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Chronic rejection in H-2 matched cardiac allografts: early emergence of vasculopathy, alloantibody, and accumulation of IFN-γ and IL-10 mRNA

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Abstract Cardiac allograft vasculopathy (CAV) is one of the crucial problems of clinical heart transplantation. We have developed a novel model of murine cardiac allograft rejection, in which chronic rejection associated with CAV occurs in its natural course. In this study we analyzed the pathogenesis of chronic cardiac allograft rejection using an H-2 matched multiple minor histocompatibility antigen-mismatched combination, AKR (H-2^k) to C3H (H-2^k) recipient mice. All the cardiac allografts survived for more than 100 days but were rejected within 260 days post-transplant (n = 13; mean survival times ± standard deviation = 189.0 ± 72.0; median = 210). The heartbeats of the graft became gradually weaker throughout the duration of the rejection process. Serial histological analyses with hematoxylin and eosin, elastica van Gieson or Masson trichrome staining revealed mononuclear cell infiltration and intimal thickening (i.e. CAV) which started in most grafts at 2 weeks post-transplant. These pathological changes eventually developed to severe graft fibrosis, and the severity of these changes correlated with the deterioration of the heartbeats. Production of anti-donor antibodies in most recipients was detectable by 2 weeks post-transplant, it peaked before day 100, and subsided before rejection was complete in most grafts. Intragraft expression of IFN-γ and IL-10 mRNA was demonstrated by reverse transcriptase-polymerase chain reaction during early periods post-transplant. In this study, we demonstrate a novel model feasible for analysis of chronic cardiac allograft rejection, in which the vascular rejection processes, including fibrosis and alloantibody production, can be tested from an early stage on, after transplantation.

Keywords Heart transplantation · Chronic rejection · Cardiac allograft vasculopathy · Cytokine

Abbreviations *Ag* Antigen(s) · *CAV* Cardiac allograft vasculopathy · *cDNA* Complementary DNA · *EVG* Elastica van Gieson · *FITC* Fluorescein-isothiocyanate · *HE* Hematoxylin and eosin · *HG* Heart grafting · *MST* Mean survival time · *MT* Masson trichrome · *RT-PCR* Reverse transcriptase-polymerase chain reaction · *SD* Standard deviation

Introduction

Accelerated cardiac allograft vasculopathy (CAV) has emerged as one of the major factors affecting long-term cardiac allograft survival [6, 12, 19]. Systematic annual coronary angiographic studies of cardiac allografts suggest that 40–60 % of grafts undergo significant vascular changes within 5 years following transplantation.

The dominant pathological findings in cardiac allografts show diffuse and luminal narrowing, which affects the entire length of the vessel wall and leads to the occlusion of small penetrating intramyocardial branches [8]. The pathogenesis of CAV has not yet been clearly demonstrated. Several reports have indicated CAV as one of the processes of chronic graft rejection after immunosuppressive therapy [2, 4, 18]. On the other hand, recent reports suggested that the initial events subsequently leading to CAV occur in the vessel endothelium [22, 23]. The endothelial injury may be caused not only by the impact of the transplantation procedures but also by alloreactive cells that produce cytokine and antibodies.

A murine model using an H-2 matched combination from DBA/2 (H-2^d) into B10.D2 (H-2^d) with which to investigate the pathogenesis of post-transplant CAV, has been recently described [13]. In this combination, 67% of cardiac allografts survived for 10 weeks after grafting without any immunosuppressive therapy and displayed CAV with interstitial and perivascular fibrosis. In this study we describe as H-2 matched combination, AKR/J (AKR; H-2^k) into C3H/He (C3H; H-2^k), using a novel model of heterotopic cervical heart transplantation technique [27] to analyze the pathogenesis of post-transplant CAV.

Materials and methods

Animals

Inbred mice of C3H/HeNSLc (C3H; H-2^k, Mls-1^b) and C57BL/6SnSLc (B6; H-2^b) strains were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). Female AKR/J (AKR; H-2^k, Mls-19) mice were obtained from the Seiwa Experimental Animal Institute, Oita, Japan. Mice 12–16 weeks old were used throughout this study.

Heart grafting

AKR mice served as donors, and C3H mice as recipients. Vascularized heart transplantation was performed heterotopically into the right cervical portion of recipients using a microsurgical cuff technique as described previously [27]. Donor brachiocephalic artery and main pulmonary artery were anastomosed to the recipient common carotid artery and external jugular vein, respectively. Ischemic time was 25–40 min. The overall success rate was higher than 90%. The function of the grafts was followed daily by palpation. Heartbeats were graded on a scale from 3–0 (3, strong; 2, moderate; 1, poor; 0, impalpable). Grafts were considered as rejected when the absence of beating was confirmed by palpation. Graft survival was expressed as the mean survival time (MST) ± standard deviation (SD) and the median survival time.

Histological analyses

Grafts were harvested at various times after transplantation. Grafts were serially sectioned to approximately 2 mm thickness and fixed with 10% formalin. They were then embedded in paraffin, 4 μm sections were prepared and stained with hematoxylin and eosin (HE), elastica van Gieson (EVG), and Masson trichrome (MT). A scoring systems was used to quantify the degree of mononuclear cell infiltration (infiltration score 0–3; 0: no infiltration, 1: mild, 2: moderate, 3: severe mononuclear infiltration) and graft fibrosis (fibrosis score 0–3; 0: no fibrosis, 1: mild, 2: moderate, 3: severe) (Fig. 2) and (Fig. 3, 4, 5) by modifying the previously described systems [3].

