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Time-dependent Expression of ICAM-1 & VCAM-1 on Coronaries of the Heterotopically Transplanted Mouse Heart

To investigate the pathogenesis of accelerated graft atherosclerosis after cardiac transplantation, a genetically well-defined and reproducible animal model is required. We performed heterotopic intraabdominal heart transplantation between the two inbred strains of mice. Forty hearts from B10.A mice were transplanted into B10.BR mice. Recipients were sacrificed at 1, 3, 5, 7, 14, 28, and 42 days after implantation. The specimens from both donor and recipient were examined with fluorescent immunohistochemistry and the serial histopathologic changes were evaluated. In the donor hearts, ICAM-1 and VCAM-1 expressions were minimal at day 1 and they gradually increased, reaching their peaks on day 5 or 7 and remained unchanged by day 42. However, there were very little expressions in the recipients' hearts. Mean percent areas of intima in the donor coronaries revealed progressive increase by day 42. However, those in the recipients occupied consistently less than 5% of the lumen. In conclusion, we demonstrated that a heterotopic murine heart transplantation model was a useful tool to produce transplantation coronary artery disease and that adhesion molecules on the cardiac allografts were activated very early and remained elevated at all time-points, nonetheless the arterial lesion was detected after day 28 and its progression was accelerated thereafter.

Key Words: *ICAM-1; VCAM-1; Heart transplantation; Coronary atherosclerosis; Graft rejection*

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INTRODUCTION

Accelerated graft arteriosclerosis (AGAS) has emerged as the leading cause of death in heart transplant recipients. Recent studies revealed the incidence of this disease goes up to 44% in three years after transplantation (1). Although the pathogenesis of AGAS is not clearly understood yet, there exist views that it could result from a subclinical sustained immunologic insult to the coronary arteries linked to MHC differences between the donor and recipient. It indiscriminately involves the entire length of the vessel (2) or it spares the recipient's arteries (3). The general belief is that AGAS involves a localized cell mediated immune response initiated by activating host T-lymphocytes by graft endothelial cells (4). T-cell activation within the vessel wall could cause local production of cytokines or growth factors that result in accumulation and proliferation of intima and smooth muscle cells of graft arteries (5). In addition to the initiating role of MHC antigens, vascular adhesion molecules also play important roles in mediating allo-immune reaction. The attachment of T-lymphocytes to other cell types is crucial to various lymphocyte functions, including antigen presentation and recognition, target cell recognition, and traversing blood vessel walls (6, 7). Vascular adhesion molecules regulate this process and may play an important role in the development of cell-mediated immune responses. Several studies using prophylactic anti-ICAM-1 and anti-VCAM-1 antibody showed inhibition of rejection and induced a state of long-term graft survival (8, 9). This study has been performed to document the time courses of two endothelial adhesion molecules expressions: intercellular adhesion molecule (ICAM)-1 (10) and vascular cell adhesion molecule (VCAM)-1 (11), and to document the progressive histopathologic changes in the donor coronary arteries in the heterotopically transplanted murine cardiac allografts.

MATERIALS AND METHODS

Heterotopic heart transplantation

Adult mice (seven to ten weeks of age) of B10.A and B10BR strains, weighing 17-22 gram, were obtained from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, U.S.A.). The B10.A (H-2D^a) and B10.BR (H-2D^k) strains differ in the D locus of the class I MHC antigen. The mice were housed under conventional conditions and fed a standard diet (Rodent laboratory chows, Cheil Jedang Company, Incheon, Korea) and water ad libitum. The surgical technique for intraabdominal heterotopic heart transplantation was a modification of the technique previously described by Corry et al. (12). Briefly, after adequate anesthesia using 4% chloral hydrate (0.1 mL/20 g of body weight, intraperitoneal injection) and methoxyflurane (inhalation), a "U"-shaped sternal lid was made and lifted cephalad. Both the right and the left superior caval veins were ligated with 5-0 silk. The donor heart was arrested with 0.5 mL cold heparinized saline (100 unit/mL of saline) delivered via the inferior caval vein. The aorta and main pulmonary artery were then transected. After transection of caval veins distal to the ligatures, the pulmonary veins were ligated en bloc and transected. The donor heart was preserved in 4℃ cold saline solution until the recipient mouse was prepared. Through a midline abdominal skin incision, the recipient's infrarenal abdominal aorta and inferior caval vein were dissected and controlled both proximally and distally with 5-0 silk ligatures. After longitudinal aortic incision, an end-to-side anastomosis between the transected end of donor ascending aorta and the recipient abdominal aorta, followed by an end-to-side anastomosis between the transected end of donor pulmonary artery and the recipient inferior caval vein using 10-0 nylon sutures. Subsequently, the proximal recipient aortic ligature was released first. After completion of heterotopic heart transplantation, the mice were allowed to recover with oxygen and local heat, and were then transferred into their cages with free access to food and water. Recipient animals were sacrificed at 1, 3, 5, 7, 14, 28, and 42 days after implantation. Five mice were included in each corresponding day.

