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ORIGINAL ARTICLE

Influence of mast cells on outcome after heterotopic cardiac transplantation in rats

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Summary

Correlative data suggest that mast cells adversely affect cardiac transplantation. This study uses a mast cell-deficient rat model to directly address the role of mast cells in cardiac allotransplantation. Standardized cardiac heterotopic transplantation with cyclosporine immunosuppression was performed in mast cell-deficient and mast cell-competent rats. Rejection, ischemia, fibrosis, fibrin deposition, numbers of T-cell receptor α/β positive cells, expression of transforming growth factor-β (TGF-β), and of endothelin-1 (ET-1) and its receptors ETA and ETB were assessed. Differences in baseline cardiac gene expression were quantified by real-time PCR in a separate group of untransplanted animals. Baseline cardiac gene expression levels of all investigated growth factors, cytokines, ET-1, ETA, and ETB were similar in mast cell-deficient and mast cell-competent rats. Surprisingly, upon heterotopic transplantation, donor heart survival was significantly reduced in mast cell-deficient rats. Moreover, in mast cell-deficient donor hearts rejection was more severe, although nonsignificant, and extracellular matrix associated TGF-β immunoreactivity was significantly lower than in mast cell-competent donor hearts. Fibrin immunoreactive area, on the other hand, was only increased in mast cell-deficient donor hearts, but not in mast cell-competent donor hearts. Histopathological changes in all donor hearts were accompanied by increased immunoreactivity for ET-1. In conclusion, this study shows that mast cells play a protective role after cardiac transplantation.

Introduction

Correlative and indirect evidence suggests a role for mast cells in cardiac allograft rejection and adverse cardiac remodeling. Mast cell hyperplasia is consistently observed during chronic rejection episodes and in areas of post-transplant cardiac fibrosis [1–3]. Moreover, mast cells regulate several processes that contribute to cardiac allograft survival, including inflammatory responses [4], thrombus formation [5–7], and extracellular matrix formation [8–10]. Conversely, recent evidence from mast cell-deficient rodent models shows that mast cells actually play a protective role in some inflammatory and fibroproliferative conditions in the heart [11,12], as well as in other organs [13,14].

Mast cells contain a plethora of biologically active mediators, some stored in granules and ready for immediate release, others synthesized on demand. Mast cells do not only produce several cytokines and growth factors, including transforming growth factor-β (TGF-β), but also

contain mast cell-specific enzymes that influence biological and chemical processes. The mast cell protease chymase, for instance, is able to release TGF-β from extracellular matrix [8,15]. A recent study has shown that mast cells are essential for T-cell tolerance after skin allograft transplantation, strongly suggesting that TGF-β is involved in this process [16].

Notably, recent evidence shows that mast cells mediate their effects in part via the endothelin system [13,17,18], by regulating levels of ET-1, both at the mRNA and at the protein level [19,20]. Increased expression of endothelin-1 (ET-1) and its two receptors ETA and ETB in human tissue specimens after transplantation and in animal models of cardiac transplantation suggest a role for the endothelin system in cardiac allograft survival [21–23].

This study used a mast cell-deficient rat model that has been used and validated extensively in our laboratory [11,12,14] to directly address the role of mast cells in cardiac allotransplantation. Interestingly, but consistent with recent findings in other inflammatory and fibroproliferative disorders, the absence of mast cells was associated with decreased graft survival, histopathological evidence of rejection, increased fibrin deposition, and decreased extracellular TGF-β accumulation. These data strongly support the notion that mast cells play a protective role in the post-transplantation period.

Materials and methods

Animals

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Male mast cell-deficient (Ws/Ws) rats and mast cell-competent (+/+) rats were obtained from Japan SLC (Hamamatsu, Japan). Ws/Ws animals originate from a rat of the BN/*f*Mai strain with a spontaneous 12-base deletion in one locus of the *c-kit* gene. This Ws/+ rat was crossed with normal (+/+) rats of the Donryu strain. The offspring of these hybrids include mast cell competent or +/+ rats, heterozygous Ws/+ rats and homozygous Ws/Ws rats [24], of which the outbred +/+ and Ws/Ws rats were used for this study. Because of the c-kit mutation, Ws/Ws rats lack functional melanocytes, mast cells, and interstitial cells of Cajal in the intestine. All animals were maintained on a 12:12 light-to-dark cycle with free access to food and water.

Assessment of major histocompatibility complex disparity

The degree of major histocompatibility complex (MHC) disparity within +/+ and Ws/Ws animals was assessed in seven animals from each group, by PCR amplification of the highly polymorphic *RT1-Bb* locus. Genomic DNA (0.2 μg) was used as template in PCR using a forward primer (0.5 μM) corresponding to sequences in the 5′ end of *RT1-Bb* exon 2 (GGCCTGTGCTACTACACCAACGG GACGCAGCGC) and a reverse primer (0.5 μM) corresponding to sequences near the 3′ end of *RT1-Bb* exon 3 (CTCCCCGCTGAGGCGTCATCTCCAGCATGACCAGG). PCR was performed by using 1 μl Elongase® (Invitrogen, Carlsbad, CA, USA) in 50 μl of 60 mM Tris–SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.2 mM dNTPs. Forty cycles of two-step thermocycling was performed at 94 °C for 30 s and 68 °C for 90 s. A portion (10 μl) of the reaction products was resolved on 0.8% agarose gels.

Heterotopic cardiac transplantation

Standardized rat heart heterotopic transplants were performed by an experienced rodent transplant surgeon. Ten mast cell-deficient recipients, at an average weight of 276 ± 5 g, received a mast cell-deficient heart from age-matched donors. Likewise, nine mast cell-competent rats, at an average weight of 286 ± 5 g, received a mast cell-competent heart. Rats were anesthetized with isoflurane inhalation, followed by intraperitoneal injection of sodium pentobarbital (70 mg/kg for mast cell-competent and 35 mg/kg for mast cell-deficient rats, because of their increased sensitivity to anesthesia). Additional isoflurane was administered to maintain anesthesia during the operation.

