, newly accumulated smooth muscle cells were observed in the adventitia just outside of acellular media after 7 months. In some cases, scattered lamellar calcium deposition was observed in the same regions. This study presents a comprehensive documentation of regeneration and acellular transformation in cryopreserved aortic allografts based on short and long-term analysis.

Key words: cryopreservation, aortic allograft, transplantation, smooth muscle cell

I. Introduction

The use of arterial or venous allografts for vascular reconstruction was introduced in the early years of modern vascular surgery and provided fair to intermediate short-term results [5, 9]. Due to the possibility of aneurysmal or occlusive degeneration, difficulties in procurement, and the availability of excellent ready-to-use synthetic grafts, vessel allografts were abandoned in the 1960s [29]. Technological improvements in cryopreservation methods have since been made that allow allografts to be stored for long periods, making procurement easier than for fresh allografts. Recently, cryopreserved aortic allografts also come to be used for treating infected synthetic grafts or mycotic aneurysms because of their superior resistance to infection compared to synthetic grafts [13, 17], as well as their excellent early and midterm results in comparison with the extremely poor results with other treatments [7, 12, 16, 20, 30]. To date, however, the histological changes in cryopreserved aortic allografts after transplantation have not been well documented.

The existence of viable smooth muscle cells and endothelial cells in cryopreserved aortic allografts after thawing has been verified by cell culture [22, 27], but no quantitative analyses have ever been reported. In cryopreserved human valves, Armiger reported that most cells of cryopreserved valves were nonviable when thawed [3], although Niwaya et al. reported that 85.9%±6.3% of fibroblastic cells were viable after the valves were thawed [25]. Therefore, the rate of viable smooth muscle cells in cryopreserved aortic allografts after thawing has yet to be satisfactorily
elucidated. On the other hand, Motomura et al. reported that
the number of smooth muscle cells in the medial muscle
layer of cryopreserved aortic allografts on the 10th day after
transplantation was maintained at approximately the same
level as that of native aortas [22].

In terms of chronic phase, some studies have reported
the presence of living donor cells remaining in explanted
cryopreserved allografts [26], while others have described
host cell ingrowth into the allografts [2, 6]. Tissue chimer-
ism in implanted allografts have also been reported in recent
years [14]. From these experimental studies, it is inferred
that implanted cryopreserved aortic allografts might trans-
form into totally different structures from native ones
through host-recipient interactions at the histological level.
The present study aimed to elucidate acute and chronic
structural changes of implanted cryopreserved aortic allo-
grafts.

II. Materials and Methods

Animals

Japanese domesticated white rabbits (Kbs:JW) breeding
in a closed colony with an average weight of 3 kg were
used. Rabbit aortas were used because their structure is
similar to human aortas, and the arterial disease found in
rabbits closely replicates that found in humans [4, 18].
Furthermore, rabbit aortas are well suited for the examina-
tion of the medial muscle layer due to their size and distinct
layer structure.

This study was approved by the Kobe University
Animal Experiment Committee, and all rabbits received
in compliance with the Guide for the Care and Use
of Laboratory Animals, Institute of Laboratory Animal
Resources, Commission on Life Sciences, National
Research Council (Washington D.C.: National Academy
Press, 1996).

Harvesting and cryopreservation of aortic grafts

The descending aortas were resected from donor
rabbits in a sterile manner, washed in saline, and then
immediately dipped into separate test tubes containing
cryopreservation medium. They were kept at 4°C for 30 min
and then frozen to −80°C at a rate of 1°C/min. The test tubes
containing the grafts were then transferred into liquid
nitrogen and preserved for 2 months, until the aortas were
used for transplantation. The cryopreservation medium
contained tissue culture medium 199 (TCM199; Nacalai
Tesque, Japan) supplemented with 10% dimethylsulfoxide
(DMSO) and 5% Hepes buffer. The procedures for cryo-
preservation used in this study were basically the same as
those currently used for human aortic allografts in Japan.

