

Peritransplant Ischemic Injury Is Associated With Up-Regulation of Stromal Cell-Derived Factor-1

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OBJECTIVES	We evaluated chimerism and stromal cell-derived factor-1 (SDF-1) expression in response to peritransplant ischemic injury following human heart transplantation.
BACKGROUND	Myocardial ischemia has been shown to trigger mobilization of stem cells to the heart in animal experiments.
METHODS	Between January 1998 and April 2002, a total of 114 male recipients received hearts from female donors. Of these 114 recipients, 26 had evidence of ischemic injury on their initial heart biopsies (ischemia group). These were compared to the remaining 88 patients (control group). Heart biopsy specimens obtained initially at one week and at one year after transplant were evaluated from 20 matched patients of each group for the presence of Y chromosome-containing nuclei. The SDF-1 messenger ribonucleic acid (mRNA) and protein expression were also evaluated on initial heart biopsy specimens.
RESULTS	At one week, Y chromosome-containing nuclei were significantly increased in the ischemia group (0.68% vs. 0.04%; $p < 0.0001$) compared to the control group. These were positive for the stem cell factor receptor c-kit. A significant 3.3-fold increased mRNA expression ($p = 0.001$) and 2.8-fold increased protein expression ($p = 0.01$) of SDF-1 was noted in the ischemia group. At one year, Y chromosome was detected in 0.29% of cardiomyocyte nuclei in the ischemia group but none in the control group. The ischemia group had poorer survival and increased vasculopathy.
CONCLUSIONS	This is the first report to describe chimerism and up-regulation of SDF-1 in human heart transplantation in response to ischemic injury. (J Am Coll Cardiol 2005;46:1029–35) © 2005 by the American College of Cardiology Foundation

Experimental evidence suggests that ischemic injury plays a pivotal role in triggering mobilization of stem cells to the site of injury (1). Stem cells have been shown to contribute to cardiac muscle repair and neovascularization after ischemic injury (2). Several endogenous and environmental factors play important roles in regulation of stem cells, including inflammatory cytokines, growth factors, surface receptors, proteolytic enzymes, nuclear proteins, and stem cell–host cell interaction (3). Stromal cell-derived factor-1 (SDF-1), a member of the chemokine CXC subfamily, is considered to play an important role in the trafficking of hematopoietic stem cells to sites of ischemic injury and contributing to the process of neovascularization (4). Recently, expression of SDF-1 has been shown to induce stem cell homing to injured myocardium in an animal model of myocardial infarction (5,6). Ischemic injury following cardiac transplantation has been shown to be associated with development of vasculopathy and to portend a poor long-term outcome (7). Cardiac chimerism, caused by migration of primitive cells from the recipient to the grafted heart, has recently been demonstrated in postmortem examinations (8) and in myocardial biopsies (9). However, the phenomenon of stem cell mobilization and expression of SDF-1 in

response to peritransplant ischemic injury has not been evaluated before. We hypothesized that peritransplant ischemic injury triggers mobilization of stem cells to the transplanted heart and is associated with increased expression of SDF-1.

METHODS

Patient population. To study the process of cardiac chimerism in response to ischemic injury, patients with mismatched hearts were evaluated. Between January 1998 and April 2002, a total of 114 consecutive male recipients received hearts from female donors. A group of 26 patients had evidence of ischemic injury on their initial heart biopsies (ischemia group). Ischemic injury related to transplantation was identified by areas of myocyte necrosis with an absence of infiltration by activated lymphocytes or macrophages. Their clinical outcome was compared to the remaining 88 patients (control group). Patients underwent serial heart biopsies and serial coronary angiograms as per the surveillance heart transplant protocol. We collected five biopsy specimens per patient and the sampling was done from the interventricular septum. Coronary artery stenosis of 50% or greater in the absence of donor coronary atherosclerosis was required for the diagnosis of transplant vasculopathy. The protocol was approved by the ethics review committee of our institution.

