Cardiac transplantation has become a well-established therapeutic modality for patients with severe end-stage cardiac failure. Early survival rate after cardiac transplantation have improved significantly as a result of progress of medical and surgical management, monitoring techniques and immunosuppressive therapy. However, long-term survival has not improved as that of early stage because the recipient brings various comorbidities, such as infection, malignancy, renal failure and cardiac allograft vasculopathy (CAV). CAV is a specific form

Characteristics of Cardiac Allograft Vasculopathy Induced by Immunomodulation in the Miniature Swine

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Purpose: We aimed to develop swine cardiac transplantation model for study of cardiac allograft vasculopathy (CAV) and to characterize the mechanisms of its formation.

Methods: Heterotrophic cardiac transplantation was performed in swine leukocyte antigen mismatched miniature swine, and CAV was induced by immunomodulation by cyclosporine A (CyA). Histology and immunohistochemistry were performed to identify cellular components of CAV. Fluorescence in situ hybridization (FISH) was developed for detection of 1 and Y-chromosome for identification of cell origin in the female donor to the male recipient heart transplantation model.

Results: CAV was successfully developed by immunomodulation of CyA. Severity of CAV revealed more prominent in the distal epicardial coronary arteries than proximal coronary arteries. Phenotype of the SMCs proliferated in the intimal thickening of CAV were mostly embryonal/secretory type. Our new chromosome specific probes for FISH method were useful for discrimination of sex of each cell, and proliferated SMCs were revealed to be mainly donor origin.

Conclusion: CAV mimicking human heart transplantation can be developed by appropriate immunomodulation in the swine. In swine CAV, proliferated SMCs seen in the intimal thickening were demonstrated to be from the donor origin.

Keywords: miniature swine, heart transplantation, cardiac allograft vasculopathy, smooth muscle cell, intimal hyperplasia

Introduction

Cardiac transplantation has become a well-established therapeutic modality for patients with severe end-stage cardiac failure. Early survival rate after cardiac transplantation have improved significantly as a result of progress of medical and surgical management, monitoring techniques and immunosuppressive therapy. However, long-term survival has not improved as that of early stage because the recipient brings various comorbidities, such as infection, malignancy, renal failure and cardiac allograft vasculopathy (CAV). CAV is a specific form
of coronary artery disease and characterized by concentric and diffuse intimal proliferation rather than the more focal lesions of conventional atherosclerosis, ultimately resulting in the development of luminal stenosis or vessel occlusion in 50% or more of heart-transplant recipients in several years after surgery. The mechanisms of development of CAV and its prevention have been widely studied. It is suggested that CAV development begins as a “response to injury,” initial endothelial dysfunction followed by intimal hyperplasia as a result of vascular remodeling in response to initial reperfusion injury and repetitive immunological transplant-related endothelial injury. These vascular remodeling and smooth muscle cell (SMC) proliferation are potentiated by inflammatory cytokines, growth factors, and chemoattractant factors produced by activated endothelial cells and migrating cells, resulting in the activation of SMC and their migration from the media to the intima. Activated SMC further proliferate and secrete cytokines, which potentiate proliferation and subsequent matrix deposition and luminal narrowing. We have already reported that many inflammatory cytokines, growth factors, and chemoattractant factors involved in CAV and developed preventive method of CAV by various gene therapy. Based on the “response to injury” theory, it is postulated that donor SMC migrate from the media to the intima and forming intimal hyperplasia. However, recent reports suggested another mechanism that recipient-derived cells such as recipient bone marrow cells may be involved.