Transplantation of aortic grafts

The test tubes containing the aortic grafts were
removed from the liquid nitrogen and kept at room tem-
perature for 10 min. They were then dipped into a water
bath at 37°C for 10 min until thawed. Subsequently, the
thawed grafts were taken from the tubes and were washed
in a TCM199 solution at 4°C for 10 min to remove the
DMSO. All recipient rabbits were anesthetized with
propofol by an initial intravenous injection (2 mg/kg body
weight) followed by a continuous infusion at a dose of 6
mg/kg/hour. Heparin (500 U/kg body weight) and flomoxef
sodium (60 mg/kg body weight) were administered intra-
venously. Local anesthesia was provided with 20 ml of
lidocaine hydrochloride solution (0.5%) injected into the
abdominal wall. After the abdominal incision, the infrarenal
abdominal aortas were replaced with a 30 mm length
section of the cryopreserved aortic allografts in an end-to-
end anastomosis with continuous 7-0 polypropylene suture.
No anticoagulants, immunosuppressants, or antibiotics were
administered postoperatively.

Sampling of transplanted allografts

Recipient rabbits were anesthetized at the sampling
periods, which were postoperative day (POD) 2, 5, 11, 60,
210, 360 and 720. They were sacrificed an hour after being
injected with 5'-bromo-2'-deoxyuridine (BrdU; 7 mg/kg),
and the allografts and the flanking native aortas were
explanted en bloc. The specimens were fixed in 10% paraformaldehyde.

Sampling of native and cryopreserved aortas

As a control, 5 rabbits were sacrificed an hour after
the injection of BrdU (7 mg/kg body weight), and the
descending aortas were harvested in a sterile manner. The
aortas were washed in saline and were cut into 2 pieces. The
5 halves of the aortas were immediately fixed in 10% paraformaldehyde and were analyzed as control native
aortas. The other 5 halves were cryopreserved and then
thawed in the same way as the transplanted allografts. They
were fixed in paraformaldehyde immediately after being
thawed. They were not transplanted, but were analyzed as
control cryopreserved aortas.

Histological analysis

The paraformaldehyde-fixed specimens (5 control
native aortas, 5 untransplanted cryopreserved aortas, 5 allo-
grafts excised on POD5, 5 allografts excised on POD11,
3 allografts explanted on POD60, 3 allografts explanted on
POD210, 2 allografts explanted POD360, and 2 allografts
explanted on POD720) were cut longitudinally and em-
bedded in paraffin. Sections 3-μm-thick were stained with
either hematoxylin-eosin or by using the immunoperoxidase
technique. The immunoperoxidase technique involves using
monoclonal antibodies against α-smooth muscle actin (α-
SMA; monoclonal mouse anti-human α-smooth muscle
actin; DAKO, Japan), CD141 (mouse anti-human endo-
thelium, MCA641; Serotec Ltd., UK), and CD45 (mouse
anti-rabbit leucocyte common antigen, AL54502; Bio-
Source International, USA) so as to detect smooth muscle
cells, endothelial cells, and leukocytes, respectively. Antibodies against Ki-67 (monoclonal antibody Ki-67
antigen, MIB-1; IMMUNOTECH S.A., France) and BrdU
(anti-bromodeoxyuridine mouse monoclonal antibody, NCL-BrdU; Novocasta Laboratories Ltd., UK) were used to detect cycling cells [21]. Ki-67 is a nuclear antigen associated with cell proliferation that is found throughout the cell cycle (G1, S, G2, and M phases) and is absent in resting (G0) cells. However, the expression of Ki-67 antigen does not always verify a cell’s proliferation, as it would also include cells arrested or in an apoptotic process. Thus, we also used BrdU to label cells so as to determine if there were cells synthesizing DNA in vivo just before the rabbits were sacrificed. BrdU is an analog of thymidine and is incorporated into the DNA of proliferating cells during the S phase if it is administered before the sample animal is sacrificed.