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Abbreviation and Acronyms	
FISH	= fluorescent in situ hybridization
mRNA	= messenger ribonucleic acid
RT-PCR	= reverse transcription-polymerase chain reaction
SDF-1	= stromal cell-derived factor-1

Fluorescent in situ hybridization (FISH). To evaluate for chimerism over time, paired heart biopsy specimens, obtained at one week and one year after transplant, were evaluated from 20 patients of the ischemia group. Paired specimens were not used in the remaining six patients because one patient had severe rejection during the first week of transplantation and, therefore, was excluded from tissue analysis to avoid potential rejection-related bias; five patients expired before the end of 12 months and, therefore, no tissue was available at 1 year. The 20 patients in the ischemia group were matched, in relation to baseline characteristics, to 20 patients from the control group who also had no acute rejection on their initial biopsies, and their myocardial biopsies were compared. Fluorescent in situ hybridization was used to determine the presence of X chromosome using X Alpha Satellite (DXZ1) probe (Qbiogene, Irvine, California) and of Y chromosome using DNA probe CEP Y satellite III (Vysis, Downers Grove, Illinois) as previously described (8). Cell number was determined by 4',6-diamidino-2-phenylindole dihy-

Table 1. Baseline Characteristics

	Control (n = 88)	Ischemia (n = 26)
Donor age (yrs)	36 ± 15	39 ± 14
Recipient age (yrs)	54 ± 9	57 ± 10
Etiology of heart disease		
ICM/DCM/congenital	47/40/1	15/11/0
Donor cause of death		
CVA/trauma/other	40/43/5	13/11/2
Ischemia time (min)	178 ± 42	180 ± 44
LVAD	18 (20%)	6 (23%)
Diabetes mellitus	16 (18%)	5 (19%)
Hypertension	39 (44%)	11 (42%)
Hyperlipidemia	35 (40%)	10 (38%)
HLA mismatch	4.7 ± 1.4	4.4 ± 1.5
PRA >20%	5 (5.7%)	2 (7.7%)
CMV status		
D-R-/D+R-/D-R+/D+R+	16/25/19/28	5/7/5/9
CMV disease	9 (10.2%)	3 (11.5%)
Mean episodes of acute cellular rejection grade ≥3A	1.5 ± 1.1	1.3 ± 1.2
Immunosuppression		
Prednisone + CSA + AZA	15 (17%)	5 (19.2%)
Prednisone + CSA + MMF	39 (44.4%)	11 (42.3%)
Prednisone + MMF + FK506	30 (34.1%)	9 (34.6%)
Prednisone + CSA + everolimus	(3.4%)	1 (3.9%)
Prednisone + CSA + rapamycin	1 (1.1%)	0 (0%)

AZA = azathioprine; CMV = cytomegalovirus; CSA = cyclosporine; CVA = cerebrovascular accident; D = donor; DCM = dilated cardiomyopathy; HLA = human leukocyte antigen; ICM = ischemic cardiomyopathy; LVAD = left ventricular assist device use as a bridge to transplant; MMF = mycophenolate mofetil; PRA = panel reactive antibody; R = recipient.

Table 2. Percentage of Y Chromosome-Containing Nuclei

	Control	Ischemia	p Value
One week	0.04 ± 0.03%	0.68 ± 0.12%	<0.0001
One year	0.07 ± 0.06%*	1.25 ± 0.39%†	<0.0001

*p = 0.058, compared to one week, in control group. †p < 0.001, compared to one week, in ischemia group.

drochloride (DAPI) staining (Vysis) (10). Two biopsy specimens from female-donor/female-transplant recipients and another three biopsy specimens from male-donor/male-transplant recipients were used as negative and positive controls, respectively.