In the donor, the vena cava was exposed through an abdominal incision. An aqueous heparin solution (300 U) was injected and the rats were exsanguinated by incising the vena cava. The thorax was opened and the heart exposed. The pulmonary artery was divided, after which the ascending aorta was clamped distally and cold crystalloid cardioplegia was infused into the aortic root. The aorta was then divided and all venous tributaries to the heart were ligated with a single braided ligature. The heart was excised and placed in cold normal saline solution.

In the recipient, a midline abdominal incision was made to expose the aorta and inferior vena cava. The aorta and vena cava were dissected from the surrounding connective tissue and clamped with a single vascular c-clamp. The donor and recipient aorta were anastomosed end to side with 8–0 prolene suture. This was repeated for the pulmonary arteries. Intraluminal thrombus, micro-debris, and air were removed from the vessel lumen prior to each anastomosis. The aortic clamp was removed to reperfuse the heart. Spontaneous beating occurred after every transplant.

Immunosuppression of the recipients was provided with a daily dose of cyclosporine A (CSA, 10 mg/kg) by oral gavage.

Abdominal heterotopic heartbeat was monitored daily by palpation. Animals with cessation of heterotopic heartbeat were euthanized humanely. All other animals were euthanized 12 weeks after transplantation.

From here on, transplanted hearts are designated 'donor' and the native hearts of the transplanted animals are designated 'native'. Twelve mast cell-competent rats and 12 mast cell-deficient rats were not operated or treated with CSA and were used to study basal characteristics of their hearts. These hearts are referred to as 'baseline'.

Tissue processing

Baseline hearts of untreated animals, as well as donor and native hearts of transplanted animals, were harvested and sectioned transversely at the time of graft failure or at termination of the experiment 12 weeks after transplantation. One-half of the hearts was fixed in methanol Carnoy's solution (60% methanol, 30% chloroform, and 10% acetic acid) and one-half was fixed in 10% neutral buffered formalin, followed by tissue embedding in paraffin.

Of six mast cell-competent and six mast cell-deficient baseline hearts, left ventricular tissue specimens were stored at −80 °C for gene expression analysis with real-time PCR.

Histology

Five micrometer sections of formalin-fixed tissue were stained with standard hematoxylin–eosin and examined for histopathological changes by an experienced transplant pathologist as described before [25,26]. Rejection was scored on a graded scale as follows: 0, no rejection; 1, mild rejection (sparse, focal or diffuse mononuclear cell infiltrate, no myocyte damage); 2, moderate rejection (focal or multifocal mononuclear cell infiltrate, myocyte damage); 3, severe rejection (diffuse mononuclear cell infiltrate, multifocal myocyte damage). Ischemia was scored on a graded scale (0–3), while thrombus formation was scored as absent or present.

For determination of fibrosis, 5-μm sections of formalin-fixed tissue were stained with standard sirius red. Fibrosis was scored on a graded scale as follows: 0: no fibrotic areas; 1: increased numbers of interstitial collagen fibers; 2: 0–10% of area is fibrotic, 3: 10–50% of area is fibrotic; 4: 50–100% of area is fibrotic.

Toluidine Blue staining was used to visualize mast cells, as mast cell granules show metachromatic staining after uptake of Toluidine Blue stain. Five micrometer sections of methanol Carnoy's-fixed tissue were stained with 0.5% Toluidine Blue in 0.5 N HCl overnight, followed by a 10-min incubation in 0.7 N HCl and light counterstaining with eosin. Per section, mast cell density was calculated as mast cell number per area.

Immunohistochemistry and computerized image analysis

Immunohistochemical stainings were performed with methods established and optimized in our laboratory. Five micrometer sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol for 30 min at room temperature. Nonspecific binding was reduced by 10% normal rabbit serum, 10% normal goat serum or 10% normal donkey serum (Vector Laboratories, Burlingame, CA, USA) in 3% dry powdered milk in TBS–Tris buffered saline for 1 h. Five micrometer sections of methanol Carnoy's fixed tissue were incubated for 2 h at 20 °C with rabbit anti-fibrin(ogen) (1:500; Dr JJ Emeis, TNO Leiden, The Netherlands), rabbit anti TGF-β (1:300; R&D Systems, Minneapolis, MN, USA), mouse anti T-cell receptor α/β (TCR α/β, 1:20; BD Biosciences, San José, CA, USA), or rabbit anti-ETA, (1:100; Abcam, Cambridge, MA, USA), or overnight at 4 °C with mouse anti-ET-1 (1:100; Calbiochem, EMD Biosciences, La Jolla, CA, USA), or rabbit anti ETB (1:100; Abcam). After 30-min incubation with the appropriate biotin-labeled secondary antibody in a 1:400 dilution [goat anti-rabbit IgG (Vector Laboratories), donkey anti-rabbit IgG, or donkey anti mouse IgG (both American Diagnostica, Stamford, CT, USA)], sections were incubated with an avidin–biotin–peroxidase complex (Vector Laboratories) for 45 min. For TCR α/β, ET-1, ETA, and ETB immunostaining, bound peroxidase was enhanced with biotin-labeled tyramide, followed by streptavidin-bound horseradish peroxidase (TSA™ Biotin System, PerkinElmer, Shelton, CT, USA). All peroxidase binding was visualized with 3,3′-diaminobenzidine.

Computerized image analysis with IMAGEPRO PLUS software (Media Cybernetics, Silver Spring, MD, USA) was used to determine areas immunoreactive for TGF-β or fibrin in 12 rectangular fields using a 40× objective. This method of quantitative immunohistochemistry has been extensively used and validated in our laboratory [27,28].

RNA isolation and real-time PCR

Frozen tissue samples from the left ventricle of baseline hearts were homogenized in Ultraspec™ RNA reagent (Biotecx Laboratories, Houston, TX, USA). Total RNA was extracted by a standard chloroform extraction protocol. After treatment with RQ-DNAse I (Promega, Madison, WI, USA) at 37 °C for 30 min, cDNA was synthesized by using the High Capacity cDNA Archive Kit™ (Applied Biosystems, Foster City, CA, USA).