**Optical microscopic observation of specimens**

Optical microscopic observation of each specimen was performed. To assess the number and proliferation ratio of the medial smooth muscle cells in the allografts and controlled aortas, the numbers of nucleated smooth muscle cells, Ki-67-positive smooth muscle cells, and BrdU-positive smooth muscle cells per 0.1 mm² were counted in 5 random portions of the media of each specimen. The numbers of each type of cell in each group were calculated (mean±standard deviation). The mean values were evaluated by performing a two-way factorial analysis of variance and a multiple comparison procedure (Scheffe’s method). Differences among the groups were considered significant when the p value was less than 0.05.

**DNA isolation and microsatellite analysis**

The origins of the medial smooth muscle cells in the transplanted cryopreserved aortic allografts were determined by analyzing the polymorphic microsatellite DNA marker data. The total DNA was isolated from the medial smooth muscle cells of the 5 allograft specimens on POD11. Using a scalpel under microscopic visualization, the media of the allografts was microdissected from the Giemsa-stained 10-μm-thick paraffin sections. To identify and determine the origin of the majority of the cells in a wide area of the media, we simply chose to employ the method using a scalpel under microscopic observation, although laser capture microdissection would have been applicable. The dissected specimens were treated with proteinase K, and the DNA was extracted with phenol-chloroform. Similarly, DNA was extracted from the livers of the donor and recipient rabbits to serve as a control.

Two microsatellite regions of the rabbit DNA were amplified by the polymerase chain reaction (PCR) using oligonucleotide primers for the OCASICG and the OCRLADF4 loci. The OCASICG locus is located in the α-S1-casein gene, and its sense and anti-sense primers were AGAGAGGAGGATGCACA and TTGGATAGGGCCAGATCTG, respectively. The OCRLADF4 locus is located in the major histocompatibility complex class II DF gene, and its sense and anti-sense primers were TTCCTTTCTGTCCTGAGA CCATG and GCAGTTGTGTCAGA, respectively [10].

The PCR amplification was performed in a programmable thermal cycler. The amplification products were electrophoresed in Spreadex EL800 gels (Elchrom Scientific AG, Switzerland) using Elchrom’s SEA 2000 submarine electrophoresis apparatus. The gels were then stained with ethidium bromide and photographed. Allelic bands were designated in alphabetical order with a coding for the shortest PCR product. The allelic patterns of the amplification products of five POD11 allografts were compared with those of the donor and recipient liver DNA. Of the 2 loci examined, the most informative combinations were selected and used for 5 analyses.

**III. Results**

**Histological changes in medial smooth muscle cells of the allografts**

The cryopreserved aortic allografts, taken sequentially up to POD720 after transplantation, were examined using an optical microscope (Fig. 1). We first focused on the behavior of the medial smooth muscle cells by using hematoxylin-eosin staining (Fig. 1A–E) and by doing an immunohistochemical analysis with α-SMA antibodies (Fig. 1F–J).

**Acute phase**

The average numbers of nucleated medial smooth muscle cells were: control native aortas, 679±217/0.1 mm²; control cryopreserved aortas, 619±142/0.1 mm²; POD2 allografts, 320±131/0.1 mm²; POD5 allografts, 471±140/0.1 mm²; and POD11 allografts, 587±129/0.1 mm² (Fig. 2, closed circles). No significant differences in the numbers of nucleated medial smooth muscle cells were found between the control native aortas and the control cryopreserved aortas (p=0.2671). The number of nucleated medial smooth muscle cells markedly decreased in the POD2 allografts (p<0.0001), then increased in the POD5 allografts (p<0.0001) and further increased in the POD11 allografts (p=0.0004). There were no significant differences in the numbers of cells found in the POD11 allografts or the numbers of cells found in the control cryopreserved aortas (p=0.8336).