Cell markers. Antibodies against c-kit, a stem cell factor receptor (11), were used to identify primitive cells (Vector Laboratories, Burlingame, California) on initial heart biopsy specimens at one week after transplantation (12). To determine the nature of cells at one year after transplantation, antibodies against cardiac myosin heavy chain (Chemicon, Temecula, California), smooth muscle alpha-actin (Sigma, St. Louis, Missouri), factor VIII (Sigma), vimentin (Sigma), CD45RB (Dako, Carpinteria, California), and CD68 (Dako) were used to identify myocytes, smooth muscle cells, endothelial cells, fibroblasts, lymphocytes, and macrophages, respectively. The immunoglobulin-G antibodies conjugated with fluorescein isothiocyanate (Jackson Immuno Research, West Grove, Pennsylvania) were used as secondary antibodies (12).

SDF-1 expression. The SDF-1 mRNA and protein expressions were evaluated on initial heart biopsy specimens using TaqMan quantitative reverse transcription-polymerase chain reaction (RT-PCR) (13) and Western blot analysis (14), respectively, as previously described. The oligonucleotide sequences of TaqMan probe and primers of SDF-1 were as follows: TaqMan probe, SDF-1-859T: CAGCAGCGCCCCCTCCCAAGA; forward primer, SDF-1-837F: TCTTCAGACACTGAGGCTCCC; reverse primer, SDF-1-903R: TATCTGAGTGCCACAGAGGCC. Rabbit antihuman SDF-1 antibody (Chemicon) was used for immunoblotting.

Biotinylated antihuman SDF-1 antibody (Peprotech, Rocky Hill, New Jersey) was used (dilution = 1:100) for immunohistochemistry staining of heart biopsy specimens examined at one week after transplant.

CXCR4 expression. We evaluated mRNA expression of CXCR4, a receptor for SDF-1, on the initial heart biopsy specimens using TaqMan quantitative RT-PCR as described before (13). The oligonucleotide sequences of TaqMan probe and primers of CXCR4 were as follows: TaqMan probe, CXCR4-P837: TTCTTCGCCTGTTGGCTGCC; forward primer, CXCR4-F814: CCACAGTCATCCTCATCCTG; reverse primer, CXCR4-R915: ACCCTTGCTTGATGATTTCC.

Statistical analysis. Data are presented as mean ± standard deviation. Categorical variables were compared by chi square. The Fisher exact test was used when the sample size

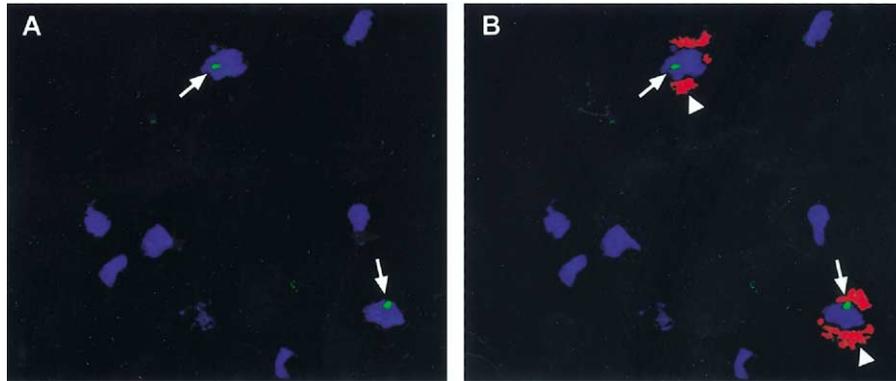


Figure 1. Fluorescent in situ hybridization and double immunofluorescence of initial heart biopsy specimen from ischemia group showing DAPI blue-stained nuclei containing Y chromosome (arrows) in green (A). (B) These cells are positive for c-kit in red (arrowheads), suggestive of stem cell origin.

of the categorical variable was five or less, such as etiology of heart disease (congenital), donor cause of death (other), diabetes mellitus, panel reactive antibody, cytomegalovirus disease, and immunosuppression. Continuous variables were compared using the Wilcoxon rank sum test. Differences were considered significant at $p < 0.05$. Survival and freedom from vasculopathy were assessed by construction of Kaplan-Meier plots and calculation of the log-rank chi-square statistic.

RESULTS

Both groups had similar baseline characteristics (Table 1). Two patients in the ischemia group had evidence of mild left ventricular dysfunction (left ventricular ejection fraction 40% and 45%, respectively) soon after transplantation that recovered within one week.