In this study, we developed a large animal model of CAV with miniature swine in which distributions and functional constrictions of coronary artery and susceptibility to atherosclerosis are similar to that of human beings by regulating blood cyclosporine A (CyA; Sandoz, Swiss) levels after heterotopic cardiac allo-transplantation. The donor heart was heterotopically transplanted into the recipient swine abdomen by infrarenal allografting. Transplant donor and recipient were fasted overnight, then anesthetized with ketamine and atropine intramuscularly for intubation. Anesthesia was maintained with 2% of halothane. First, the donor was heparinized and the donor heart was harvested after cardiac standstill induced by cold cardioplegic solution (Miotector; Mochida, Tokyo, Japan), and no further cardioprotection was performed. The recipient was placed in the left lateral decubitus position and systemic heparinization. The donor pulmonary artery was sutured to the recipient infrarenal inferior vena cava with end-to-side anastomosis. Next, the ascending aorta of the donor heart was anastomosed to the recipient’s infrarenal abdominal aorta in a similar manner. The graft function was daily checked by palpation and echocardiography was performed at 2 times a week. At the time of transplantation, blood samples were obtained for analysis of swine leukocyte antigen (SLA) class II antigen by polymerase chain reaction methods (PCR) and others.

Regimen of cyclosporine A
CyA was intramuscularly injected to the recipient starting on the day of operation (postoperative day 0: POD 0) and continuing until POD 12 and orally administered from POD 13 to POD 90. The blood levels of CyA were measured by cloned enzyme donor immunossay, and were maintained between 400 and 800 ng/ml from POD 1 to 12, between 200 and 600 ng/ml from POD 13 to 60, and between 100 and 200 ng/ml from POD 61 to 90. Blood levels of CyA were measured at 2 times a week, and doses of CyA were adjusted to the aimed blood level.

Histology and immunohistochemistry
After transplanted grafts were harvested at POD 90, tissues were collected from the proximal and distal portion of each epicardial left anterior descending artery (LAD), left circumflex artery (LCX) and right coronary artery (RCA). Formalin-fixed and paraffin-embedded
tissues were used for histology and immunohistochemistry. Each paraffin-embedded tissue was cut into 3 μm sections, deparaffinized in xylene, rehydrated in phosphate buffered saline (PBS), and stained with hematoxylin-eosin (HE) and elastica-van-Gieson (EVG) for evaluation of percent stenosis. The sections were photographed and blindly video-digitized in an image analysis system (NIH image). The area encompassed by the lumen and internal elastic lamina (IEL) was traced carefully, and the cross-sectional area of luminal stenosis was calculated by the formula: percent stenosis = (IEL area – luminal area)/IEL area. In immunohistochemical analysis, smooth muscle cells in the intimal thickening were identified by markers for α-actin (α-SMA) and embryonalsecretory type of smooth muscle (SMemb).16)

Fluorescence in situ hybridization

We developed a simultaneous detection system of chromosome Y- and 1-bearing swine cells by FISH. A conventional polymerase chain reaction (PCR) was performed using a set of oligonucleotide primers (5'- GTTGCACCTTTACGGACCAG -3’ and 5'-CTAGCCATTGCTGCCAATG-3’) for 244 bp fragment of porcine male-specific DNA sequence for X12695 and (5'- AATCCACCATACCTCATGGACC -3’ and 5'-TTTCTCCTGTATCCTCCTGC-3’) for 377 bp fragment of porcine Y-chromosome DNA sequence for X51555 as a positive control. Chromosome Y- and 1-specific DNA probes were produced by PCR. DNA fragment specific to chromosome Y was labeled by TRITC/ Cy3 fluorescence and chromosome 1 was labeled by FITC fluorescence. The hybridization probe mixture of labeled Y-chromosome and chromosome 1-specific DNA was applied to the preparation.12)

Statistical analysis

Data were expressed as mean ± SD. Differences were compared using the un-paired t test for comparisons between 2 groups. Differences with values of p <0.05 were considered significant.

Results

Among 36 transplanted recipients, 14 recipients survived throughout the experiment. SLA class II antigen of 5 survived recipients were matched to the donor, thus mismatched 9 survived recipients (7 male to male transplantations and 2 female to male transplantations) were evaluated in this study.

The ischemic times were 186.6 ± 30 minutes. Blood concentrations of CyA were maintained almost at the aimed levels as 585.3 ± 271.5 ng/ml at POD7, 168.2 ± 60.7 ng/ml at POD 50 and 84.0 ± 28.1 ng/ml at the end of experiment. The heart rates gradually decreased 85.4 ± 23.3 bpm on POD 7 to 60.7 ± 19.7 bpm on POD 90 (P <0.05) (Fig. 1). Fractional shortening gradually increased up to POD 42 and decreased thereafter, but did not show any significant change (Fig. 1).