Immunohistochemistry

On each corresponding day, all mice were sacrificed and grafts were snapfrozen in liquid nitrogen containing isopentane and stored at -70℃. The specimens were embedded in OCT compound (Tissue-Tek, Miles Inc., Elkhart, IN, U.S.A.). Cryosections (4 μm thickness) were mounted on silane coated glass slides and fixed in -20℃ acetone for five min and air dried. The slides were washed three times in PBS. Before addition of the primary antibody, non-specific binding was blocked with PBS containing 0.15% normal horse serum (Vector, U.S.A.) for 30 min. The sections were then incubated with the primary antibody (for ICAM-1: purified hamster anti-mouse CD54 monoclonal antibody, Pharmigen, U.S.A. dilute 1:50 in blocking serum, for VCAM-1: purified anti-mouse CD106 monoclonal antibody, Pharmigen, dilute 1:100 in blocking serum) for two hr in a humidified chamber at room temperature. The slides were dipped in PBS in 0.1% Triton X-100 for three min in order to reduce background noise and washed additional three times with PBS. Then the specimens were incubated with FITC-labeled secondary antibodies (for ICAM-1: FITC conjugated anti-hamster IgG, Pharmigen, dilute 1:50 in blocking serum, for VCAM-1: FITC conjugated rabbit anti-rat IgG, Pharmigen, dilute 1: 100 in blocking serum) for 30 min at room temperature in the dark. Following subsequent washes in PBS with 0.05% Tween 20, slides were counterstained with propodium iodide/antifade (Oncor, U.S.A.), and mounted in fluorescence mounting media (Dako, Denmark). The sections were examined and scored on a scale of 0 to 3 by two of the authors in a blind manner (Table 1). Photomicrographs were taken with a fluorescence microscope (Olympus, Japan).

Morphometric measurement

To demonstrate the serial changes of intimal hyperplasia and medial hypertrophy, percent areas of intima and media were calculated by following equations. {Percent area of intima=$[1-(D_L^2/D_{IEL}^2)]\times 100$, percent area of media=$[1-(D_{IEL}^2/D_{EEL}^2)]\times 100$, where D_L=diameter of lumen, D_{IEL}=diameter of internal elastic lamina, D_{EEL}=diameter of external elastic lamina}. Percent luminal patency was calculated by the equation, $[(D_L^2/D_{IEL}^2)\times 100]$.

Statistical analysis

The mean±standard error values were calculated for each group of mice and AVONA with single factor analysis was used to determine significance.

Table 1. Grading scale of the semi-quantitative method in measuring ICAM-1 and VCAM-1 in cardiac tissues

Grade	Extent of immunofluorescent staining
0	Negative for immunofluorescence
1	Equivocal staining for immunofluorescence
2	Definite positive staining of intramuscular arteriole for immunofluorescence
3	Positive staining of myocardium and interstitium in addition to arteriole

RESULTS

All allografts showed palpable contractions at the time of harvest. Cold ischemic time ranged from 40 to 61 min (mean 49±8 min). ICAM-1 and VCAM-1 expressions in cardiac tissues were demonstrated in Table 2 and Table 3. In the donor heart, ICAM-1 was expressed on the coronary arteries with a mean grade of 1.1±0.1 at day 1. The intensity of expressions increased gradually, peaking on either day 5 or 7 (mean grade of 2.3±0.3 at day 7), and remained unchanged thereafter (mean grade of 2.4±0.2 at day 42). However, the recipient's coronary arteries did not show such dramatic increases of expression except milder degrees of elevation for initial 3-5 day ($p<0.05$, ANOVA) (Fig. 1, Table 2). VCAM-1 expression was present from day 1 at a mean grade of

Table 2. ICAM-1 expressions in cardiac tissues were demonstrated. The intensity of expressions increased gradually, peaking on day 5, and remained unchanged thereafter. However, the recipients' coronary arteries did not show such dramatic increases in expression

	Donor	Recipient	*p* value
Day 1	1.1±0.1 (n=7)	1.1±0.2 (n=10)	0.43
Day 2	1.0±0.3 (n=5)	1.5±0.5 (n=5)	0.22
Day 3	1.2±0.2 (n=9)	1.7±0.4 (n=9)	0.14
Day 5	2.6±0.2 (n=7)	2.0±0.6 (n=4)	0.14
Day 7	2.3±0.3 (n=7)	1.4±0.4 (n=9)	0.05
Day 14	2.6±0.3 (n=9)	1.1±0.4 (n=10)	0.005
Day 28	2.8±0.2 (n=9)	1.3±0.4 (n=8)	0.001
Day 42	2.4±0.2 (n=10)	0.8±0.2 (n=9)	0.001

Table 3. VCAM-1 expression was increased by day 5 and remained unchanged thereafter. On the contrary, in the recipient heart it was expressed to a lower degree