Vascular analyses

The severity of intimal thickening (% area of luminal occlusion) as well as the frequency of diseased arteries was measured for two sections from each graft. Microscopic images of each elastin-stained vessel were taken, and areas of luminal occlusion were tabulated by tracing internal elastic lamina and open lumen with the Mac SCOPE software (Mitani Corp. Fukui, Japan). Vessels with > 0% luminal occlusion were defined as diseased vessels. Grafts were tested for each group. Mean area of occlusion ± SD is reported for each group.

Flow cytometry for anti-donor antibodies

To analyze the production of anti-donor (AKR) antigen (Ag) antibodies, recipient sera were collected at various times after transplantation. Spleen cell suspensions from AKR mice (1×10^7/ml) were prepared as described previously [26] and used as target Ags. One million AKR spleen cells were incubated with 10 μl of recipient sera for 30 min at 4°C and then washed twice. To block nonspecific binding of antibodies to the FcγR of splenocytes, 20 μl of undiluted culture supernatant of 2.4G2 (rat anti-mouse FcγR mAb, 17) was added during the first incubation. To detect cell-bound anti-donor antibodies, cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated rat anti-mouse IgG1 (PharMingen, San Diego, Calif.), IgG2a (PharMingen), IgG2b (PharMingen), IgG3 (PharMingen) mAbs for 30 min at 4°C. Dead cells were excluded by gating out low forward scatter/high propidium iodide-retaining cells. The data were expressed as a mean ± SD of the mean fluorescence channel of histogram per 10^4 cells analyzed.

RT-PCR

To compare cytokine mRNA expression, RT-PCR analysis was carried out as described previously [14] with minor modifications. Total RNA was extracted from grafted or normal hearts using TRIzol RNA isolation reagent (Gibco BRL, Gaithersburg, Md.) according to the manufacturer's instructions. To synthesize the first strand cDNA, 3 μg of RNA was reverse transcribed with Superscript reverse transcriptase (Gibco BRL) and random hexamer at 42°C for 50 min then incubated at 95°C for 5 min to inactivate the enzyme. The cDNA was amplified by PCR in 100 μl reaction volume with β-actin or cytokine sense and antisense primers and AmpliTaq DNA polymerase (Elmer Cetus, Norwalk, Conn.) with 30 PCR cycles consisting of incubation at 94°C for 1 min, 54°C for 1 min, and 72°C for 30 s. The amount of cDNA in each PCR reaction was standardized by amplification of serially diluted cDNA with β-actin primers and by comparing of the intensity of the am-

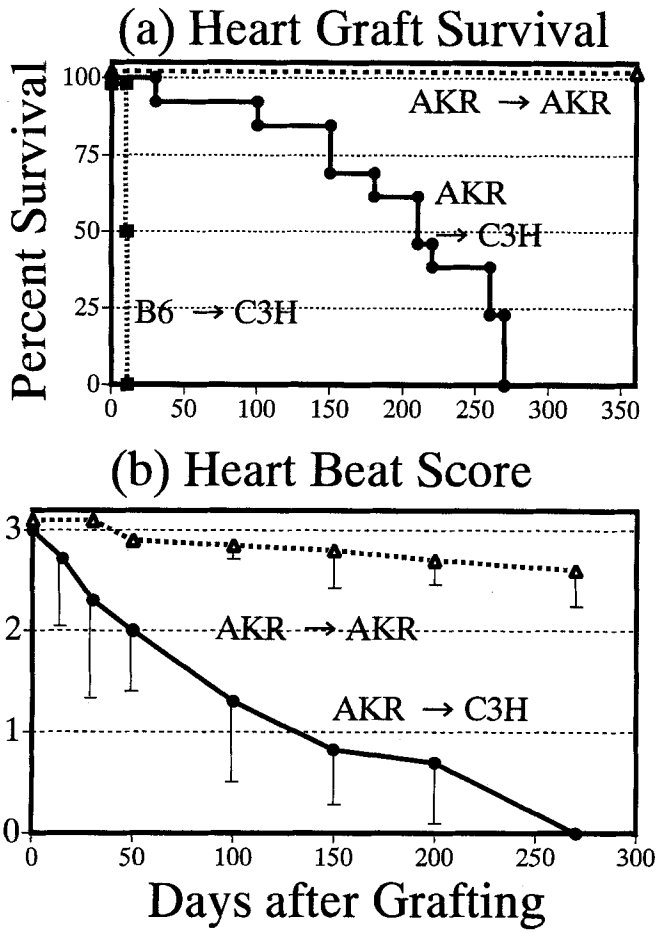

Fig. 1 Cardiac allograft survival (**a**) and heartbeat score (**b**). Heterotopic AKR (H-2^k) or B6 (H-2^b) heart grafts were performed in the C3H (H-2^k) mice (**a**). MST ± SD and median survival days of AKR (n = 13) and B6 (n = 6) cardiac allografts were 189 ± 72.0, and 210 days, and 10.6 ± 0.6 and 10 days, respectively. The function of the heart graft was followed daily by palpation (**b**). The beat of AKR syngrafts was well-palpable for 260 days (n = 5). The beat of allografts became impalpable by 260 days after grafting (n = 13)