Steady-state mRNA levels were measured with real-time quantitative PCR (TaqMan™) by using the ABI Prism 7700 Sequence Detection System, TaqMan mastermix and TaqMan polymerase, and the following predesigned TaqMan Gene Expression Assays™: rat interleukin-1β (IL-1β, Rn00580432_m1), IL-4 (Rn01456866_m1), IL-6 (Rn00561420_m1), tumor necrosis factor-α (TNF-α, Rn00562055_m1), TGF-β1 (Rn00572010_m1), connective tissue growth factor (CTGF, Rn00573960_q1), ET-1 (Rn00561129_m1), ETA (Rn00561137_m1) and ETB (Rn00569139_m1). Eukaryotic 18S rRNA (Hs99999901_s1) was used as endogenous control (all Applied Biosystems).

Relative mRNA levels were calculated with the ABI PRISM 700 SDS software by using the ΔΔCt method.

Statistical analysis

Statistical tests were performed with NCSS 2000 (NCSS, Kaysville, UT, USA) and SPSS 14.0 (SPSS, Chicago, IL, USA). Cardiac allograft survival was compared with the Kaplan–Meier test. Differences in mast cell densities were assessed by using two-way ANOVA, with Tukey *post hoc* test. Differences in gene expression were tested with one-way ANOVA. Differences in histopathological scores were tested with the Mann–Whitney test. The criterion for significance was set at $P < 0.05$.

Results

Baseline gene expression

The two rat types did not differ in baseline cardiac gene expression levels of any of the investigated mediators: TGF-β1, CTGF, IL-1, IL-4, IL-6 and TNF-α (Table 1). Although baseline gene expression of ET-1 and ETA was slightly higher in mast cell-deficient rat hearts, the differences were not significant (Table 1).

Table 1. Steady-state gene expression levels of pro-fibrogenic and pro-inflammatory mediators and of components of the endothelin-system in baseline hearts of mast cell-deficient rats, relative to baseline hearts of mast cell-competent rats. No significant differences were found between mast cell-deficient and mast cell-competent animals.

	Mast cell-competent	Mast cell-deficient
Transforming growth factor-β1	1.00 ± 0.23*	1.09 ± 0.27
Connective tissue growth factor	1.00 ± 0.50	0.89 ± 0.70
Interleukin-1	1.00 ± 0.29	1.08 ± 0.12
Interleukin-4	1.00 ± 0.24	1.27 ± 0.24
Interleukin-6	1.00 ± 0.28	0.87 ± 0.45
Tumor necrosis factor-α	1.00 ± 0.52	1.03 ± 0.62
Endothelin-1	1.00 ± 0.27	1.36 ± 0.30
Endothelin receptor A	1.00 ± 0.14	1.29 ± 0.13
Endothelin receptor B	1.00 ± 0.35	0.84 ± 0.19

*Values are expressed as average ± SEM. $n = 6$.

Assessment of MHC disparity

Polymerase chain reaction amplification of the highly polymorphic *RT1-Bb* locus revealed several different size PCR amplification products, although all close in size to the predicted product of the well-characterized *RT1-Bb*^{n} allele, suggesting variation in intron 2 length. Thus, by this analysis, there appears to be a measurable degree of MHC disparity between donors and recipients.

Graft survival after transplantation

Of nine mast cell-competent recipients, one animal had to be euthanized on postoperative day 12 with signs of paraplegia and aortic thrombosis. The start-weight of this animal was smaller than average, being 228 g at transplantation. Of 10 mast cell-deficient recipients, two animals died spontaneously on postoperative days 1 and 2. One animal had to be euthanized on day 4 with paraplegia. As it cannot be excluded that these early deaths were due to technical failures, the four above mentioned rats were omitted from further analysis.

In the seven remaining mast cell-deficient and eight remaining mast cell-competent recipients, abdominal heterotopic heartbeat was monitored daily by palpation. Of seven mast cell-deficient recipients, only three exhibited palpable donor heartbeat until the predetermined termination of the study at 12 weeks. In contrast, all eight mast cell-competent donor hearts survived until the end of the study (Fig. 1, $P = 0.015$).

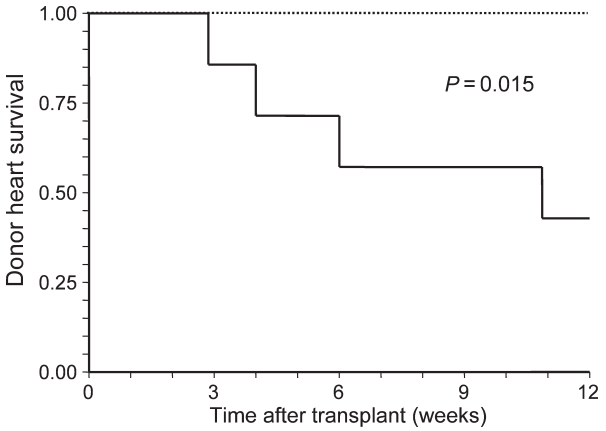

Figure 1 Donor heart survival after cardiac heterotopic transplantation in mast cell-competent rats (dotted line, $n = 8$) and mast cell-deficient rats (solid line, $n = 7$). The difference in survival between the two types of donor hearts was statistically significant ($P = 0.015$).

Animals with cessation of heterotopic heartbeat were euthanized humanely where after donor and native hearts were collected for histopathology and immunohistochemistry. All other recipients were euthanized 12 weeks after transplantation, where after donor and native hearts were collected for histopathology and immunohistochemistry.

Histopathology of baseline, native and donor hearts

Table 2 gives scores for rejection, ischemia, fibrosis, and whether or not thrombus formation was observed, in donor hearts of the seven mast cell-deficient and eight mast cell-competent rats that were included in histopathological analyses.

Signs of rejection were found in three out of eight mast cell-competent and three out of seven mast cell-deficient donor hearts, indicating that there was no difference in the incidence of rejection between the two types of donor hearts. Rejection scores were slightly, but not significantly, higher for mast cell-deficient donor hearts compared with mast cell-competent donor hearts.

No thrombi were detected in baseline and native hearts of either rat type. Thrombus formation was observed in seven out of eight mast cell-competent donor hearts and five out of seven mast cell-deficient donor hearts. Therefore, no significant difference was found in incidence of thrombus formation between the two types of donor hearts.