	Donor	Recipient	*p* value
Day 1	1.6±0.4 (n=7)	1.1±0.3 (n=8)	0.20
Day 2	1.7±0.3 (n=5)	1.2±0.3 (n=5)	0.14
Day 3	1.8±0.5 (n=6)	1.7±0.4 (n=8)	0.45
Day 5	3.0±0.0 (n=7)	1.6±0.4 (n=7)	0.01
Day 7	2.5±0.3 (n=7)	1.6±0.4 (n=6)	0.08
Day 14	2.8±0.1 (n=10)	1.5±0.3 (n=8)	0.001
Day 28	2.6±0.2 (n=8)	1.4±0.4 (n=8)	0.004
Day 42	3.0±0.0 (n=10)	1.1±0.2 (n=10)	0.001

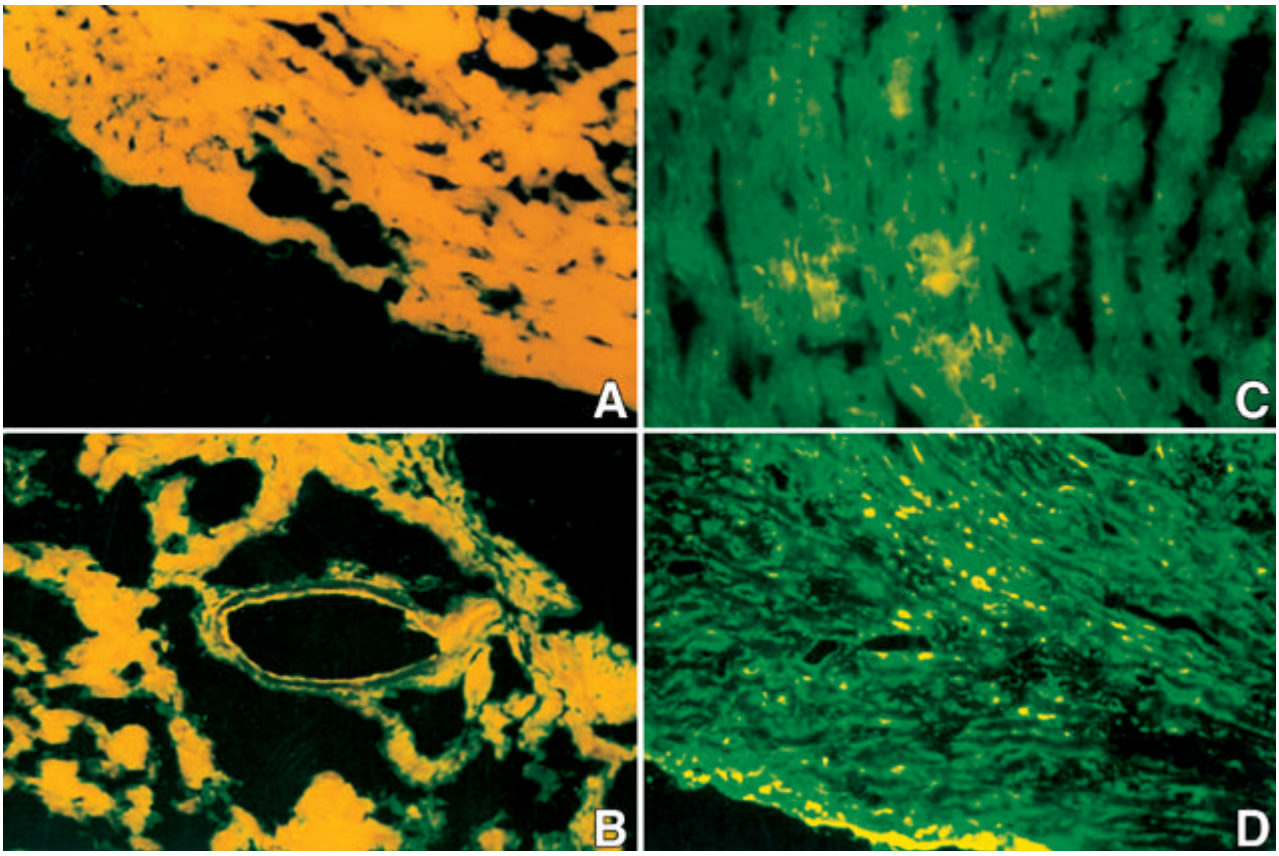

Fig. 1. ICAM-1 and VCAM-1 expression in cardiac tissues. A: ICAM -1 expression in recipient heart. B: In day 7 donor coronary artery, ICAM-1 is expressed in mean grade 2.3 that is, definitely positive staining of intramyocardial arteriole for immunofluorescence. C: VCAM -1 expression in recipient heart. D: VCAM-1 expression in day 42 donor heart, VCAM-1 is expressed in mean grade 3.0 that is, definitely positive staining of myocardium and interstitium in addition to intramyocardial arteriole (×400).