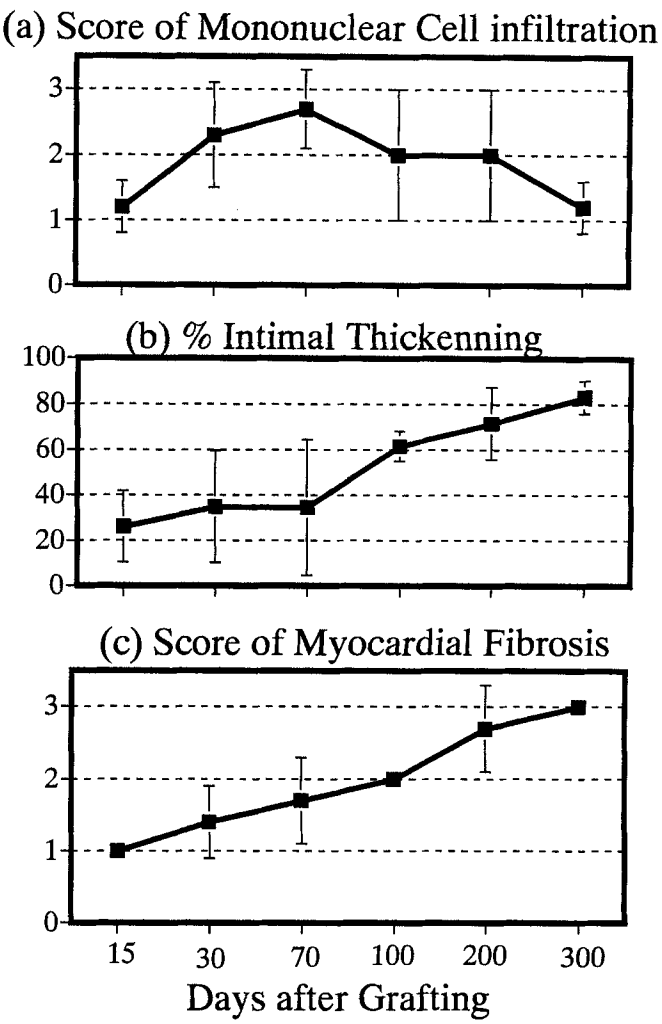

Fig. 2 Mononuclear cell infiltration (**a**), post-transplant CAV (**b**), and fibrosis (**c**) in AKR cardiac grafts. The AKR hearts were grafted to the recipients on day 0, harvested on day X, and then histologically analyzed (**a**). Allografts were scored (0 to 3; 0 no infiltration, 1 mild, 2 moderate, 3 severe). Score of infiltration of lymphoid cells was calculated. Both data from mean ± SD are shown (n = 28). (**b**) The percentage of intimal thickening was tabulated by tracing the internal elastic lamina and the lumen. Either data from mean ± SD is shown (n = 28). (**c**) Allografts were scored (0 to 3; 0 no fibrosis, 1 mild, 2 moderate, 3 severe). Score of myocardial fibrosis was calculated. Both data from mean ± SD are shown (n = 28). On the other hand, no histological changes were observed in AKR syngeneic cardiac grafts at various times

plified bands. The diluted cDNAs giving the same intensity of β-actin were considered to contain equivalent amounts of cDNA. The following sense and anti-sense primers were used: IL-2 sense (TGATGGACCTACAGGAGCTCCTGAG), antisense (GAGTCAAATCCAGAACATGCCGCAG); IFN-γ sense (AGCGGCTGACTGAACT CAGATTGTTAG), antisense (GTCACAGTTTTCAGCTGTATAGGG); IL-4 sense (CGAAGAACACCACAGAGAGTGAGCT), antisense (GACTCATTCATGGTGCAGCTTATCG); IL-10 sense (TACCTGGTAGAAGTGATGCC), antisense (TACCTGGTAGAAGTGATGCC); β-actin sense (TGGAATCCTGTGGCATCCATGAAAC), antisense (TAAAACGCAGCTCAGT AACAGTCCG). The PCR products were electrophoresed through 1.8% agarose gel, stained with ethidium bromide, and photographed. The length of PCR products is as follows: IL-2 (167 bp), IFN-γ (213 bp), IL-4 (180 bp), IL-10 (255 bp), and β-actin (348 bp).

Statistics

The data were statistically analyzed with a by Mann-Whitney U-test for non-parametric data. For parametric data, Student's t test was used. A P value < 0.05 was considered to be significant.