Six out of seven mast cell-deficient and six out of eight mast cell-competent donor hearts exhibited signs of ischemia. Ischemia in mast cell-deficient donor hearts (median score of all seven hearts: 2) did not differ from ischemia in mast cell-competent donor hearts (median score of all eight hearts: 1.5). Similarly, there was no significant difference in fibrosis between mast cell-deficient (median score: 4) and mast cell-competent donor hearts (median score: 3).

Table 2. Scores for rejection (0–3), ischemia (0–3), fibrosis (0–4), and whether (+) or not (–) thrombus formation were observed, in donor hearts of mast cell-deficient and mast cell-competent rats.

Rat type	Observation time (days)	Rejection score	Thrombus formation (+/–)	Ischemia score	Fibrosis score
Competent	83	1	+	0	1
Competent	83	1	+	0	1
Competent	83	0	+	1	3
Competent	83	0	+	2	3
Competent	83	1	+	2	3
Competent	83	0	–	3	4
Competent	83	0	+	3	4
Competent	83	0	+	1	4
Deficient	20	0	+	0	1
Deficient	28	0	+	1	4
Deficient	42	3	+	3	4
Deficient	76	0	–	2	2
Deficient	83	0	–	1	1
Deficient	83	3	+	3	4
Deficient	83	1	+	3	4

Mast cell densities

As expected, all baseline, native and donor hearts from mast cell-deficient rats were devoid of mast cells, as demonstrated by absence of metachromatic staining with Toluidine Blue. Mast cell density in baseline hearts was 0.70 ± 0.40 per mm^2. Mast cell density in the native hearts of the transplant recipients was 2.18 ± 0.82 per mm^2 and thereby slightly, but not significantly, higher than the density in baseline hearts. Mast cell density of the donor hearts in these animals, on the other hand, was 15.54 ± 6.44 per mm^2 and thereby considerably higher ($P < 0.001$).

T-lymphocyte accumulation

Table 3 shows numbers of TCRα/β immunoreactive cells in comparable areas of baseline, native, and donor hearts. More TCRα/β positive cells were found in mast cell-competent donor hearts compared with mast cell-deficient donor hearts.

Extracellular matrix-associated TGF-β and fibrin

Transforming growth factor-β immunoreactive staining in donor hearts was less pronounced in ischemic areas than in viable areas. Image analysis was used to measure areas of extracellular matrix-associated TGF-β immunoreactivity in viable tissue only of baseline, native, and donor hearts (Fig. 2a). In mast cell-competent rats, a significant increase in TGF-β immunoreactivity was shown in donor hearts compared with native hearts ($P = 0.005$). In mast cell-deficient rats, this increase was considerably less and nonsignificant. TGF-β immunoreactive areas in viable

Table 3. Numbers of baseline, native and donor hearts, categorized by their total number of TCRα/β immunoreactive cells. Higher numbers of TCRα/β immunoreactive cells were found in mast cell-competent than in mast cell-deficient donor hearts.

			Numbers of TCRα/β immunoreactive cells			
Rat type	Heart type	*n**	0–10	10–50	50–100	>100
Competent	Baseline	6	6	–	–	–
Deficient	Baseline	6	6	–	–	–
Competent	Native	5	5	–	–	–
Deficient	Native	5	5	–	–	–
Competent	Donor	8	–	3	2	3
Deficient	Donor	7	5	1	–	1

*n = total number of hearts.

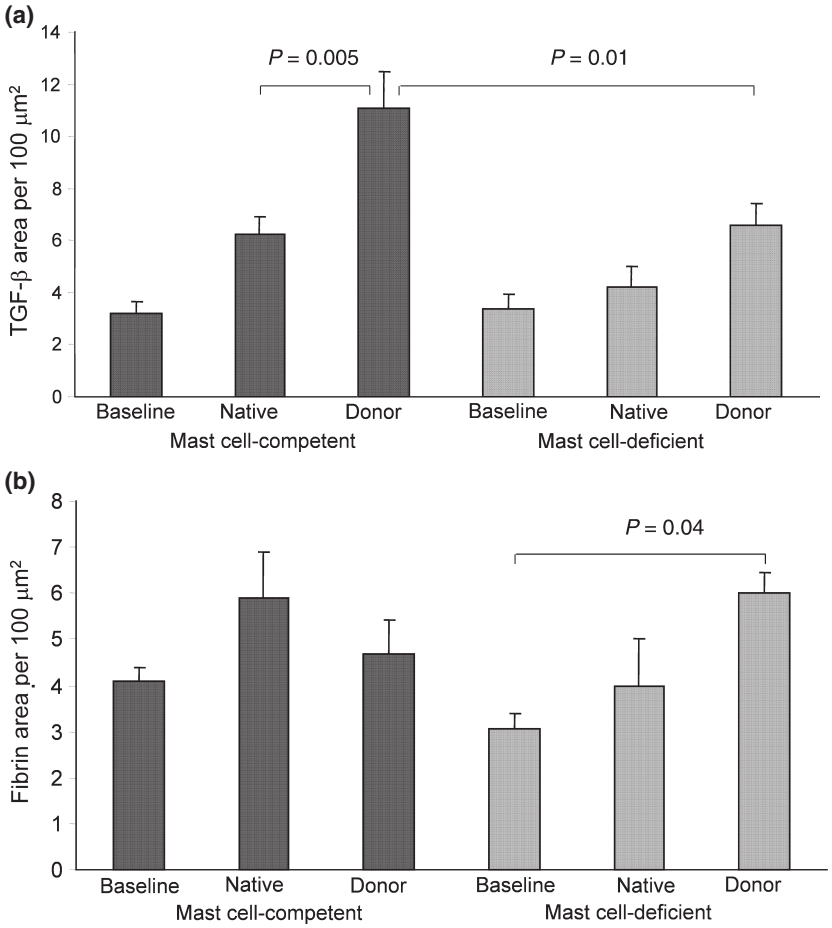

Figure 2 Fibrin and extracellular matrix-associated transforming growth factor-β (TGF-β) in baseline, native, and donor hearts (μm² per 100 μm² total area), measured by quantitative immunohistochemistry. (a) Increases in TGF-β immunoreactivity, measured in viable tissue only, were more severe in mast cell-competent donor hearts than in mast cell-deficient donor hearts (average ± SEM, $n = 4$). (b) Significant increase in fibrin deposition, in mast cell-deficient donor hearts (average ± SEM, $n = 5$–9).

tissue correlated with mast cell-density (linear regression: $R^2 = 0.3$, $P = 0.009$).