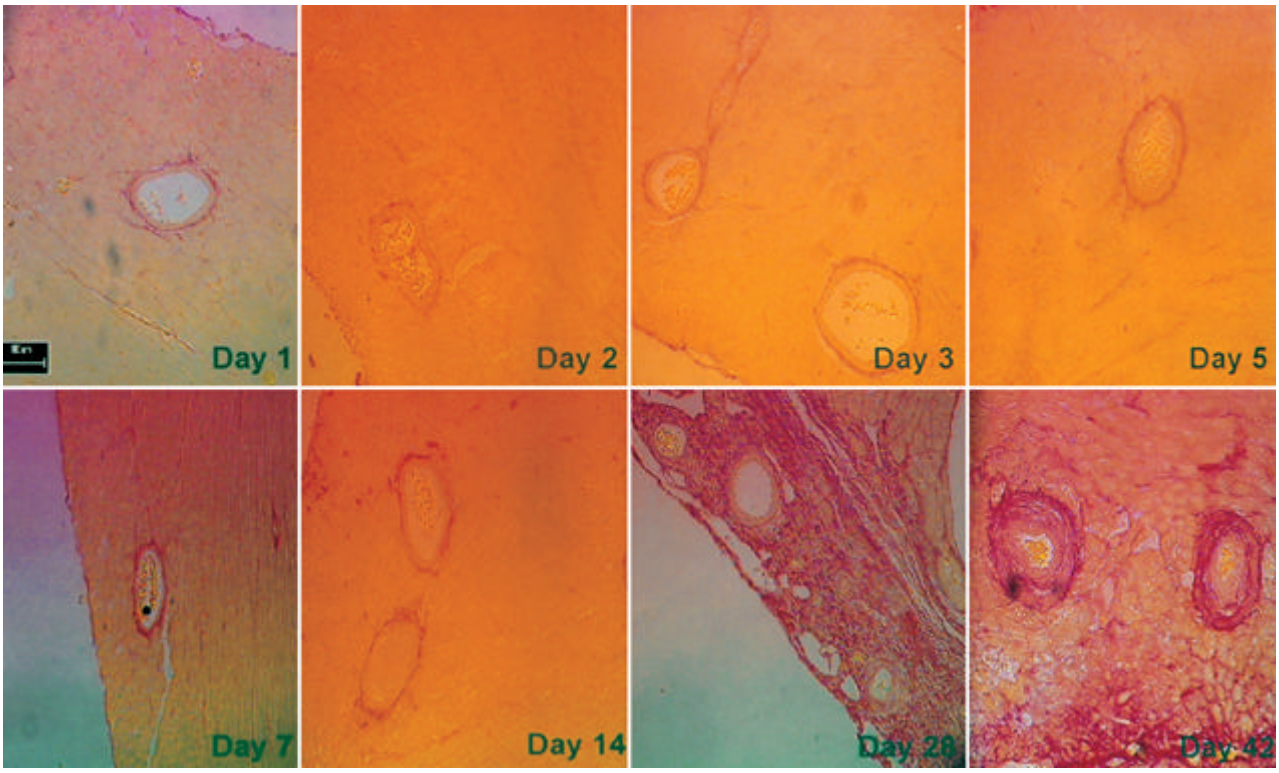

Fig. 2. Serial representative histopathologic changes in donor coronary arteries. Day 42 specimen has characteristic atherosclerotic change in comparison with early specimen.