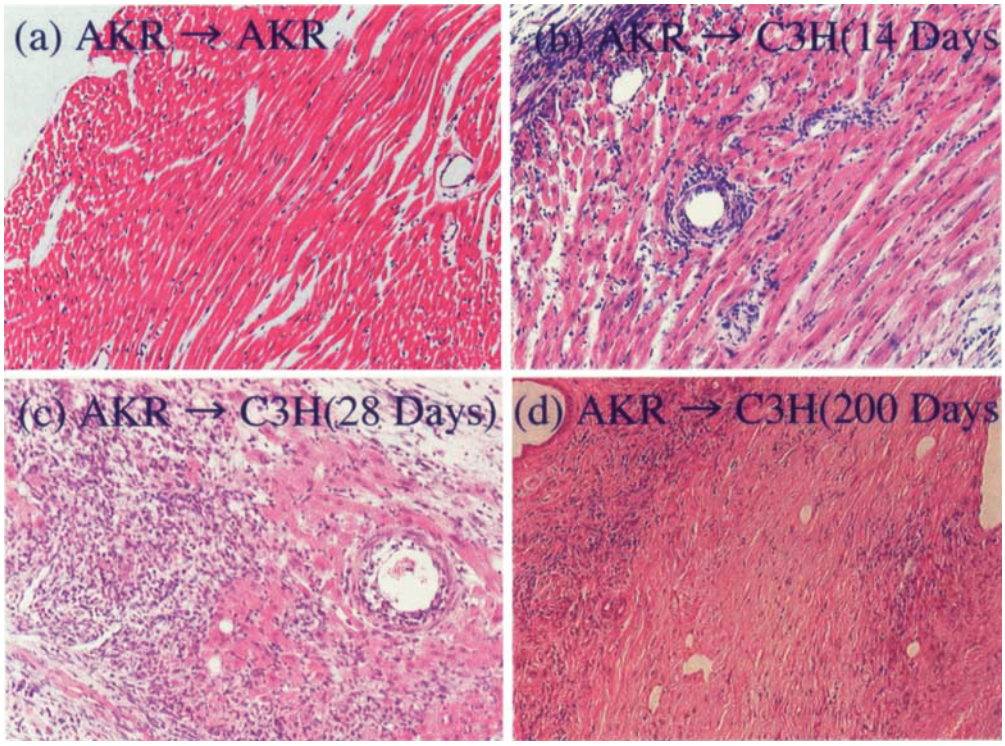

Fig. 3a–d H&E staining of allografts after cardiac transplantation. (**a**) Section of a syngraft at 200 days after transplantation. (**b**) Section of an allograft at 14 days. (**c**) At 28 days. (**d**) At 200 days. Photomicrographs, × 100

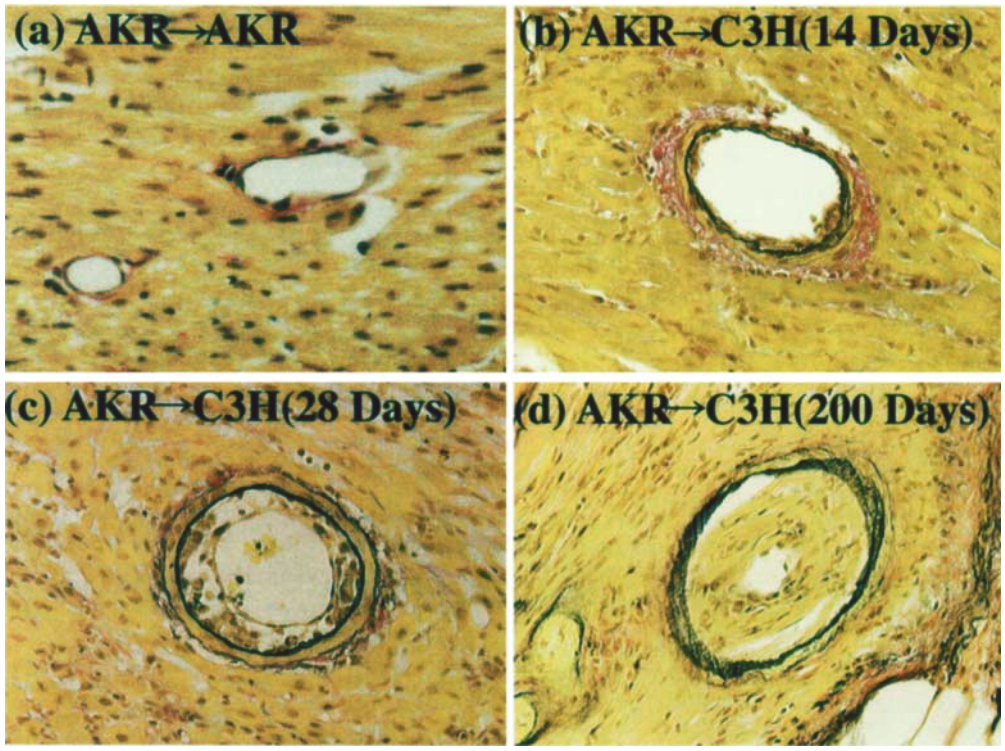

Fig. 4a–d EVG staining of allografts after cardiac transplantation. (**a**) Section of a syngraft at 200 days after transplantation. (**b**) Section of an allograft at 14 days. (**c**) At 28 days. (**d**) At 200 days. Photomicrographs, × 200