Epicardial coronary arteries showed CAV from mild to severe lesions by concentric cellular proliferation. SMCs in the intimal thickening were identified by markers for α-actin (α-SMA) and embryonalsecretory type of smooth muscle (SMemb).16)

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Specificity of the developed DNA probes of FISH for discrimination of swine sex was confirmed in each male and female swine tissue samples as shown in the Fig. 4.

Analysis of cellular origin of CAV in the male recipient by FISH revealed proliferated cells were mostly positive to chromosome 1 DNA probe and very few positive to Y chromosome probe (Fig. 5).

Discussion

Chronic rejection remains a major cause of late morbidity and mortality in patients receiving organ transplantation. In heart transplantation, to clarify the mechanisms of CAV is crucial for developing effective diagnosis, prevention and therapeutic strategies.

The findings of previous experimental and clinical studies have suggested that immunologic events involving allo-reactive T cells and the humoral immune system, as well as non-immunologic factors induce CAV. The animal models of CAV were reported from rodents to large animals. The distinctive differences between rodents and swine are immune systems and cardiovascular systems. The swine major histocompatibility complex has been well-characterized, and unlike that in rodents, swine coronary artery endothelium constitutively express class II antigen. And also the swine heart can be treated like human beings for repeated biopsies, angiography and intravascular ultrasound, thus we developed the swine heart transplantation model for study of CAV. In the present study, we successfully developed swine CAV lesions identical to those observed in clinical human transplantations by modulating the blood CyA levels. Severity of CAV revealed more prominent in the distal epicardial coronary arteries than proximal coronary arteries. And phenotype of the SMC proliferated in the intimal thickening of CAV were mostly embryonal/secretory type. Our new chromosome specific probes for FISH method were useful for discrimination of sex of each cell, and using this method, proliferated SMCs seen in the intimal thickening were demonstrated to be from the donor origin.

In swine experimental transplantation, previous studies demonstrated that development of CVA is achieved by selection of partially SLA mismatched pairs or by immunomodulation of immunosuppressant drugs. In the present study, we successfully developed swine CAV lesions identical to those observed in clinical human transplantations by modulating the blood CyA levels. We did pilot study before determination of final protocol. In the pilot study, we found difficulties in controlling...
blood CyA levels at aimed levels by the pre-determined doses. Thus, we employed the different protocol of changing the CyA dose adjusting to the blood CyA levels measured 2 times a week instead of dose response study. We selected SLA type II mismatched pairs and CyA for immunosuppressive therapy. Our protocol of controlling blood CyA levels for formation of CVA in the beating transplanted hearts was effective to form various degrees of CVA from mild stenosis to severe intimal thickening indistinguishable from CVA lesions observed in human cardiac recipients.\(^{14,15}\) However, in the present study, mortality rate of recipients before 3 months was high. Madsen, et al. reported that MHC matched recipients survived 42–56 days, thus it is very difficult to keep alive recipients in swine cardiac transplantation model for a long-time.\(^{14}\) We did not experienced any apparent complications suggesting of side effects of CyA, our experimental model may be useful to study post-transplant CAV.