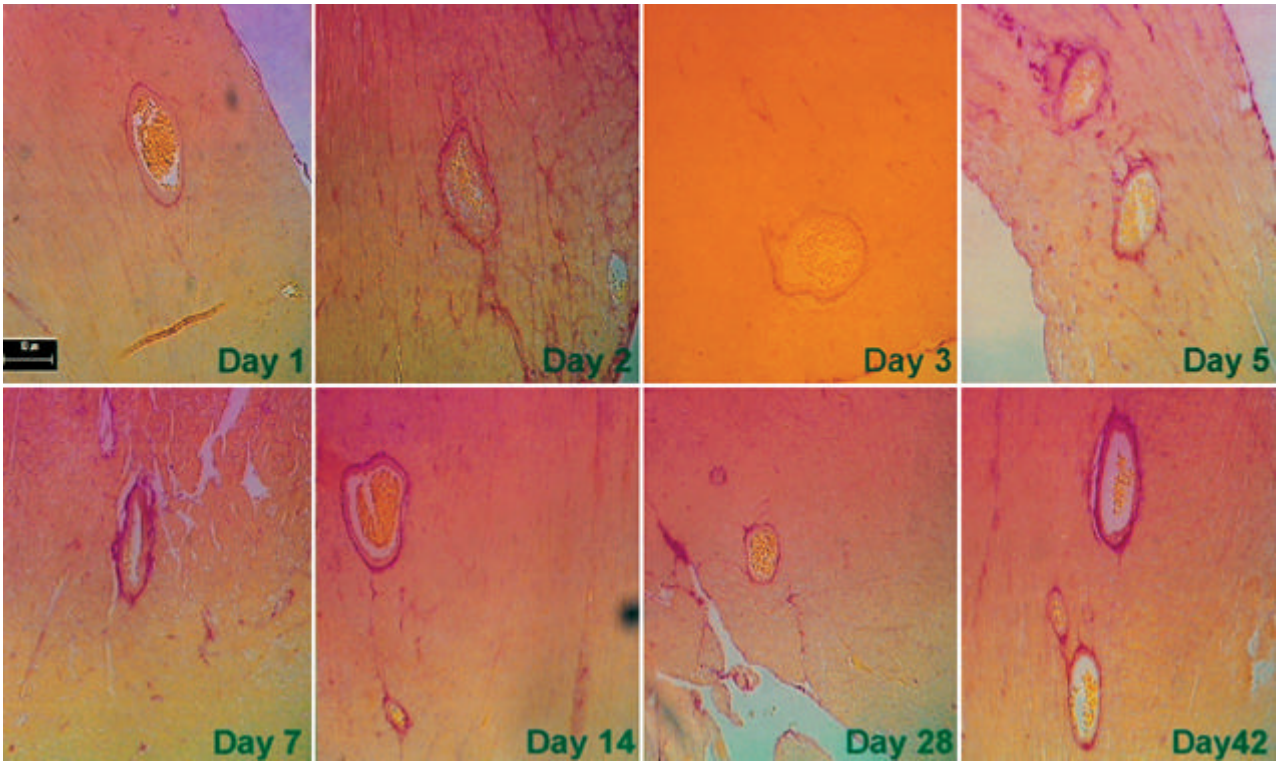

Fig. 3. Serial representative histopathologic changes in recipient coronary arteries. Thickness of intima and media remained unchanged throughout the observed experimental period.

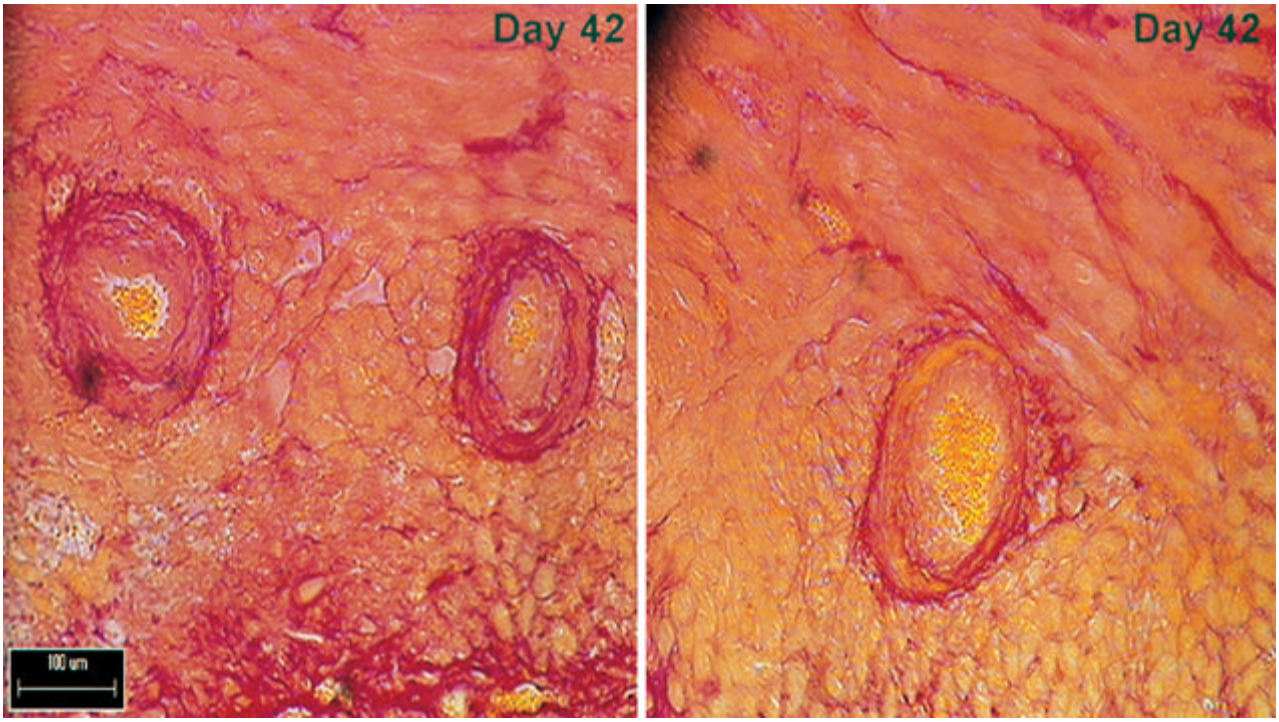

Fig. 4. Severe arteriosclerotic narrowing similar to accelerating graft atherosclerosis is observed in donor artery at day 42. These findings are characterized by prominent intimal and medial hyperplasia, and preserved internal elastic lamina with diffuse involvement.