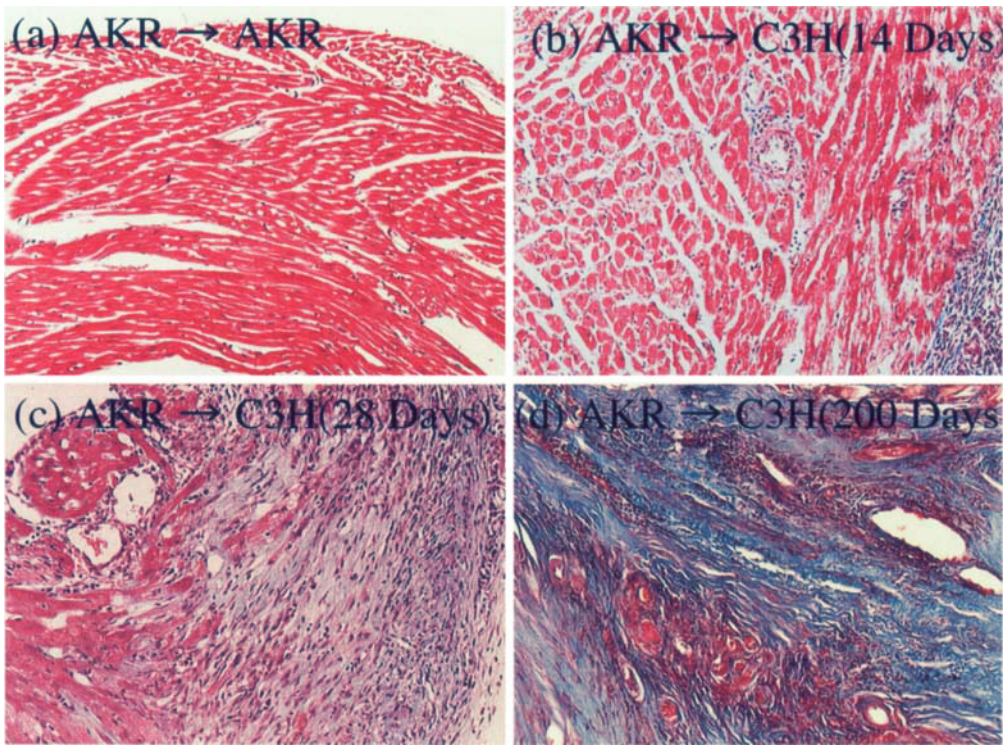

Fig. 5 a–d MT staining of allografts after cardiac transpantation. (**a**) Section of a syngraft at 200 days after transplantation. (**b**) Section of an allograft at 14 days. (**c**) at 28 days. (**d**) At 200 days. Photomicrographs, × 100

Results

Discrepancy in survival between heart- and skin grafts in H-2 identical AKR (H-2^k) into C3H (H-2^k) combination

Fully H-2 disparate B6 (H-2^b) hearts were rejected by C3H mice within 11 days (n = 6, MST ± SD: 10.6 ± 0.6, median: 10 days) (Fig. 1 a). In contrast, all H-2 matched AKR heart grafts survived for long periods but were rejected by 260 days post-transplant (n = 13, MST ± SD: 189 ± 72.0, median: 210 days). When skin grafting was performed on C3H mice, both B6 and AKR skin grafts were rejected within 14 days as described previously [26].

Heartbeat score

The function of heart grafts (n = 13) was monitored daily by palpation. We have graded the data observed on a scale from 0–3. The function of the transplanted hearts became gradually weaker and the heartbeating ceased by 260 days after grafting (Fig. 1 b).

Temporal assessment of mononuclear cell infiltration, post-transplant CAV and fibrosis in AKR cardiac grafts

In order to elucidate the histological aspects of chronic rejection of AKR heart grafts in C3H recipients, grafts were retrieved at various times and scored for infiltration of mononuclear cells, the severity of intimal thickening, and the degree of fibrosis (Fig. 2).

Infiltration of lymphoid cells became detectable by 14 days after grafting, and the infiltration Score reached 2.7 at day 70, but began to decrease subsequently. Representative HE staining profiles are shown in (Fig. 3 a). Mononuclear cells were detected around the coronary arteries in some of the allografts at 14 days after grafting (Fig. 3 b) and infiltration into myocardium began to be observed at day 28 (Fig. 3 c). At 200 days after grafting, few graft cardiomyocytes were remaining (Fig. 3 d).

It has been shown that post-transplant CAV was initiated within 14 days post-transplant although the degree of intimal thickening in each individual was different. The degree of intimal thickening had gradually advanced, and stenoses had reached high levels of 71.7 ± 15.8 % at 200 days after grafting. Representative EVG staining profiles are shown in Fig. 4. Similar results were observed in terms of development of post-transplant graft fibrosis. At 200 days post-transplant, the majority of graft cardiomyocytes had been replaced with fibrosis (Fig. 5).