Deposition of fibrin was measured by quantitative immunohistochemistry in baseline, native, and donor hearts (Fig. 2b). ANOVA with Tukey *post hoc* testing showed a significant difference in fibrin deposition, only between mast cell-deficient control hearts and mast cell-deficient donor hearts ($P = 0.04$).

Immunohistochemical analysis of the cardiac endothelin system

In baseline and native hearts, ET-1 immunoreactivity was found in a few cardiomyocytes and on mast cells. The ET-1 receptors ETA and ETB were predominantly expressed by vascular smooth muscle cells and cardiac mast cells. In donor hearts, increased ET-1 immunoreactivity was found in tissue surrounding ischemic areas (Fig. 3a), whereas ET-1 expression was less prominent in donor hearts with little or no ischemia. Similar to baseline and native hearts, cardiac mast cells in donor hearts expressed ET-1 (Fig. 3a). In donor hearts, immunoreactivity for ETA and ETB was found on vascular smooth muscle cells, mast cells (Fig. 3b,c), as well as on mononuclear inflammatory cells (Fig. 3d). Cardiac immunoreactivity of the components of the endothelin system was similar in mast cell-deficient and mast cell competent rats, with the obvious exception of immunoreactive mast cells in the latter.

Discussion

Mast cells contain a large number of inflammatory, vasoactive, chemotactic, and mitogenic mediators and are a significant source of inflammatory and fibrogenic cytokines. In addition to their role in hypersensitivity reactions, mast cells are being increasingly implicated in

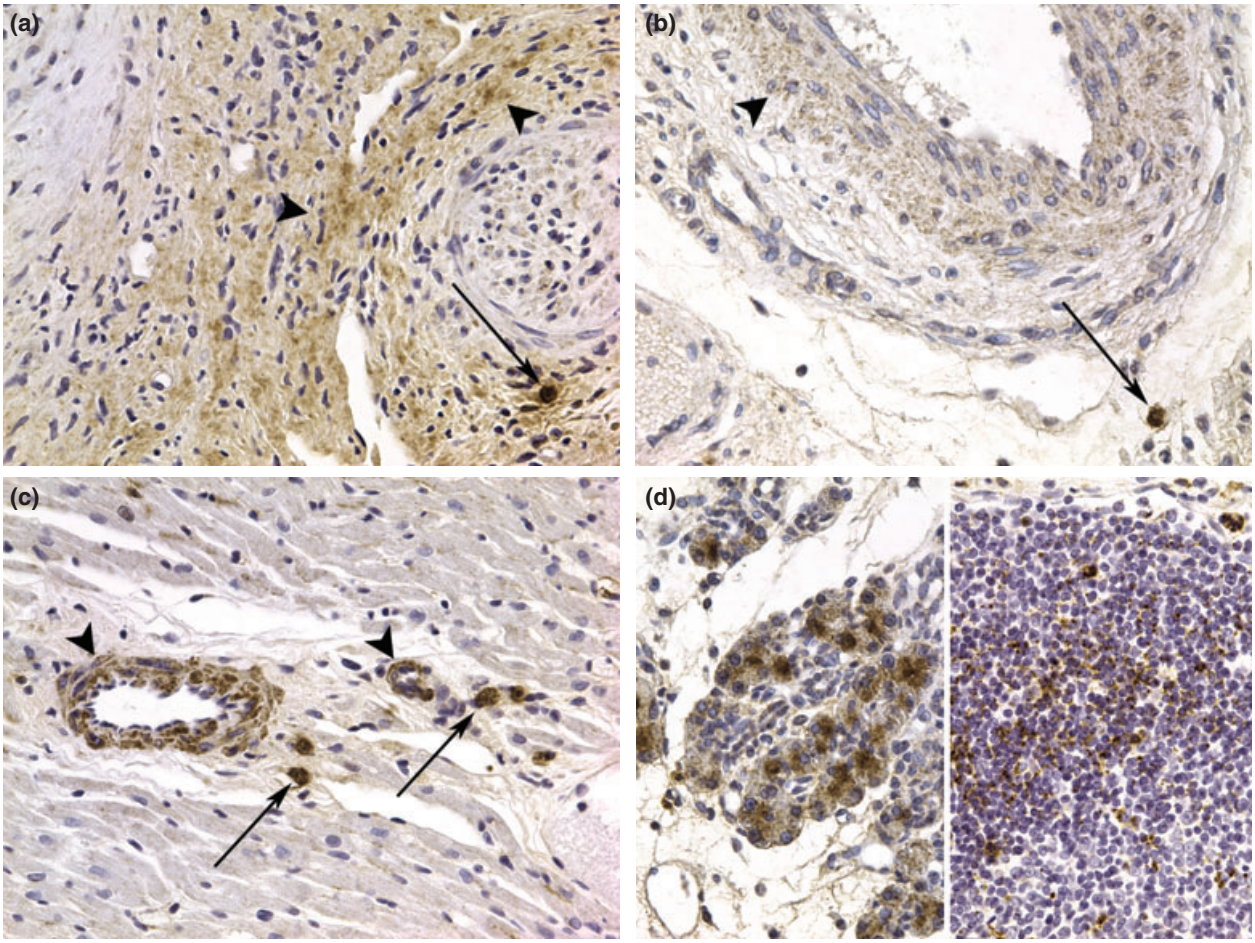

Figure 3 Representative images of immunoreactivity for components of the endothelin system in mast cell-competent donor hearts (40× objective). (a) Increased endothelin-1 (ET-1) immunoreactivity was found in tissue surrounding ischemic areas (arrowheads) and on mast cells (arrow). Immunoreactivity for (b) ETA and (c) ETB was found in vascular smooth muscle cells (arrowheads) and cardiac mast cells (arrows). (d) Some mononuclear inflammatory cells expressed ETA (left panel) and/or ETB (right panel).