All the areas of the media in the control native aortas and the control cryopreserved aortas were relatively evenly stained by antibody against α-SMA (Fig. 1F, G), while in the POD2 allografts, the α-SMA-positive medial cells showed a patchy distribution, indicating the partial loss of smooth muscle cells (Fig. 1H).

To confirm that the increase in the number of cells from POD2 through to POD11 was caused by cellular proliferation, immunoperoxidase staining using antibodies against Ki-67 and BrdU was performed (Fig. 1K–T).

There were no Ki-67-positive cells in either the control native or the control cryopreserved aortas before transplantation. The numbers of Ki-67-positive cells in the media were 189±160/0.1 mm² in POD2 allografts, 58±31/0.1 mm² in POD5 allografts, and 9±11/0.1 mm² in POD11 allografts.
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(Fig. 2, open squares). The increase in the number of Ki-67 positive cells in POD2 allografts was statistically significant ($p<.0001$). The ratio of Ki-67-positive cells among nucleated medial smooth muscle cells was 59% on POD2, 12% on POD5, and 1.5% on POD11. Similarly, BrdU-positive smooth muscle cells were not found in the media of the media.

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**Fig. 1.** Optical microscopy of the allografts in acute phase: Native aorta (A, F, K, and P); cryopreserved aorta not used as a graft (B, G, L, and Q); and the transplanted allografts excised on POD2 (C, H, M, and R), 5 (D, I, N, and S), and 11 (E, J, O, and T). All tissues were stained with hematoxylin-eosin (A–E) and with the anti-α-SMA (F–J), Ki67 (K–O) and BrdU (P–T) antibodies. Positive cytoplasmic staining for α-SMA indicates that most of the cells in the media are of smooth muscle cell origin. Positive nuclear staining for Ki-67 and BrdU indicate the proliferating rate of the smooth muscle cells. “i” indicates the region corresponding to the intima, and “m” indicates the region corresponding to the media. Bar=50 μm (A–T).
control native and the control cryopreserved aortas. Their numbers in the media of the transplanted allografts were 61±30/0.1 mm² (POD2), 28±29/0.1 mm² (POD5), and 4±5/0.1 mm² (POD11) (Fig. 2, asterisk). The increase in the numbers of BrdU-positive cells by POD2 was statistically significant (p<.0001). The ratio of BrdU-positive cells to nucleated cells was 19% on POD2, 6% on POD5, and 0.8% on POD11. These ratios are similar to those found in Ki-67 positive cells.

Chronic phase

To further examine the outcome of the proliferating smooth muscle cells in the acute phase of transplantation, similar experiments were performed in extended time course (POD60, 210, 360, and 720). The average numbers of nucleated medial smooth muscle cells of POD60 allografts were 162±68/0.1 mm², which were drastically decreased compared to that of POD11. Furthermore, no medial smooth-muscle cell was detected in the allografts explanted on POD210, 360 and 720, retaining well-preserved frames of elastic fiber (Fig. 3). In addition, neither Ki-67 nor BrdU staining gave signals in any of the allografts on POD210, 360, and 720 (Fig. 2). Thus, medial smooth muscle cells transiently proliferated after transplantation but were all eliminated eventually resulting in an acellular medial structure with the intact elastic frameworks.

On the other hand, smooth muscle cells were accumulated as stratified layers in the adventitia just outside of acellular media in all three allografts at POD210 (Fig. 3D). Similar accumulation of smooth muscle cells were observed in all four allografts on POD360 and 720, although the numbers of the accumulated cells were less. Neither Ki-67 nor BrdU gave any signals in these newly accumulated smooth muscle cells (data not shown), indicating that these cells are not actively proliferating.

In accordance with the proliferation of smooth muscle cells in the adventitia, vasa vasorum infiltration was also observed in the similar sites, but not in the media of all ten allografts on POD210, 360, and 720, assessed by immunohistochemical staining with CD34 antibody (data not shown).