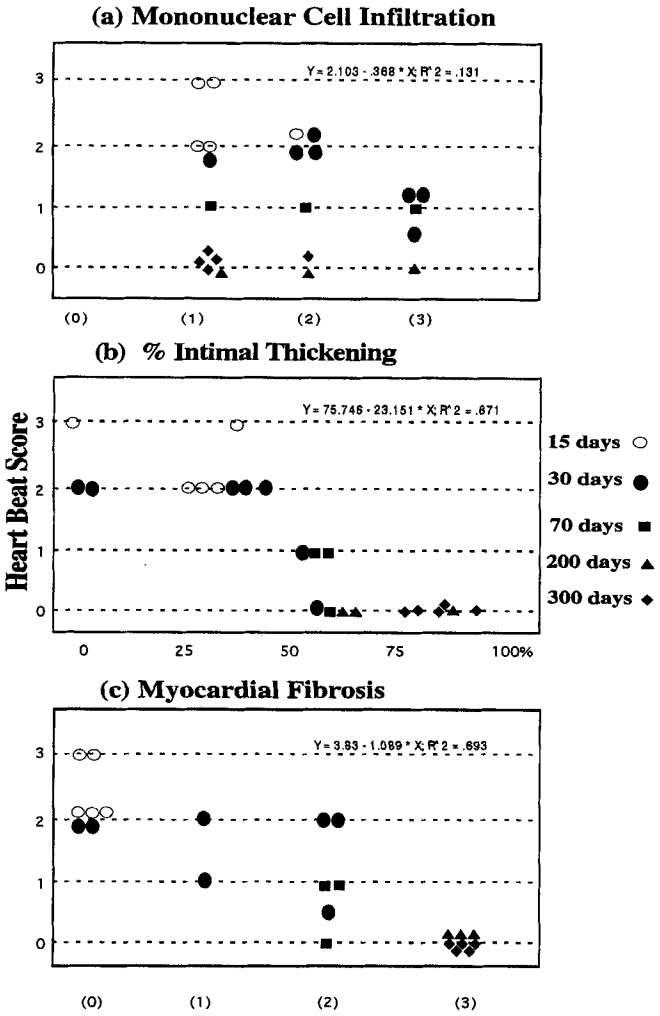

Fig. 6 Correlation between the mononuclear infiltration (**a**), % intimal thickening (**b**), and myocardial fibrosis (**c**) and heartbeat score, as determined from histological sections 14, 28, 70, 200, and 300 days after transplantation. Allografts were scored 0–3 as described in the Materials and methods section. The individual values are shown. There is strong correlation between heartbeat score and % luminal occlusion ($r2 = 0.671$) (**b**) or allograft fibrosis ($r2 = 0.69$) (**c**) and heartbeat score

We also analyzed the relationship between heartbeat score and mononuclear infiltration, % intimal thickening or allograft fibrosis at each time point. As shown in Fig. 6, the correlation between heartbeat score and % intimal thickening ($r2 = 0.671$) or allograft fibrosis ($r2 = 0.69$) is strong, suggesting that factors such as arterial occlusion or interstitial fibrosis are affecting the strength of beating.

Generation of anti-AKR Ab in C3H mice grafted with AKR heart graft

In order to test the occurrence of humoral immune responses after heart grafting in this chronic rejection model, we collected serum from recipient mice at various time points and examined the levels of anti-AKR Ab (Fig. 7). AKR spleen cells (1×10^6/0.1 ml) were incubated with 10 µl of serum for 30 min. Anti-AKR Ab coated AKR spleen cells were detected with FITC-conjugated rat anti-mouse IgG isotype mAbs as secondary antibodies. All data were collected at the same time and expressed by mean fluorescence channel.

IgG2a, IgG2b, and IgG3 isotype anti-AKR Abs were had already been generated in C3H recipient mice by 15 days after heart grafting, except for IgG1. The levels of anti-AKR Ab showed higher levels at day 30 and reached the peak levels at days 30–70, even though more than 80% of AKR allograft were functioning. Anti-AKR Ab declined to low levels by 200 days after heart grafting, while more than 50% of AKR allografts were still functioning. IgG1 isotype anti-AKR Ab became detectable at 4 weeks. Representative profiles of anti-donor Abs staining are shown in Fig. 8.

Expression of cytokine mRNA

In order to further elucidate the mechanisms of chronic heart allograft rejection, the levels of IL-2, IFN-γ, IL-4 and IL-10 mRNA expression were analyzed in AKR cardiac allografts by RT-PCR. Total mRNA was extracted from grafted hearts retrieved 1 and 2 weeks after grafting (Fig. 9). IL-2 and IL-4 mRNAs were hardly detected 1 and 2 weeks after transplantation. In contrast, IFN-γ and IL-10 mRNA were apparent in these grafts. The same results were obtained 4 and 10 weeks after grafting (data not shown).

Discussion

Post-transplant CAV has been a leading cause of long term graft failure despite the use of modern immunosuppressive regimens. There have been many of studies concerning post-transplant CAV, however, the pathophysiology remains poorly understood. Most of the previous animal models required long term immunosuppressive treatments to prevent acute rejection and to develop long term CAV.

In the current study, we have developed a novel model of long term CAV in vascularized murine cardiac transplantation using an H-2 identical combination, AKR ($H\text{-}2^k$) to C3H ($H\text{-}2^k$), without any immunosuppressive regimen. The arterial lesions in this combina-