many conditions associated with inflammation and excessive collagen deposition. Despite correlative data suggesting that mast cells are involved in cardiac allograft rejection [1–3], to our knowledge, this is the first study that directly addresses the role of mast cells in the outcome of cardiac allo-transplantation using an outbred mast cell-deficient animal model. The mast cell-deficient and mast cell-competent rats used in this study showed no differences in basal cardiac collagen and fibrin deposition, or basal cardiac gene expression of several pro-fibrogenic and pro-inflammatory mediators. Upon transplantation, however, significantly reduced donor heart survival, accompanied by exacerbated structural and molecular changes, was observed in mast cell-deficient rats. These findings strongly suggest a beneficial role for mast cells during the post-transplant period and are consistent with recent demonstrations of a beneficial role for mast cells in other cardiac conditions [11,12] and of a role of mast cells in induction of T-cell tolerance [16].

In accordance with former studies [1–3], mast cell-competent donor heart showed increased densities of mast cells compared with native hearts and baseline hearts. Moreover, the presence of degranulating mast cells in donor hearts was indicative of mast cell activation [1,2]. Mast cells are not only involved in inflammatory responses, but also in several other processes, including tissue remodeling and thrombus formation. For example, while resting mast cells are mainly pro-fibrinolytic, resulting in degradation of fibrin, activated mast cells exhibit pro-thrombotic characteristics and inhibit degradation of fibrin [7]. In this study, there was a net increase in fibrin in mast cell-deficient compared with mast cell-competent donor heart. While this increase in fibrin was not accompanied by an increase in thrombus formation, it may still

represent a risk factor for graft coronary artery disease and graft failure [29,30].

The present study showed lower TGF-β levels in mast cell-deficient compared with mast cell-competent donor hearts. Mast cells not only produce TGF-β [8], but also regulate the local levels and activity of TGF-β by releasing it from the extracellular matrix [15]. While TGF-β has potent pro-fibrogenic properties, it is also strongly immunosuppressive [31]. The increased TGF-β levels in mast cell-competent hearts were associated with lower scores for rejection, consistent with the notion that TGF-β may help prevent cardiac allograft rejection [32,33]. Moreover, a recent study has shown that mast cells are essential for T-cell tolerance after skin transplantation and suggests that TGF-β is involved in this process [16].

In this study, rejection was partly abrogated by relatively high doses of CSA. Hence, it is possible that a different (likely greater) difference in some parameters such as TGF-β immunoreactivity had been observed if the study had been performed without exogenous immunosuppression, or with a different immunosuppressive agent.

Fibrosis did not differ between mast cell-deficient and mast cell-competent donor hearts in this study, probably because the observation time was limited to 12 weeks. In fact, it is possible that mast cells, while exerting a beneficial effect during the early to mid-term post-transplantation period, may promote development of delayed graft fibrosis by increasing the levels of TGF-β. Results from other studies performed in our laboratory are consistent with this notion [14].

TCRα/β-positive T-lymphocytes play an important role in cardiac allograft rejection [34–36]. Mast cells modulate a T-cell-dependent immune response by antigen presentation to T-cells and by modulating migration, proliferation, and differentiation of T-cells [37]. In the present study, fewer T-lymphocytes were found in mast cell-deficient donor hearts, consistent with the notion that, upon transplantation, mast cells may be involved in the recruitment and/or proliferation of these cells. Lower numbers of TCRα/β T cells in mast cell-deficient donor hearts coincided with higher scores for rejection. This observation is in contrast with former studies, showing that lower numbers of TCRα/β expressing T-cells, or blocking the function of these cells, reduce cardiac allograft rejection and benefit cardiac allograft survival [35,36]. Mast cell-deficient (Ws/Ws) rats can develop normal numbers of T-lymphocytes [38], suggesting that the particular mutation in the c-kit receptor that causes the mast cell-deficiency may not affect the proliferation and differentiation of the relevant hematopoietic cell lineage. In the present study, however, most donor hearts were examined 12 weeks after transplantation, which can be considered a mid-term post-transplantation time point. T-cell numbers and signs of rejection could have been different in the first few weeks after transplantation. Moreover, T-cell activation status was not investigated, but may be different in the absence or presence of mast cells [16]. Moreover, it cannot be excluded that the decreased number of T-lymphocytes in mast cell-deficient donor hearts is caused by an increased sensitivity to CSA [1].

The ET-1 receptors ETA and ETB are expressed on a wide variety of cell types, including vascular smooth muscle cells, cardiac fibroblasts, mast cells, erythrocytes and mononuclear inflammatory cells [23,39,40]. The endothelin system affects allograft survival in experimental models of cardiac transplantation. For example, inhibition of endothelin-converting enzyme improved cardiac allograft survival, reduced rejection, and ameliorated myocardial fibrosis in a rat heterotopic transplant model [41]. Moreover, administration of the combined ETA and ETB receptor antagonist bosentan [23], or pretreatment with ET-1 antisense oligodeoxynucleotides [42] reduced the formation of atherosclerotic lesions in chronic cardiac transplant rejection. Interestingly, the beneficial effect of mast cells on tissue homeostasis appears to be related to their ability to reduce ET-1 induced toxicity [13]. In the present study, similar immunoreactivity levels of components of the cardiac endothelin system were observed in mast cell-competent and mast cell-deficient donor hearts, suggesting that the difference in graft survival was not related to ET-1 or its receptors.

Mast cells contain a plethora of biologically active mediators. Besides the factors examined in this study, other mast cell mediators might be involved in cardiac allograft survival and remodeling. For instance, basic fibroblast growth factor expression by mast cells is correlated with cardiac collagen contents [43].