FISH. The female-donor/female-recipient biopsies showed no evidence of Y chromosome and the male-donor/male-recipient biopsies showed evidence of Y chromosome in 46% of the 2,000 nuclei studied, yielding a sensitivity and specificity comparable to other studies (8,9,15). The ischemia group had significant increased proportion of Y chromosome-containing nuclei compared to the control group, at both one week and one year after transplantation (Table 2). These cells included all cells that contained the Y chromosome and were positive for c-kit marker, suggesting stem cell in origin (Fig. 1). The increase in the proportion

of Y chromosome-containing nuclei from one week to one year was also significant in the ischemia group (Table 2). None of the patients in the control group had evidence of chimerism in the cardiomyocytes on their biopsies. The Y chromosome was detected only in lymphocytes and macrophages in this group of patients (Fig. 2). However, in the ischemia group, Y chromosome was detected in $0.29 \pm 0.19\%$ of 49,421 cardiomyocyte nuclei evaluated (Fig. 3). No 3-X chromosomes were identified in the nuclei, thus excluding the possibility of cell fusion as an explanation for the chimerism phenomenon observed. In addition to infiltrating cells, such as lymphocytes, macrophages, and fibroblasts, Y chromosome was detected also in vascular smooth muscle cells ($0.12 \pm 0.09\%$) and capillary endothelial cells ($0.08 \pm 0.07\%$) in the ischemia group (Fig. 4).

SDF-1 expression. Compared to the control group, the ischemia group had significant increased protein expression (2.8-fold; $p = 0.01$) (Figs. 5 and 6) and significant increased mRNA expression (3.3-fold; $p = 0.001$) (Fig. 7) of SDF-1, suggesting the presence of up-regulation of SDF-1 in response to ischemic injury. Interestingly, the highest level of chimerism in the myocytes ($0.56 \pm 0.53\%$) was noted in a subgroup of four patients who had greater than three-fold increased protein expression of SDF-1.

CXCR4 expression. Compared to the control group, the ischemia group had significantly increased mRNA expression of CXCR4 (3.1-fold; $p < 0.001$), suggesting the

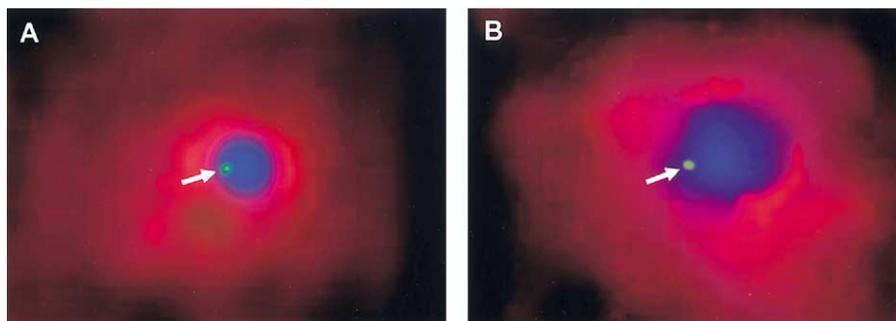


Figure 2. A heart biopsy specimen from the control group, obtained at one year after transplantation, showing immunostaining for CD45RB (A) and CD68 (B) in red. Y chromosome (arrows) is detected in green.

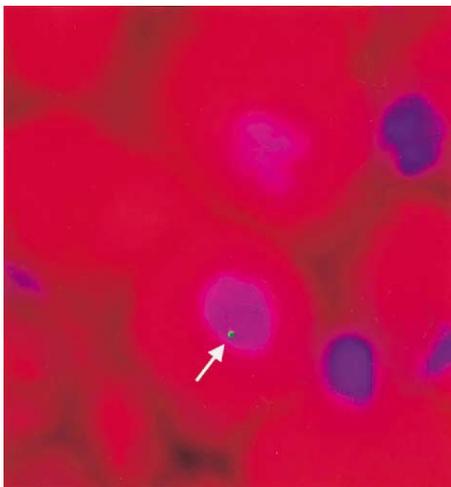


Figure 3. A heart biopsy specimen from the ischemia group, obtained at one year after transplantation, showing immunostaining for myosin heavy chain. Y chromosome is detected (arrow) in a cardiomyocyte nucleus.

presence of up-regulation of this receptor to SDF-1 in response to ischemic injury.