1.6±0.4, and was increased by day 5 (mean grade of 5 day=3.0±0) and remained at mean grades over 2.5 by day 42 in the donor arteries. On the contrary, in the recipient heart it was expressed to a lower degree, following mild degree of increase for initial three days ($p<$ 0.05, ANOVA) (Fig. 1, Table 3).

The representative histopathologic changes in donor and recipient coronary arteries were demonstrated in Fig. 2 and 3. Severe arteriosclerotic narrowing was observed in the day 42 group (Fig. 4). Means of percent area of intima in donor arteries revealed less than 25% by the 14 day group, then it progressively increased thereafter (means of percent area of intima at 42 day, 68.5±3.5%). On the contrary, the intima of the recipients' coronary arteries occupied less than 5% of the lumens (Fig. 5). Mean percent area of media in donor arteries remained

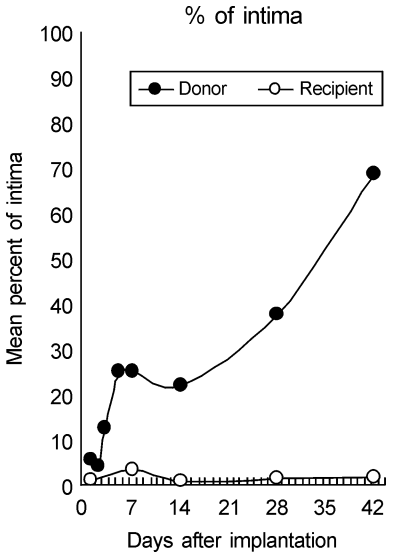

Fig. 5. The serial changes of percent areas of intima was calculated.

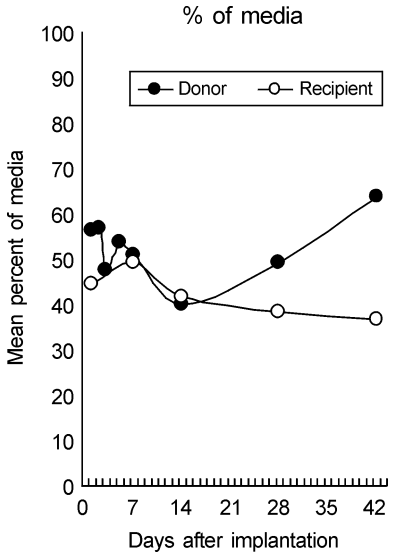

Fig. 6. Serial changes of percent areas of media were calculated.

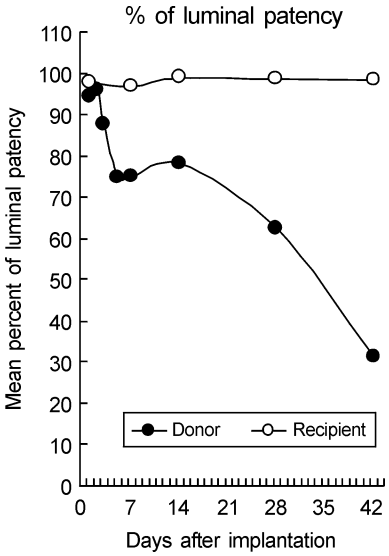

Fig. 7. Serial changes of percent luminal patency were calculated.

constant compared to the corresponding recipient specimens by the 28 day, followed by a modest increase in the 42 day group (Fig. 6). Mean percent luminal patencies were over 75% by two weeks after implantation, then gradually dropped thereafter to 31.5±19.2% at 42 day (Fig. 7).

DISCUSSION

This study documented the long-term serial progressive histopathologic changes of donor coronary arteries in a murine model of accelerated graft arteriosclerosis, and observed the time course of the adhesion molecule expression including ICAM-1 and VCAM-1. The availability of mouse strains with predetermined different histocompatibility provides an opportunity to examine the AGAS. However, a strict restriction of histocompatibility difference between donor and recipient is required to produce the mature vascular obstructive lesions, otherwise the entire transplanted heart is likely to be destroyed due to acute-type rejection. The strain combination, B10A and B10BR, in this study differs in D locus of class I MHC antigen and previously reported as the strain pair with long-term donor heart survival (13, 14) and progressive intimal thickening of coronary arteries had been demonstrated (14, 15). In addition, post-transplantation immunosuppressive agent was not required in these strain pairs. Percent area of intima was greater in donor coronary arteries than in the recipient at any point in time. The intimal hyperplasia in early specimens may represent early activation of thickening process after transplantation or early low grade intimal hyperplasia due to an inflammatory reaction to intimal injury occurred during the harvest. It has been reported that severe intimal proliferation with occlusion of the lumen as early as three months after heart transplantation in patients with unremitting heart rejection (16).