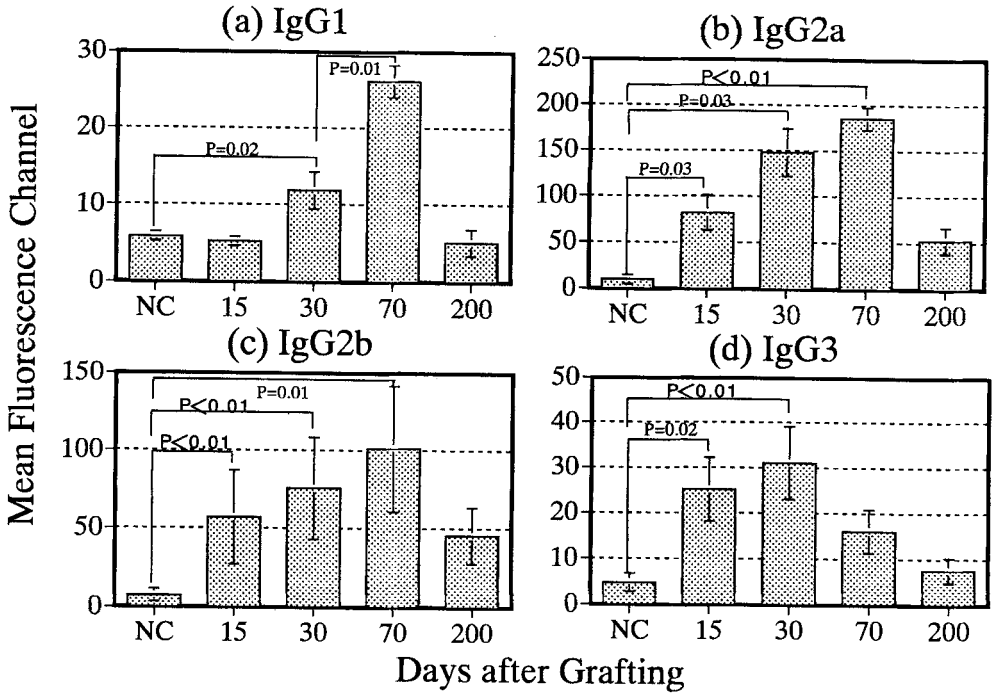

Fig. 7a–d Generation of Anti-AKR Abs in sera of recipient C3H mice. Mean fluorescence channels of FCM staining profiles are shown for normal AKR spleen cells incubated with sera of recipient C3H mice followed by FITC-conjugated anti-mouse IgG1 (**a**), IgG2a (**b**), IgG2b (**c**), and IgG3 (**d**) mAbs. NC (negative control) shows the mean fluorescence channel of normal AKR spleen cells incubated with sera of normal C3H mice followed by FITC-conjugated anti-mouse IgG1 (**a**), IgG2a (**b**), IgG2b (**c**), and IgG3 (**d**) mAbs

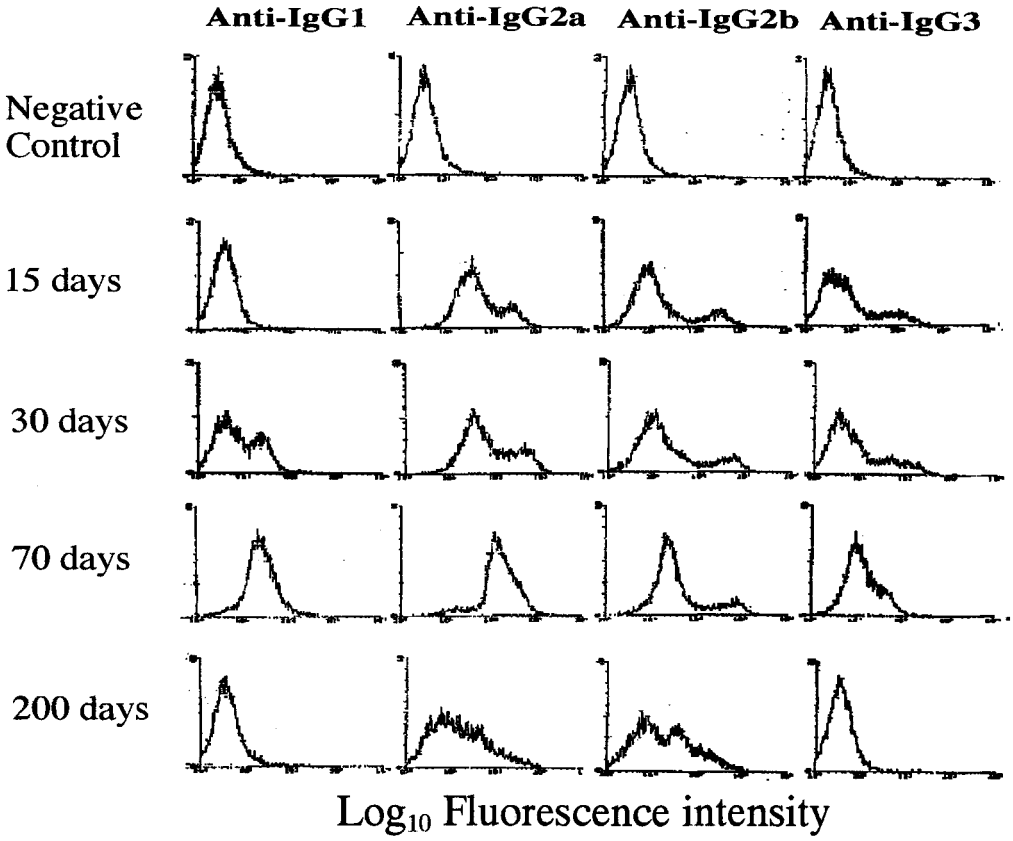

Fig. 8 Anti-AKR Ab IgG1, IgG2a, IgG2b, and IgG3 formation in recipient C3H from 15 to 200 days after heart grafting