Vasculopathy and survival. The ischemia group had increased angiographic evidence of transplant vasculopathy (Fig. 8). At one year, three patients in the ischemia group, but none in the control group, had evidence of graft dysfunction ($p = 0.01$). The left ventricular ejection fractions were 35%, 40%, and 45%, respectively. Freedom from vasculopathy was 41% in the ischemia group compared to 95% in the control group (Fig. 8) at five years ($p < 0.001$). Further, the ischemia group experienced worse four-year survival (Fig. 8), 64% versus 85% ($p = 0.047$).

DISCUSSION

Our study has three main findings. First, ischemic injury is associated with increased chimerism. Second, ischemic injury is associated with up-regulation of SDF-1 and its receptor, CXCR4. Third, ischemic injury is associated with worse vasculopathy and survival.

Mobilization of stem cells has been described in animal

models of ischemia (1–6). Following experimental myocardial infarction, stem cells migrate to the damaged area in an attempt to promote repair and contribute to regeneration of ischemic cardiac muscle and vascular endothelium (10). These stem cells could have originated directly from the bone marrow and implanted the heart, or circulating hematopoietic cells could have homed to the heart (8,10,16). Cardiac chimerism has been reported in response to chronic rejection in the rat transplant model (17). The investigators noted that the majority of the stem cells exhibited fibroblast phenotypes. Our study reports chimerism in response to another phenomenon, an ischemia-induced myocardial injury, a well defined histopathologic pattern, observed soon after transplantation (18,19). Several factors play a role in the pathogenesis of ischemic injury, including hemodynamic status of the donor, inotropic support required by the donor, graft ischemia time, the quality of graft preservation, and myocardial damage due to reperfusion (20). As a result, injured endothelial cells could, in turn, promote atherosclerosis through a number of mechanisms including release of growth factors and cytokines, platelet adhesion, expression of adhesion molecules, and proliferation of vascular smooth muscle cells (21). The phenomenon of chimerism in transplanted human hearts has been a subject of controversy (22). Some investigators found no evidence for chimerism in the cardiac myocytes (15,23,24) or reported a low level of chimerism, an average of 0.04% of cardiomyocytes, mostly in association with regions of acute rejection (25). However, other investigators have reported a higher degree of chimerism, 0.16% (9) and 9% (8) in the cardiomyocytes. Several factors have been proposed as an explanation of this variation in results, including methodology, limitations of the measurements, timing of the observations, and the criteria used to identify chimerism (22). In our study, we evaluated patients at two specific points of time to exclude the timing factor as a confounding variable. The sensitivity and specificity of our technique was comparable to others. We also excluded patients with acute rejection during the first week to avoid this potential bias as suggested by some investigators

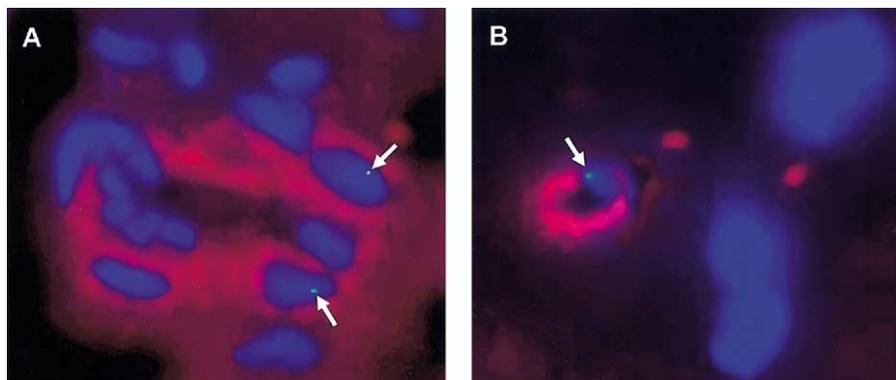


Figure 4. Heart biopsy specimens from the ischemia group, obtained at one year after transplantation, showing immunostaining for smooth muscle alpha-actin (A) and factor VIII (B). Y chromosomes (arrows) are detected in vascular smooth muscle cell nuclei in intramyocardial arteriole (A) and capillary endothelial cell (B).