Adhesion of leukocytes to vascular endothelium is a necessary step leading to the migration of cells into the target tissue. Vascular adhesion molecules regulate this process and may play a role in the graft rejection. The subsequent release of cytokines enhances adhesion and migration through endothelial cells into the tissue. Within the tissues cytokines cause upregulation of class I MHC antigen and ICAM-1, which makes the endothelium a target for cytotoxic damage by leukocyte functioning antigen (LFA)-1, CD8 positive T-cells (17). ICAM-1 (CD54) is a member of the immunoglobulin superfamily whose constitutive expression on the endothelial cells is upregulated during the inflammatory process by various cytokines (IL-1, TNF, lymphotoxin). Its specific ligand is LFA-1 (10). Tanio et al. demonstrated that ICAM-1 expression is strongly correlated with severity of allograft rejection (18). Normal or non-rejecting capillary endothelial cells are constitutively capable of ICAM-1 expression (18). Tanaka et al. (19) observed that ICAM-1 expression starts two days after transplantion in rabbit cardiac allograft. Our study found that this upregulated ICAM-1 expression remains high in all groups during six-week observation both in endothelium and media of the donor coronary arteries. This might suggest that medial cells could also trigger the immune response.

VCAM-1 is a cell adhesion molecule induced on the vascular endothelial cells at the site of inflammation and is one of the ligands for very late antigen (VLA-4). VCAM-1 expression has been identified in human allograft rejection (20), at the site of chronic inflammation in rabbit (21), and at the site of viral myocarditis in mice (22). These observations suggest that VCAM-1 may promote leukocyte recruitment at inflammatory site as well as rejecting allografts. Pelletier (22) observed that endothelial VCAM-1 reactivity develops in the third day of post-transplant in allograft recipient in mice, which is related to the time of significant T-cell infiltration. We show here that high-grade VCAM-1 expression was detected from one wk to six wks after transplantation both in endothelium and media of coronary arteries of the allograft. The role of endothelial VCAM-1 in the development of allograft rejection is still not well understood. Tanaka et al. (19) observed that vascular smooth muscle cells expressed both VCAM-1 and ICAM-1 in later stages of acute rejection in rabbit cardiac allografts, suggesting that such events may contribute to the later development of accelerated graft atherosclerosis in transplanted heart. They also found that expression of VCAM-1 preceded that of ICAM-1 on endothelial cells during acute rejection. Ferran (23) reported VCAM-1 expression in endomyocardial biopsies obtained two wks before histologic evidence of rejection was apparent in clinical transplantation. Orosz et al. (8) noted that the inflammatory enhancement of endothelial ICAM-1 expression is observed in both non-rejecting cardiac isografts and rejecting cardiac alloraft. In contrast, the inflammatory induction of endothelial VCAM-1 is observed only in rejecting cardiac allograft. These observations may suggest that VCAM-1 can serve as an early marker of allograft rejection. Our results revealed that VCAM-1 expression stays high during the six wks of observation.

In our study, both ICAM-1 and VCAM-1 were expressed in donor arteries from day 1 and they gradually increased by day 7. Their intensities remained high thereafter. These findings imply that it takes several days or even a week until adhesion molecules are fully activated and the cardiac allograft alloimmunity status is unchanged thereafter. Initial three to five-day mild eleva-

tion of expressions were present in the recipients' heart as in the donors'. However, this initial up-regulation in the recipient heart is likely to be due to a surgery-related inflammatory reaction.

Determinant of progression rate of the arterial lesion is multifactorial. The possible causative factors include species difference, histocompatibility difference, severity and number of rejection episodes, donor harvesting techniques, and serum cholesterol levels. Thus our experiment represents a set of models which consists of an exchange between the mice with a minor MHC disparity, a 60-min ischemia for donor heart, and normocholesterolemia. Our results demonstrated the time sequence of the arterial lesions in the donor hearts and these might be used as a control for comparison in further studies related to cardiac allograft vasculopathy.

In conclusion, we demonstrated that a heterotopic murine heart transplantation model was a useful tool to produce transplantation coronary artery disease. This model could be helpful for future studies investigating the pathogenesis of chronic cardiac allograft vasculopathy and developing a potential new therapeutic modality for AGAS in cardiac transplantation. In addition, we demonstrated the time course of ICAM-1 and VCAM-1 expression, and the sequential histopathological changes in the development of AGAS in cardiac allografts between murine inbred strains, that is adhesion molecules on the cardiac allografts were activated very early and remained elevated state at all period, however the significant arterial lesion was detected by day 28 and severely progressed thereafter.