We rarely found leukocytes in the media of any hematoxylin-eosin stained specimen on optical microscopic observation. Furthermore, immunoperoxidase staining using antibodies against CD45 detected no significant number of CD45-positive cells in the media of any aortic allograft, although they were occasionally found in the adjacent adventitia (data not shown).

Calcium depositions were observed in all seven allografts after POD60, but not observed in any of the eight allografts within 2 months. Scatter calcifications with lamellar shape were observed only in the adventitia adjacent to these regions of migrated smooth muscle cells. Leukocyte infiltrations induced from inflammatory reactions were not observed around calcium depositions. Although surrounded by calcium depositions, the structural frames of elastic lamellae of the media were well preserved (Fig. 4).

The origins of proliferating medial smooth muscle cells in transplanted allografts

The allelic patterns of the medial smooth muscle cells of POD11 allographic specimens coincided with those of the donor DNA in all 5 analyses, indicating that donor cells were present in the media of the allografts on the 11th day after transplantation (Fig. 5 and Table 1). In analyses 1, 2, and 5, the recipient-derived allelic bands were not detected in allographic DNA, because there were not enough recipient cells in the media of the allograft to be

![Fig. 2. The concentration of medial smooth muscle cells: Mean numbers of nucleate (closed circles), Ki-67-positive (squares), and BrdU-positive (asterisk) smooth muscle cells per 0.1 mm² in the media of native aortas, untransplanted cryopreserved aortas, and transplanted allografts excised on POD2, 5, 11, 60, 210, 360, and 720.](image-url)
Fig. 3. Optical microscopy of the allografts in chronic phase: No viable cell in the media was detected in the allografts explanted at POD210, 360, and 720 (A, B and C: Hematoxylin and eosin stain). The absence of medial smooth muscle cells was also confirmed (D, E and F: α-SMA immunostain). Accumulation of the smooth muscle cells in the adventitia just outside of acelluar media was noted (D, E and F; arrows). See the results in the text for each SD. Bar=50 μm (A–F).

Fig. 4. Calcification in the allografts show semicircumferential and lamellar pattern with scattered granular deposits in the adventitia of the allograft explanted on POD720 (arrows). The presence of calcium precipitation is proved by von Kossa stain (A: Hematoxylin and eosin stain, B: von Kossa stain). Bar=50 μm (A and B).
detected. On the other hand, in analyses 3 and 4, the donor-derived bands partly resembled those of the recipient. Therefore the possible involvement of the recipient cells cannot be completely ruled out. However, most of the medial smooth muscle cells in the allografts were considered to be donor-derived, based on the predominant density of the allelic bands.

**Fig. 5.** Microsatellite analyses: **A**, Allograft excised on postoperative day 11 and stained with Giemsa. **B**, Medial lesions “m” were microdissected from paraffin sections and used for DNA extraction. Intima and adventitia were excluded. Bar=300 μm (**A** and **B**). **C**, Gel electrophoretic patterns of the microsatellite DNA fragments from medial smooth muscle cells of the allograft excised on POD11 (1), and from the livers of donor (2) and recipient (3) rabbits in analysis 2. Microsatellite regions were amplified by PCR using oligonucleotide primers for the OCRLADF4 locus. Alleles were designated in alphabetical order with *a* coding for the shortest PCR product.

**Table 1.** Microsatellite analyses of the origins of the proliferated medial smooth muscle cells in the cryopreserved aortic allografts excised 11 days after transplantation

| Analysis | Allelic pattern* at OCASICG or OCRLADF4 locus | allograft | donor | recipient |
|----------|-----------------------------------------------|---------|------|-----------|
| 1        | **a**                                         | **a**   | **b**| **b**     |
| 2        | **a, c**                                      | **a, c**| **b**| **b**     |
| 3        | **a, b**                                      | **a, b**| **b**| **b**     |
| 4        | **a, b**                                      | **a, b**| **b**| **a**     |
| 5        | **a**                                         | **a**   | **a, b**| **b**     |

* Alleles were designated in alphabetical order with *a* denoting the shortest PCR product.
* The OCASICG locus was studied in analysis 1, 3, 4, and 5. In analysis 2, data regarding the OCASICG locus was not informative and the OCRLADF4 locus was used instead.