The pathogenesis of CAV involves both immunological and non-immunological factors, such as an older donor age, cytomegalovirus infection, hyperlipidemia, hypertension, ischemia reperfusion injury and the intensity of acute rejection.\(^{18}\) Recent findings demonstrated the initial pivotal role of the endothelial cells, resulting in release of cytokines and multiple mediators, recruitment of circulating leucocytes and lymphocytes, proliferation of vascular SMCs, and deposition of extracellular matrix proteins. CAV is a concentric intimal thickening due to proliferation of SMCs in the intimal layer of coronary arteries. SMCs have been reported to be classified into 3 types in accordance with myosin heavy chain (MHC) isoforms such as SMemb, SM1 and SM2.\(^{16}\) SMemb is expressed in the early stage of development, and then gradually changed to adult phenotypes of SM1 and SM2. Previously, we reported that SMemb along with SM1 positive SMCs existed in the neointima with accumulation of lymphocytes in acute rabbit heart transplantation model, indicating that phenotype modulation of SMCs occurred in response to allogenic stimuli.\(^{19}\) Cellular proliferation appears to be central to the pathogenesis of CAV, leading researchers to develop inhibitors of such proliferation. We already reported that CVA can be prevented by modulating various multiple mediators and cytokines by gene therapy such as Egr-1 (antisense), MMP (ribozyme), NFkappa-B (Decoy), E2F (Decoy), PCNA (antisense), and Bcl-x and Bax (antisense) in rodents heart transplantation models.\(^{8}\) In the present study, severity of epicardial coronary artery lesions are prominent in the distal portion compared to that of proximal portion is identical to previous studies of CVA.\(^{6}\) While on the contrary, Lin reported the equal distribution and severity of coronary artery lesions by comparing proximal and distal portion of the left anterior descending coronary artery.\(^{20}\) These differences in preference and severity of CVA may be due to the episodes of acute cardiac rejection, duration after transplantation, medication, species difference and coronary risk factors such as hypertension and hyperlipidemia.

The origin of the SMCs in the neointimal lesions is controversial.\(^{21}\) Many possible mechanisms have been proposed from the experimental and clinical studies of atherosclerosis and transplant associated CAV. Shi suggest the significance of vascular adventitial fibroblasts in the process of arterial repair using porcine coronary artery injury model.\(^{22}\) They postulated that following coronary artery injury, adventitial fibroblasts translocated from adventitia to neointima, changing their phenotype to myofibroblasts to form neointima. Campbell postulated that medial SMCs transformed its phenotype from adult type to embryonal or secretional type, and migrate into intimal lesions and proliferate in the neointima.\(^{23,24}\) Other report suggested the importance and involvement of circulating progenitor cells from bone marrow in the development of CVA.\(^{25}\) The presence of chimerism in the transplanted heart is controversial. Quaini demonstrated
the high incidence of chimerism in the male recipients' hearts transplanted from the female donors by analysis of gender specific chromosome.\textsuperscript{25} In other report using the same method of sex-mismatched human cardiac transplantation by Glaser, the chimerism in the coronary artery is very few (host origin 2.6\%), and no cardiac myocyte originated from the recipient were found in the transplanted myocardium.\textsuperscript{26} Recent study of the 34 autopsied cases by Devitt, in 28 cases of the epicardial intimal lesions contained 2 clearly definable layers overlying the media. The layer most adjacent to the media was SMC-rich, with the SMC oriented longitudinally along the vessel length and containing few macrophages.\textsuperscript{3} In contrast, the more luminal layer showed that SMC
(or SMC-like cells) are sparsely present. Those SMCs present circumferential orientation, reminiscent of the cells of the media. They proposed that the layer most adjacent to the media was carry-over donor-derived intimal proliferation and the more luminal layer resembled naturally occurring atherosclerosis, and that generation of the de novo lesions involves the perturbation and activation of this carry-over donor intimal thickening, the deposition of lipid resulting in the development of accelerated graft atherosclerosis. We developed a new chromosome specific probes for FISH method demonstrated SMCs in CAV lesions are mainly donor origin in which no pre-existing intimal thickening or atherosclerosis are present in contrast to human donor heart. The implications of this FISH method are not only for medical study but also for discrimination of sex in stockbreeding.\(^{12}\)

**Limitation**

The limitation of our study is that swine has no coronary risk factors such as hyperlipidemia, hypertension and others, thus participation of the process of natural atherosclerosis after transplantation cannot be evaluated in this study.

**Conclusion**

We successfully developed swine CAV lesions identical to those observed in clinical human transplantations by modulating the blood CyA levels. Severity of CAV revealed more prominent in the distal epicardial coronary arteries than proximal coronary arteries. And phenotype of the SMC proliferated in the intimal thickening of CAV were mostly embryonal/secretory type. Our new chromosome specific probes for FISH method were useful for discrimination of sex of each cell, and using this method, proliferated SMCs seen in the intimal thickening were demonstrated to be from the donor origin.

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**Disclosure Statement**

There is no conflict of interest to declare.

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