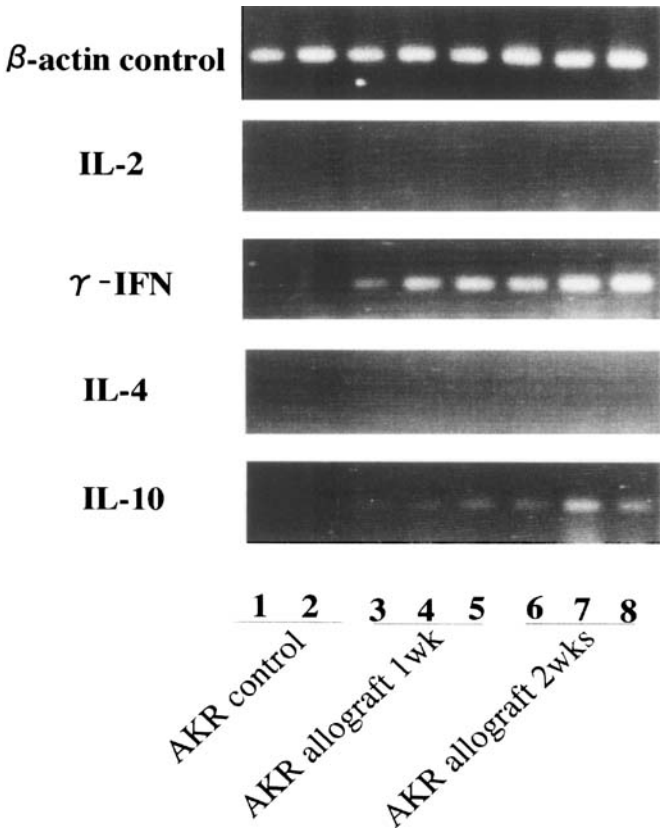

Fig. 9 Expression of cytokine mRNA in cardiac allografts. Cardiac allografts from recipient C3H mice were harvested 1 and 2 weeks after grafting and RNA was isolated and reverse transcribed. Concentrations of cDNA were adjusted as mentioned in Materials and Methods. The figure shows representative data of RT-PCR on expression of IL-2, γ-IFN, IL-4, and IL-10 in the AKR allografts. *Lanes 1, 2* Control AKR cardiac grafts on AKR mice. *Lanes 3, 4, 5* AKR allograft on the C3H mice at 1 week. *Lanes 6, 7, 8* AKR allograft on the C3H mice at 2 weeks

tion seem to have many similarities to human CAV and also to vascular lesions described in previous animal experiments [1, 13]. Intimal thickening of the affected graft coronary arteries in this model showed concentric narrowing of the lumen in a patchy distribution; however, no typical atheromatous plaque was observed. No apparent differences were noted in the incidence and severity of vessel disease among small, medium and large arteries (unpublished observations). Although most of the AKR heart grafts survived over 100 days, the process of post-transplant CAV accompanied with perivascular and interstitial fibrosis and mild mononuclear cell infiltration had initiated since as early as 2 weeks after grafting. We have also clearly shown that the strength of cardiac contraction decreased gradually, suggesting that the rejection of these AKR allografts was not an acute onset after long term survival but had chronically progressed. This decline in strength of cardiac impulse clearly correlated with the severity of arterial obstruction and graft fibrosis. These results suggest that the gradual decrease of functioning cardiomyocytes, hardening of the grafts and reduction in blood supply caused by intimal thickening and myocardial fibrosis, resulted in slow deterioration of cardiac contraction and slow progression of cardiac dysfunction, as shown in the results.

There have been few reports regarding the distinct role of cellular and humoral immunity in the development of chronic allograft disease. We have shown the early onset of vasculopathy and fibrosis accompanied by mononuclear cell infiltration and alloantibody production. Although both the cellular infiltration and alloantibody production reached peak levels before day 100 post-transplant and declined subsequently, more than 80% of the allografts were still functioning at day 100. At day 200, production of alloantibody had returned to near background levels, and more than 50% of the allografts were still functioning. These observations suggest that continual allo Ab production is not required for progression of whole duration of chronic allograft disease. A previous report showed the inability of mice lacking both T cell- and humoral immunity to develop chronic vasculopathy in an aortic graft model, whereas mice only lacking humoral immunity developed chronic vasculopathy, suggesting that humoral mechanisms may not be required [7]. Another report has shown that mice deleted of FasL were unable to develop CAV but had high titers of alloantibodies from a previous period, suggesting that CAV is initiated by cellular immunity with its perpetuation facilitated by alloantibodies [24]. Several studies have been conducted to also test the effects or roles of alloantibodies or humoral immunity on chronic graft disease [11, 20, 21]. Humoral immunity may not be required for the development of CAV, but may be triggering the initiation and facilitating, optimizing or accelerating the progression of CAV mediated by cellular immunity. These problems in CAV remain to be tested.

We have also tested the intragraft expression of several cytokines in this model. We observed the expression of IFN-γ and IL-10 mRNA in allografts retrieved as early as 1 week. IFN-γ is an inflammatory cytokine and its role in acute rejection of allografts has been documented in many studies [10]. It has been reported that IFN-γ stimulates allograft endothelium to express adhesion molecules and to produce cytokines. It is also known that intragraft expression of MHC class I and class II antigens are enhanced by IFN-γ. Recent studies have suggested the role of IFN-γ in inducing expression of chemokines such as IP-10 and Mig which recruit effector T cells into allografts to mediate graft rejection [15, 16]. We propose that IFN-γ also contributes to increasing graft allogenecity, recruiting effector T cells into the grafts by inducing cytokines and chemokines in

our chronic rejection model, but these facets of the model remain to be tested. Expression of IL-10 mRNA also suggests its involvement in the chronic rejection process. Our recent study investigating cyclophosphamide-induced tolerance to allografts showed complete suppression of these Th1 and Th2 cytokines during tolerance induction [28]. Some in vitro studies have also shown the inhibitory effects of IL-10 on Th1 cytokine synthesis, APC function or cell-mediated immunity. In contrast, IL-10 might promote allograft rejection because this multifunctional peptide can stimulate the generation of cytotoxic T cells as well as antibody production [5, 9]. In support of this, high expression of IL-10 has been observed in renal allografts during clinical acute rejection [25]. The role of IL-10 in our model still remains to be tested.

In summary, we have developed a novel model of chronic rejection of heart allografts in the absence of immunosuppressive treatment. This model will be useful for testing mechanisms of CAV as discussed above. Further study is ongoing in our laboratory to elucidate the mechanisms of post-transplant CAV.

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