**IV. Discussion**

The present study demonstrates that the media of cryopreserved rabbit aortic allografts can be restored by the proliferation of donor-derived smooth muscle cells in the acute phase of transplantation. This is important as it proves that the viability of the transplanted allografts is maintained by donor-derived cells in the acute period.

We believe that the drastic increase of the number of smooth muscle cells in the media from POD2 to POD11 was caused by the proliferation of smooth muscle cells, since the Ki-67- and BrdU-positive cell numbers were consistent with the extent of the increase seen in the number of smooth muscle cells. On POD11, the number of medial smooth muscle cells in the allografts reached the same level as in the native aorta, and the proliferation of these cells ceased, as judged by the paucity of detectable BrdU- and Ki67-positive cells. Previous experimental studies of balloon- or suture-injured vascular models have demonstrated a proliferation of medial smooth muscle cells, though in mature vessels these medial smooth muscle cells are at rest and only rarely proliferate under normal conditions [1, 8, 32]. In these vascular-injury models, the incorporation of H³-thymidine into the smooth muscle cells in the arterial media was vigorous within 2 or 3 days after injury, but declined by the 5th day [8, 32]. This time
sequence of cell cycling after vascular injury was similar to that which was observed in our cryopreserved aortic allografts after transplantation.

Theoretically, the rapidly proliferating medial smooth muscle cells could have been derived from either donor or recipient. However, the replication of recipient cells is unlikely in the media of the allograft in this phase, because no heterogeneous band patterns were seen, and only donor-specific band patterns were demonstrated in the microsatellite analysis of the medial smooth muscle cells on POD11. According to the number of medial smooth muscle cells seen on POD2 and POD11, the cells that replicated after POD2 seemed to account for at least more than half of the medial smooth muscle cells seen on POD11. This fact led us to conclude that the residual donor-derived smooth muscle cells were replicating in the acute phase restoration of the medial layer.

In fact, several recent studies have shown that the smooth muscle cells in the thickened neointima after transplantation were derived from the recipient [11, 19, 28]. Since these previous studies analyzed the effects on intimal alteration, none of them focused on the repair of the allograft media. Therefore, our finding that the replicating cells in the media were of donor origin is not inconsistent with these reports.

According to the previous reports of histological alterations of cryopreserved aortic allografts after the acute transplantation phase, the number of medial smooth muscle cells in transplanted cryopreserved aortic allografts was found to either decrease to one-fifth the number by the 6th month after transplantation and remain low until 1 year afterward [23], or to decrease 3 months after transplantation and increase again 2 years later [24], although on POD10, the number of cells was almost the same as that in the native aortas [23, 24]. To elucidate the origin of the cells in the allograft, Neves et al., using sheep cryopreserved aortic allografts, demonstrated that, 1 or 2 years after transplantation, the cells in the allografts were either a mixture of donor- and recipient-derived cells or recipient-derived cells only [24]. These reports suggest that the restored medial muscle layer might get denatured, and that a further remodeling process occurs over a longer period.