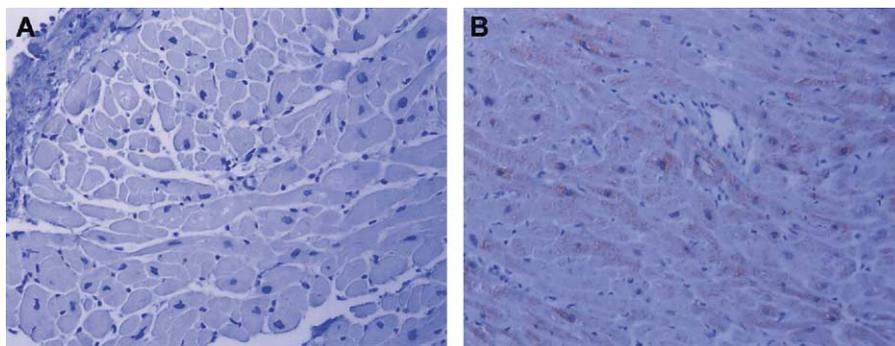


Figure 5. Stromal cell-derived factor-1 immunohistochemistry of initial heart biopsy specimens from the control (A) and ischemia (B) groups.

(22). We noted a significant increase in the Y chromosome-containing nuclei from one week to one year in the ischemia group. However, we cannot determine from our study when the peak of chimerism has occurred. It has been reported that the highest level of chimerism in myocytes, 15%, was noted between 4 and 28 days after transplantation (8). Further, it is possible that some of the stem cells could have undergone apoptosis in the interim period. Our findings of chimerism in the cardiomyocytes in patients with ischemic injury and its absence in the control group suggest that ischemia plays a key role in triggering mobilization of stem cells to the site of injury. This phenomenon may represent another factor that explains the variation in results reported among different investigators regarding speculation about the presence or absence of chimerism in the myocytes.

The increased expression of the chemokine SDF-1 and its receptor, CXCR4, is another major finding of our study. Both CXCL1 and CXCL5 are among the chemokine genes noted to be highly induced in the early phase in response to ischemia reperfusion injury in animal models of cardiac transplantation (26). In earlier studies, a dual pattern of

chemokine induction was reported in response to ischemia-reperfusion injury: an early phase of macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 production and a late phase of lymphotactin, RANTES (regulated upon activation normal T-cell expressed and secreted), and interferon-inducible protein-10 expression, which correlated with the subsequent development of cardiac allograft vasculopathy (27,28). The SDF-1 interaction with its receptor, CXCR4, is reported to play an important role in numerous biological processes including hematopoiesis, cardiogenesis, vasculogenesis, neuronal development, and immune cell trafficking (29). SDF-1 is up-regulated in hypoxic tissues (30) and may represent a physiologically relevant chemoattractant for the recruitment of circulating progenitor cells to sites of ischemia (4,31). It is expressed in myocytes (32), vascular endothelial cells (33), and fibroblasts (34).

The SDF-1 has been shown to modulate the homing of stem cells to its site by mediating chemotaxis (35). The effect of SDF-1 on stem cell homing and tissue regeneration in animal models of myocardial infarction has been recently well illustrated (5,6). The systemic overexpression of SDF-1 to induce stem cell mobilization has been described (5,36–38). In human cardiac transplantation, SDF-1 was found to play no significant role in allograft rejection (32). Our study highlights the functional role of SDF-1 in response to ischemic injury; the highest level of chimerism in myocytes was in the subgroup of patients who had a greater than three-fold increase in protein expression of this chemokine.