Other than the deficiency of mast cells, melanocytes, and interstitial cells of Cajal in the intestine, the physiological consequences of the c-kit mutation in the rat model are less than in the more commonly used mast cell-deficient mouse models (e.g. W/W-v mice), which exhibit rather severe anemia [44]. It is still possible that the mast cell-deficient rats may have other changes that could influence their response to various challenges. For example, the c-kit mutation may influence c-kit expressing vascular endothelial cells, subsets of natural killer cells, or cardiac progenitor cells involved in regeneration and repair of the myocardium [45,46]. The definitive experiment to elucidate the role of mast cells in a particular response would be to reconstitute the mast cell population in mast cell-deficient animals, similar to what is commonly done in mast cell-deficient mice [47]. However, mast cell-deficient (Ws/Ws) and mast cell-competent (+/+) rats are the F2 generation of two hybrid Donryu/ BN/fMai animals. Hence, while the rat model has several advantages for research on cardiac graft survival after allogeneic

transplantation, long-term mast cell reconstitution in these noninbred rats has not been feasible [48,49].

Experiments using mast cell-stabilizing compounds in mast cell competent rats may help clarify the mechanisms by which the mast cells affect cardiac pathophysiology to some extent. On the other hand, because these compounds generally work by inhibiting degranulation of mast cells, they address only part of the complex role that mast cells play in immunopathology and fibroproliferative disorders. Moreover, it is interesting to speculate about the potential role of resident versus bone marrow-derived mast cells in cardiac transplant pathology. Future experiments in which mast cell-deficient hearts are transplanted into mast cell-competent recipients, and vice versa, may clarify these issues.

In conclusion, this study demonstrated that the absence of mast cells is associated with significantly reduced cardiac allograft survival after heterotopic transplantation. These data, consistent with studies in other types of inflammatory and fibroproliferative disorders of the heart and other organs, strongly point toward a beneficiary role of mast cells during the post-transplantation period.

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References

1. Koskinen PK, Kovanen PT, Lindstedt KA, Lemstrom KB. Mast cells in acute and chronic rejection of rat cardiac allografts – a major source of basic fibroblast growth factor. *Transplantation* 2001; **71**: 1741.
2. Li QY, Raza-Ahmad A, MacAulay MA, *et al.* The relationship of mast cells and their secreted products to the volume of fibrosis in posttransplant hearts. *Transplantation* 1992; **53**: 1047.
3. Zweifel M, Hirsiger H, Matozan K, Welle M, Schaffner T, Mohacsi P. Mast cells in ongoing acute rejection: increase in number and expression of a different phenotype in rat heart transplants. *Transplantation* 2002; **73**: 1707.
4. Boyce JA. Mast cells: beyond IgE. *J Allergy Clin Immunol* 2003; **111**: 24.
5. Sillaber C, Baghestanian M, Bevec D, *et al.* The mast cell as site of tissue-type plasminogen activator expression and fibrinolysis. *J Immunol* 1999; **162**: 1032.
6. Valent P, Baghestanian M, Bankl HC, *et al.* New aspects in thrombosis research: possible role of mast cells as profibrinolytic and antithrombotic cells. *Thromb Haemost* 2002; **87**: 786.
7. Wojta J, Kaun C, Zorn G, *et al.* C5a stimulates production of plasminogen activator inhibitor-1 in human mast cells and basophils. *Blood* 2002; **100**: 517.
8. Crivellato E, Beltrami CA, Mallardi F, Ribatti D. The mast cell: an active participant or an innocent bystander? *Histol Histopathol* 2004; **19**: 259.
9. Hara M, Ono K, Hwang MW, *et al.* Evidence for a role of mast cells in the evolution to congestive heart failure. *J Exp Med* 2002; **195**: 375.
10. Shiota N, Rysa J, Kovanen PT, Ruskoaho H, Kokkonen JO, Lindstedt KA. A role for cardiac mast cells in the pathogenesis of hypertensive heart disease. *J Hypertens* 2003; **21**: 1935.
11. Boerma M, Wang J, Wondergem J, *et al.* Influence of mast cells on structural and functional manifestations of radiation-induced heart disease. *Cancer Res* 2005; **65**: 3100.
12. Joseph J, Kennedy RH, Devi S, Wang J, Joseph L, Hauer-Jensen M. Protective role of mast cells in homocysteine-induced cardiac remodeling. *Am J Physiol Heart Circ Physiol* 2005; **288**: H2541.
13. Maurer M, Wedemeyer J, Metz M, *et al.* Mast cells promote homeostasis by limiting endothelin-1-induced toxicity. *Nature* 2004; **432**: 512.
14. Zheng H, Wang J, Hauer-Jensen M. Role of mast cells in early and delayed radiation injury in rat intestine. *Radiat Res* 2000; **153**: 533.
15. Wang Y, Shiota N, Leskinen MJ, Lindstedt KA, Kovanen PT. Mast cell chymase inhibits smooth muscle cell growth and collagen expression in vitro: transforming growth factor-beta1-dependent and -independent effects. *Arterioscler Thromb Vasc Biol* 2001; **21**: 1928.
16. Lu LF, Lind EF, Gondek DC, *et al.* Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* 2006; **442**: 997.
17. Ehrenreich H, Burd PR, Rottem M, *et al.* Endothelins belong to the assortment of mast cell-derived and mast cell-bound cytokines. *New Biol* 1992; **4**: 147.
18. Hultner L, Ehrenreich H. Mast cells and endothelin-1: a life-saving biological liaison? *Trends Immunol* 2005; **26**: 235.
19. Metsarinne KP, Vehmaan-Kreula P, Kovanen PT, *et al.* Activated mast cells increase the level of endothelin-1 mRNA in cocultured endothelial cells and degrade the secreted Peptide. *Arterioscler Thromb Vasc Biol* 2002; **22**: 268.
20. Wypij DM, Nichols JS, Novak PJ, Stacy DL, Berman J, Wiseman JS. Role of mast cell chymase in the extracellular processing of big-endothelin-1 to endothelin-1 in the perfused rat lung. *Biochem Pharmacol* 1992; **43**: 845.
21. Aharinejad S, Krenn K, Paulus P, *et al.* Differential role of TGF-beta1/bFGF and ET-1 in graft fibrosis in heart failure patients. *Am J Transplant* 2005; **5**: 2185.