REFERENCES

1. Uretski BF, Murali S, Reddy PS, Rabin B, Lee A, Griffith BP, Hardesty RL, Trento A, Bahnson HT. *Development of coronary artery disease in cardiac transplant patients receiving immunosuppressive therapy with cyclosporin and prednisone. Circulation 1987; 76: 827-34.*
2. Hosenpud JD, Shipley GD, Wagner CR. *Cardiac allograft vasculopathy: current concepts, recent developments, and future directions. J Heart Lung Transplant 1992; 11: 9-23.*
3. Salomon RN, Hughes CCW, Shoen FJ, Payne DD, Pober JS, Libby P. *Human coronary transplantation arteriosclerosis. Evidence for a chronic immune reaction to activated graft endothelial cells. Am J Pathol 1991; 138: 791-8.*
4. Libhy P, Salomon RN, Payne DD, Schoen FJ, Pober JS. *Functions of vascular wall cells related to the development of transplantation associated coronary arteriosclerosis. Transplant Proc 1989; 21: 3677-84.*
5. Hruban RH, Beschorner WE, Baumgartner WA, Augustine SM, Ren H, Reitz BA, Hutchins GM. *Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. Am J Pathol 1990; 137: 871-82.*
6. Bier BE, Burakoff SJ. *T-cell adhesion molecules. FASEB J 1988; 2: 2584-90.*
7. Dun SM, Hillam AJ, Stomski F, Jin BQ, Lucas CM, Boyd AW, Krissansen GW, Burns GF. *Leukocyte adhesion molecules involved in inflammation. Transplant Proc 1989; 21: 31-4.*
8. Orosz CG, Ohye RG, Pelletier RP, van Buskirk AM, Huang E, Morgan C, Kincade PW, Ferguson RM. *Treatment with antivascular cell adhesion molecule-1 monoclonal antibody induces long-term murine cardiac allograft acceptance. Transplantation 1993; 56: 453-60.*
9. Sadahiro M, McDonald TO, Allen MD. *Reduction in cellular and vascular rejection by leukocyte adhesion molecule receptors. Am J Pathol 1993; 142: 675-83.*
10. Marlin SD, Springer TA. *Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function - associated antigen-1 (LFA-1). Cell 1987; 51: 813-9.*
11. Osborn L, Hession C, Tizard R, Vassalio C, Luhowskyl S, Chi-Rosso G, Lobb R. *Direct expression cloning of vascular adhesion molecule 1, a cytokine induced endothelial protein that binds to lympocytes. Cell 1989; 59: 1203-11.*
12. Corry RJ, Winn HJ, Russell PS. *Primary vascularized allografts of hearts in mice. The role of H-2D, H-2K and non-H-2 antigens in rejection. Transplantation 1973; 16: 343-50.*
13. Burdick JF, Clow LW. *Rejection of murine cardiac allografts. II. Evidence that splenocytes bearing Lyt2 inhibit responsiveness in long-term heart graft recipients. Transplantation 1987; 43: 509-14.*
14. Russell PS, Chase CM, Winn HJ, Colvin RB. *Coronary atherosclerosis in transplanted mouse hearts; I. Time course and immunogenetic and immunopathological considerations. Am J Pathol 1994; 144: 260-74.*
15. Ardehali A, Billingsley A, Laks H, Drinkwater Jr DC, Sorensen TJ, Drake TA. *Experimental cardiac allograft vasculopathy in mice. J Heart Lung Transplant 1993; 12: 730-5.*
16. Starnes VA, Stinson EB, Oyer PE. *Cardiac transplantation children and adults. Circulation 1987; 76(suppl V): 43-7.*
17. Taylor PM, Rose ML, Yacoub MH, Pigott R. *Induction of vascular adhesion molecules during rejection of human cardiac allografts. Transplantation 1992; 54: 451-7.*
18. Tanio JW, Basu CB, Albelda SM, Eisen HJ. *Differential expression of the cell adhesion molecules ICAM-1, VCAM-1, and E-selectin in normal and posttransplantation myocardium; cell adhesion molecule expression in human cardiac allograft. Circulation 1994; 89: 1760-8.*
19. Tanaka H, Sukhova GK, Swanson SJ, Cybulsky MI, Schoen FJ, Libby P. *Endothelial and smooth muscle cells express leukocyte adhesion molecules heterogenously during acute rejection of rabbit cardiac allografts. Am J Pathol 1994; 144: 938-51.*
20. Briscoe DM, Schoen FJ, Rice GE, Bevilacqua MP, Ganz P,

Pober JS. *Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografs. Transplantation 1991; 51: 537-9.*

21. Cybulsky MI, Gimbrone MA. *Endothelial expression of mononuclear leukocyte adhesion molecule during atherogenesis. Science 1991; 251: 788-91.*
22. Pelletier RP, Morgan CJ, Sedmak DD, Miyake K, Kincade PW, Ferguson RM, Orosz CG. *Analysis inflammatory endothelial changes, including VCAM-1 expression, in murine cardiac grafts. Transplantation 1993; 55: 315-20.*
23. Ferran C, Peuchmaur M, Desruennes M, Ghoussoub JJ, Cabrol A, Brousse N, Cabrol C, Bach JF, Chatenoud L. *Implication of de novo ELAM-1 and VCAM-1 expression in human cardiac allograft rejection. Transplantation 1993; 55: 605-9.*