In the present study, a rapid decrease in the number of medial smooth muscle cells by POD2 was observed in the transplanted cryopreserved aortic allografts, although the number in the non-transplanted cryopreserved aortas was almost the same as in the native aortas when assessed microscopically. There are two possible mechanisms that could explain the rapid decrease in the number of smooth muscle cells after transplantation. First, viable cells could have become non-viable because of cellular injury caused by the various stresses of the transplantation procedure itself, including wall ischemia, denervation, and mechanical trauma. However, cell injury due to the freeze-thaw procedure is unlikely because control experiments using a fresh allograft without cryopreservation showed no obvious loss of medial smooth muscle cells for 11 days after transplantation (data not shown). The second possible mechanism for explaining the rapid decrease in the number of smooth muscle cells after transplantation is that the cells were already non-viable but appeared to be viable in the non-transplanted cryopreserved aortas, because the staining patterns of the nuclei and α-SMA can be preserved for a short time after cells lose their viability.

In our study, acellularity of the media had been maintained from 7 months after the implantation up to 2 years. Vogt et al. reported in 1999, 27 explanted human cardiovascular allografts (2 weeks to 7 years) were almost acellular with only a few donor fibrocytes [31]. Loss of medial smooth muscle cells in chronic phase was also reported by others [15, 23]. However, it remains unclear why the medial cells gradually decreased in number and finally vanished away. Immunological rejection was unlikely to contribute, because no significant mononuclear cell infiltration was detected in any stages. Although medial acellularity in chronic phase was recognized as an unfavorable change, it was noteworthy that the media was kept away from not only an entry of inflammatory cells and bacteria but also newly-formed vasa vasorum infiltration for a long time, preserving the elastic fiber framework intact. The absence of reconstituted vasa vasorum might play some protective roles against unfavorable invaders. Abandonment of the vessel-associated route might provide allografts some defensive candidate against infection. In fact, our previous report on the human cryopreserved aortic allograft explanted from active infectious site demonstrated that the medium was not involved at all in inflammatory process in spite of severe suppurative inflammation in the adjacent adventitia [33].

Accumulation of smooth muscle cells in the adventitia of the allografts after 7 months was also notable. However, there were no Ki-67 or BrdU positive cells. This is probably because these smooth muscle cells in the adventitia grow extremely slowly. The significance of the accumulation of these smooth muscle cells is unknown, but one possible explanation is that, like normal medial smooth muscle cells, these smooth muscle cells in the adventitia have some contributory role in the tolerance for blood pressure.

Calcification was also an important characteristic of the implanted aortic allografts. Calcification in cryopreserved aortic allografts were also detected mainly in the inner layer of the media by Motomura et al. in 1995 [23]. However, in our study, vascular calcification was observed in explanted allografts after 7 months with lamellar shape in the adventitia. We speculate that some correlation exists between the accumulated smooth muscle cells in the adventitia and the allograft calcification because of their coincidental association in the adventitia.

The current study shows acute and chronic histological changes in rabbit aortic allografts. Medial smooth muscle cells of donor origin in cryopreserved aortic allografts have the potential to proliferate after the transient crisis in the acute phase of the postoperative course, and thus to rapidly restore the medial muscle layer. In contrast acellular trans-
formation of media in chronic phase of transplantation might be considered as one of the terminal forms in cryopreserved aortic allografts, and may play some role in the maintenance of cryopreserved aortic allografts.

V. Acknowledgments

We wish to thank Mari Kojima and Tsutomu Obata for their technical assistance.