22. Ferri C, Properzi G, Tomassoni G, *et al.* Patterns of myocardial endothelin-1 expression and outcome after cardiac transplantation. *Circulation* 2002; **105**: 1768.
23. Sihvola RK, Pulkkinen VP, Koskinen PK, Lemstrom KB. Crosstalk of endothelin-1 and platelet-derived growth factor in cardiac allograft arteriosclerosis. *J Am Coll Cardiol* 2002; **39**: 710.
24. Niwa Y, Kasugai T, Ohno K, *et al.* Anemia and mast cell depletion in mutant rats that are homozygous at ''white spotting (Ws)'' locus. *Blood* 1991; **78**: 1936.
25. Vriens PW, Blankenberg FG, Stoot JH, *et al.* The use of technetium Tc 99m annexin V for in vivo imaging of apoptosis during cardiac allograft rejection. *J Thorac Cardiovasc Surg* 1998; **116**: 844.
26. Stewart S, Winters GL, Fishbein MC, *et al.* Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *J Heart Lung Transplant* 2005; **24**: 1710.
27. James JD, Hauer-Jensen M. Effects of fixative and fixation time for quantitative computerized image analysis of immunohistochemical staining. *J Histotechnol* 2001: **22**: 109.
28. Wang J, Zheng H, Hollenberg MD, Wijesuriya SJ, Ou X, Hauer-Jensen M. Up-regulation and activation of proteinase-activated receptor 2 in early and delayed radiation injury in the rat intestine: influence of biological activators of proteinase-activated receptor 2. *Radiat Res* 2003; **160**: 524.
29. Labarrere CA, Nelson DR, Park JW. Pathologic markers of allograft arteriopathy: insight into the pathophysiology of cardiac allograft chronic rejection. *Curr Opin Cardiol* 2001; **16**: 110.
30. Torry RJ, Bai L, Miller SJ, Labarrere CA, Nelson D, Torry DS. Increased vascular endothelial growth factor expression in human hearts with microvascular fibrin. *J Mol Cell Cardiol* 2001; **33**: 175.
31. Kehrl JH, Wakefield LM, Roberts AB, *et al.* Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* 1986; **163**: 1037.
32. Chan SY, Goodman RE, Szmuszkovicz JR, Roessler B, Eichwald EJ, Bishop DK. DNA-liposome versus adenoviral mediated gene transfer of transforming growth factor beta1 in vascularized cardiac allografts: differential sensitivity of CD4+ and CD8+ T cells to transforming growth factor beta1. *Transplantation* 2000; **70**: 1292.
33. Qin L, Chavin KD, Ding Y, *et al.* Multiple vectors effectively achieve gene transfer in a murine cardiac transplantation model. Immunosuppression with TGF-beta 1 or vIL-10. *Transplantation* 1995; **59**: 809.
34. Honjo K, Xu X, Bucy RP. CD4+ T-cell receptor transgenic T cells alone can reject vascularized heart transplants through the indirect pathway of alloantigen recognition. *Transplantation* 2004; **77**: 452.
35. Exner BG, Que X, Mueller YM, Domenick MA, Neipp M, Ildstad ST. Alpha beta TCR+ T cells play a nonredundant role in the rejection of heart allografts in mice. *Surgery* 1999; **126**: 121.
36. Heidecke CD, Hancock WW, Jakobs F, *et al.* Alpha/beta-T cell receptor-directed therapy in rat cardiac allograft recipients. Treatment prior to alloantigen exposure prevents sensitization and abrogates accelerated rejection. *Transplantation* 1995; **59**: 78.
37. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as ''tunable'' effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 2005; **23**: 749.
38. Miyazawa S, Hotta O, Doi N, Natori Y, Nishikawa K, Natori Y. Role of mast cells in the development of renal fibrosis: use of mast cell-deficient rats. *Kidney Int* 2004; **65**: 2228.
39. Rivera A, Rotter MA, Brugnara C. Endothelins activate Ca(2+)-gated K(+) channels via endothelin B receptors in CD-1 mouse erythrocytes. *Am J Physiol* 1999; **277**: C746.
40. Sakashita K, Oonishi T, Ishioka N, Uyesaka N. Endothelin-1 improves the impaired filterability of red blood cells through the activation of protein kinase C. *Jpn J Physiol* 1999; **49**: 113.
41. Simonson MS, Robinson AV, Schulak JA, Hricik DE. Inhibition of endothelin-1 improves survival and vasculopathy in rat cardiac transplants treated with cyclosporine. *Transplantation* 2002; **73**: 1054.
42. Yamaguchi A, Miniati DN, Hirata K, Hoyt EG, Robbins RC. Ex vivo blockade of endothelin-1 inhibits graft coronary artery disease in a rodent cardiac allograft model. *J Heart Lung Transplant* 2002; **21**: 417.
43. Akgul A, Skrabal CA, Thompson LO, *et al.* Role of mast cells and their mediators in failing myocardium under mechanical ventricular support. *J Heart Lung Transplant* 2004; **23**: 709.
44. Cynshi O, Satoh K, Higuchi M, Imai N, Kawaguchi T, Hirashima K. Effects of recombinant human erythropoietin on anaemic W/Wv and Sl/Sld mice. *Br J Haematol* 1990; **75**: 319.
45. Ashman LK. The biology of stem cell factor and its receptor C-kit. *Int J Biochem Cell Biol* 1999; **31**: 1037.
46. Matos ME, Schnier GS, Beecher MS, Ashman LK, William DE, Caligiuri MA. Expression of a functional c-kit receptor on a subset of natural killer cells. *J Exp Med* 1993; **178**: 1079.
47. Perdue MH, Masson S, Wershil BK, Galli SJ. Role of mast cells in ion transport abnormalities associated with intestinal anaphylaxis. Correction of the diminished secretory response in genetically mast cell-deficient W/Wv mice by bone marrow transplantation. *J Clin Invest* 1991; **87**: 687.
48. Santos J, Yang PC, Soderholm JD, Benjamin M, Perdue MH. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. *Gut* 2001; **48**: 630.
49. Yang PC, Berin MC, Yu L, Perdue MH. Mucosal pathophysiology and inflammatory changes in the late phase of the intestinal allergic reaction in the rat. *Am J Pathol* 2001; **158**: 681.