We have previously shown that ischemic injury is associated with increased progression of allograft coronary vasculopathy and poorer survival (7). The present study confirms similar poor clinical outcome in gender-mismatched hearts in response to ischemic injury. Immune and nonimmune factors have been implicated in the pathogenesis of cardiac allograft vasculopathy (39,40). We have previously shown up-regulation of $\alpha v\beta 3$ (vitronectin receptor) and tissue factor, activation of the matrix metalloproteinase induction system, and increased myocardial fibrosis in response to peritransplant ischemic injury (14) that may contribute to the subsequent development of allograft vasculopathy. The present study adds to these observations. In summary,

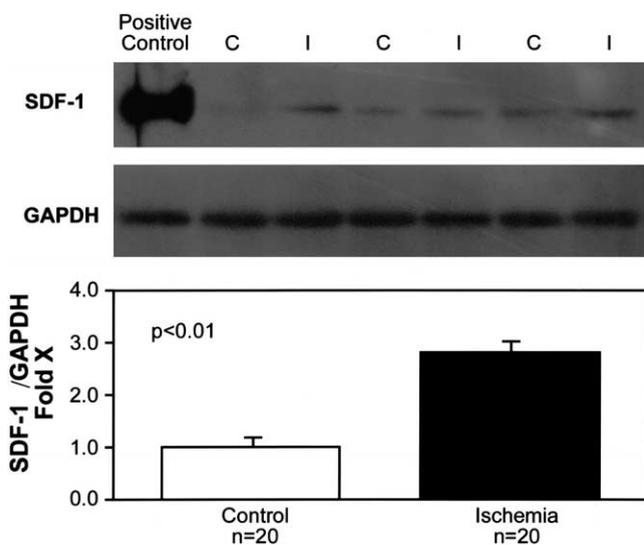


Figure 6. Stromal cell-derived factor-1 (SDF-1) immunoblotting showing increased myocardial protein expression (normalized to glyceraldehyde phospho dehydrogenase [GAPDH]) in the presence of ischemic injury (I) compared to the control group (C). Liver cirrhotic tissue was used as a positive control.