VI. References

1. Allaire, E. and Clowes, A. W. (1997) The intimal hyperplastic response. Ann. Thorac. Surg. 63; 582–591.
2. Armiger, L. C., Gavvin, J. B. and Barratt-Boyes, B. G. (1983) Histological assessment of orthotopic aortic valve leaflet allografts: its role in selecting graft pre-treatment. Pathology 15; 67–73.
3. Armiger, L. C. (1995) Viability studies of human valves prepared for use as allografts. Ann. Thorac. Surg. 60 Suppl; 118–121.
4. Buja, L. M., Kita, T., Goldstein, J. L., Watanabe, Y. and Brown, M. S. (1993) Cellular pathology of progressive atherosclerosis in the WHHL rabbit. An animal model of familial hypercholesterolemia. Arteriosclerosis 3; 87–101.
5. Carrel. A. (1912) Ultimate results of aortic transplantations. J. Exp. Med. 15; 389–392.
6. Cavin, J. B., Barratt-Boyes, B. G., Hitchcock, G. C. and Herdson, R. (1996) Using aortic allograft material to treat mycotic transplant arteriosclerosis. J. Clin. Invest. 107; 1014–1021.
7. Chiesa, R., Astore, D., Piccolo, G., Melissano, G., Jannello, A., Frigerio, D., Agrifoglio, G., Bonalumi, F., Corsi, G., Brancadoro, S. C., Novali, C., Locati, P., Odero, A., Pirrelli, S., Cuqnasca, M., Bigioli, P., Sala, A., Polvani, G., Guarin, A., Biasi, G. M., Minquazzini, P., Scalamoqna, M., Mantero, S., Spina, G., Prestipino, F. and Sirchia, G. (1998) Fresh and cryopreserved arterial homografts in the treatment of post-transplant graft infections: experience of the Italian collaborative vascular homograft group. Ann. Vasc. Surg. 12; 457–462.
8. Clowes, A. W., Reidy, M. A. and Clowes, M. M. (1983) Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. Lab. Invest. 49; 327–333.
9. Gross, R. E., Hurwitt, E. S., Bill, A. H. and Peirce, E. C. (1948) Preliminary observations on the use of human arterial grafts in the treatment of certain cardiovascular defects. N. Engl. J. Med. 239; 578–579.
10. Haeringen, W. A., Bieman, M., Zutphen, L. F. M. and Lith, H. A. (1996–1997) Polymorphic microsatellite DNA markers in the WHHL rabbit. An animal model of familial hypercholesterolemia. Cardiovasc. Pathology 6; S225–232.
11. Kuwahara, M., Jacobsson, J., Kuwahara, M., Kagan, E., Ramwell, P. W. and Foegh, M. L. (1991) Coronary artery ultrastructural changes in cardiac transplant atherosclerosis in the rabbit. Transplantation 52; 759–765.
12. Kuwahara, M., Jacobsson, J., Kuwahara, M., Kagan, E., Ramwell, P. W. and Foegh, M. L. (1991) Coronary artery ultrastructural changes in cardiac transplant atherosclerosis in the rabbit. Transplantation 52; 759–765.
13. Kuwahara, M., Jacobsson, J., Kuwahara, M., Kagan, E., Ramwell, P. W. and Foegh, M. L. (1991) Coronary artery ultrastructural changes in cardiac transplant atherosclerosis in the rabbit. Transplantation 52; 759–765.
14. Kuwahara, M., Jacobsson, J., Kuwahara, M., Kagan, E., Ramwell, P. W. and Foegh, M. L. (1991) Coronary artery ultrastructural changes in cardiac transplant atherosclerosis in the rabbit. Transplantation 52; 759–765.
K., Ruef, C., Debatin, J., Seifert, B., Kiowski, W. and Turica, M. I. (1998) Cryopreserved arterial allografts in the treatment of major vascular infection: a comparison with conventional surgical techniques. *J. Thorac. Cardiovasc. Surg.* 116; 965–972.

31. Vogt, P. R., Stallmach, T., Niederhauser, U., Schneider, J., Zund, G., Lachat, M., Kunzli, A. and Turina, M. I. (1999) Explanted cryopreserved allografts: a morphological and immunohistochemical comparison between arterial allografts and allograft heart valves from infants and adults. *Eur. J. Cardiothorac. Surg.* 15; 639–644.

32. Webster, W. S., Bishop, S. P. and Geer, J. C. (1974) Experimental aortic intimal thickening. *Am. J. Pathol.* 76; 265–284.

33. Yamada, A., Okada, K., Takahashi, R. and Okita, Y. (2006) Pathologic features of cryopreserved aortic allograft implanted in the active infection. *J. Thorac. Cardiovasc. Surg.* 132; 203–204.