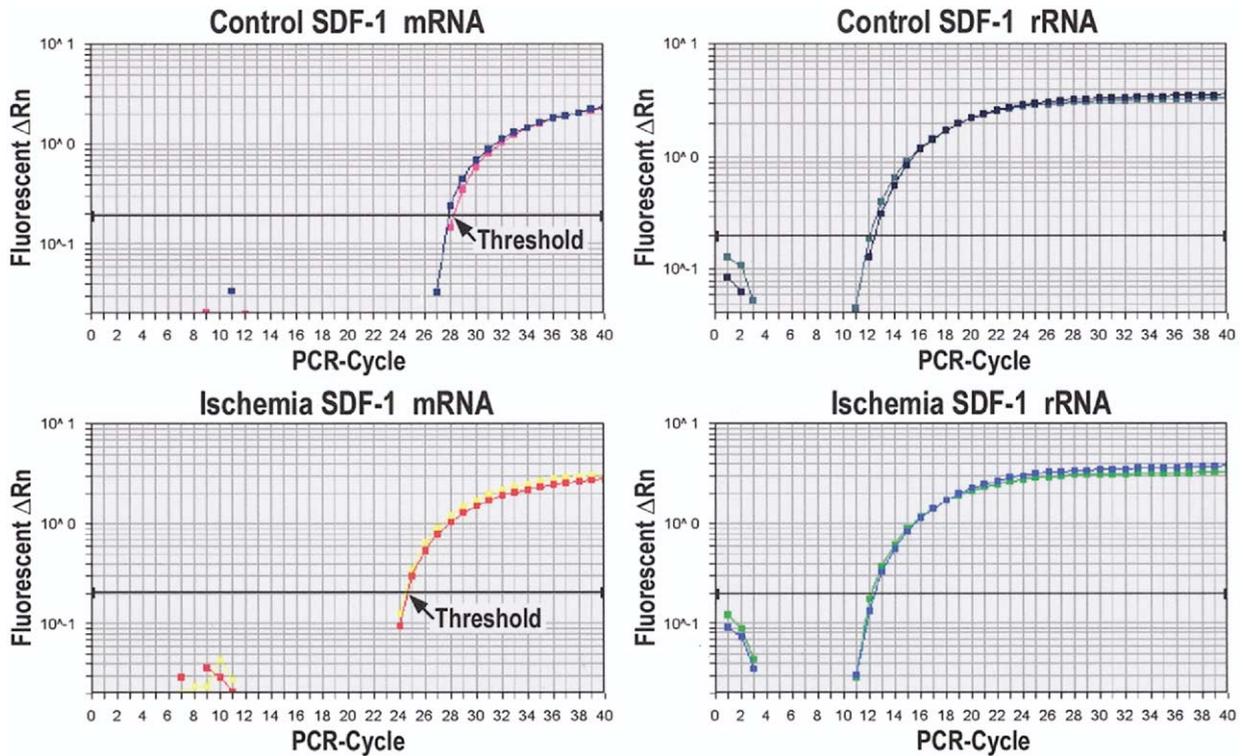


Figure 7. TaqMan polymerase chain reaction (PCR) showing messenger ribonucleic acid (mRNA) expression (left) of stromal cell-derived factor-1 (SDF-1) in the control (upper panel) and ischemia (lower panel) groups with the corresponding ribosomal ribonucleic acid (rRNA) (right) as internal control. The ischemia group demonstrates a shift of the mRNA curve to the left, indicating an earlier threshold of detection on the PCR thermal cycle and thus an increased mRNA expression of SDF-1. ΔRn = change in ribonucleic acid activity.

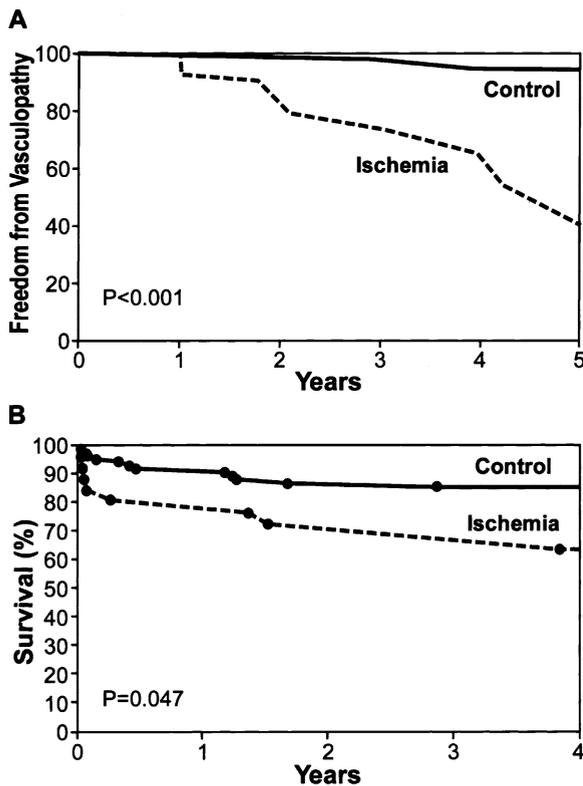


Figure 8. Kaplan-Meier curves of freedom from vasculopathy (A) and survival (B) in the control (solid line) and ischemia (dashed line) groups.

peritransplant ischemic injury induces a complex array of responses, including chimerism and increased cardiac expression of SDF-1. It is also associated with increased vasculopathy and diminished survival. Whether poor outcome is related to insufficient stem cell recruitment or related to a direct effect of stem cell mobilization can not be concluded from this study, because chimerism is not a selective process, its presence in the myocytes may intuitively suggest an attempt for a regenerative process, but its presence in the inflammatory cells and vasculature may suggest an adverse impact on allograft vasculopathy. Mechanistic studies are required to address these important issues.

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