Markers of Endothelial Function in Heart Transplant Recipients and Associations with Cardiac Allograft Vasculopathy

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Abstract

Cardiac allograft vasculopathy (CAV) is a major limitation to long-term survival in heart transplant recipients (HTR) accounting for almost 30% of deaths after 5 years. Early non-invasive detection remains a major challenge due the insensitivity and invasiveness of current diagnostic tests. Small vessel disease and endothelial dysfunction are key players in the pathophysiology of CAV. We hypothesize that in HTR there is an impairment of endothelial cellular repair and changes in arterial elasticity, especially in small arteries, resulting in a reduction of small artery elasticity (SAE), an increase in the number of circulating endothelial cells (CEC), and increased CEC activation. In addition, these changes are significant in HTR who develop CAV.

Methods: Ninety-seven HTR and 22 normal controls were included in this study. SAE was measured from the radial artery. CEC (CD146+ cells) were enumerated and assessed for activation based on VCAM expression. Continuous variables were analyzed using t-test and dichotomous variables using Chi-square. Logistic regression using stepwise selection was performed to evaluate determinants of CEC, CEC activation, and SAE.

Results: The median age was 61years(range, 18-76). The mean duration of transplant was 5.4 ± 5.3 year. 77 % were male and 57% had CAV. HT was associated with significantly lower SAE ($p<0.0001$) and increased CEC activation ($p=0.0004$) when compared to healthy controls. We also found that CAV was significantly associated with SAE and CEC ($p = 0.04$ and 0.01, respectively). On stepwise regression, hypertension treatment and duration of transplant were associated with CAV.

Conclusion: Heart transplant is characterized by endothelial activation and dysfunction as evidenced by a reduction in SAE and increased CEC activation. Prospective studies to evaluate these markers as predictors of risk are needed for further evaluation.

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INTRODUCTION

Heart transplantation is now the definitive cure for end-stage heart failure. Since the first heart transplant was performed in 1967, there have been great strides in the management of heart transplant recipients (HTR), immunosuppressive therapy, monitoring techniques, and surgical strategies. As a result, the median survival of heart transplant recipients has improved significantly to approximately 10-13 years, primarily due to an improvement in short-term survival. Long-term survival, however, remains poor. Cardiac allograft vasculopathy (CAV), a form of coronary artery disease affecting heart transplant recipients, is a major limitation to long-term success in cardiac transplantation. Despite preventive measures such as statin therapy and risk factor modification, CAV remains a leading cause of graft failure and death, accounting for up to 30% of deaths after 5 years. CAV is clinically apparent in 50% of HTR at 5 years, however intimal thickening, a predictor of CAV, develops earlier and is present in 58% of HTR one year after transplant. [1, 2] Ten percent of patients die within the first 12 months after the diagnosis of CAV. [3, 4]

BACKGROUND

Cardiac allograft vasculopathy and endothelial function

The initial event in cardiac allograft vasculopathy is theorized to be a subclinical endothelial cell injury in the graft. This injury causes upregulation of cytokines, complement, adhesion molecules, and growth factors, creating a state of

inflammation and endothelial activation, ultimately resulting in endothelial dysregulation. Cellular and humoral responses to human leukocyte antigens (HLA) and vascular endothelial cell antigens propagate this process which ultimately results in intimal proliferation and development of the vascular lesion associated with CAV.[5,6] In short, endothelial dysfunction precedes the development of CAV. This has been demonstrated clinically by Davis and colleagues who showed that early abnormal coronary responses to acetylcholine predicted the development of intimal thickening at one year. [7]

Risk Factors for CAV

The development of CAV is variable after transplant and is determined by multiple factors affecting the transplant recipient, such as immunologic activation, comorbid conditions, and exposure to cytomegalovirus, as well as "classical" risk factors for coronary artery disease, such as hypertension and hyperlipidemia.

Immunologic risk factors

Ischemia/reperfusion injury occurring during removal of the donor heart, during storage, and after engraftment in the recipient, induces an immunologic response which has been shown to cause CAV in an experimental model. [8] Brain death is associated with hemodynamic instability, altered loading conditions, decreased coronary perfusion, and apoptosis, resulting in immune activation and elaboration of inflammatory cytokines. In an animal model, reduced coronary blood flow and

abnormal coronary vasomotor response to acetylcholine was demonstrated after brain death. Mehra and colleagues showed that heart transplant recipients who received hearts from donors who died of explosive or traumatic brain death had significantly more intimal thickening and reduced survival. [9] [10, 11] CAV has been linked with the duration and number of episodes of cellular rejection as well as asymptomatic humoral rejection. [12-14] [11, 15, 16] Donor age greater than 35 years and transplant of a female donor into a male recipient are associated with increased intimal thickening on intravascular ultrasound.[12, 17, 18] Finally, cytomegalovirus infection, and subsequent eNOS dysregulation, is associated with abnormal coronary endothelial function and reduction in survival from CAV. [19-21,22]

Classical Risk Factors

Classical cardiovascular risk factors, such as smoking, diabetes, hypertension, obesity, and dyslipidemia are not uncommon in heart transplant recipients; Ischemic cardiomyopathy is the reason for transplant in approximately 40% of recipients. According to data from the International Society of Heart and Lung Transplant Registry (2009), 76% of transplant recipients have hypertension at year one, 27% have diabetes, and 79% have hyperlipidemia. At 10 years, 98% have hypertension, 37% have diabetes, and 93% have hyperlidemia. These pre-existing conditions can be exacerbated by immunosuppressants, particularly steroids and calcineurin inhibitors, however 28% of heart transplant recipients

develop new hypertension, 49% new hyperlipidemia, and 20% new diabetes. These traditional risk factors can increase the risk of CAV. [23, 24]

Challenges in the diagnosis of CAV

Early detection of CAV is limited due to the lack of sensitive diagnostic studies, variable and silent clinical presentation, and the lack of consistent nomenclature that imparts prognostic information. Coronary angiogram is used routinely to screen for CAV, but it is insensitive and does not detect disease in up 50% of HTR. [25-28,2] Furthermore, coronary angiogram is invasive and repeatedly exposes HTR, already at elevated risk for renal dysfunction due to immunosuppressant therapy and co-morbidities, to potentially nephrotoxic contrast. While it has not yet been demonstrated that early detection will improve outcomes, CAV often presents silently as sudden death or advanced disease complicated by heart failure. There is a pressing need for a sensitive, noninvasive screening study to detect patients at risk for cardiac allograft vasculopathy.

Noninvasive Assessment of Endothelial Function

A variety of techniques can be used to evaluate endothelial function, including invasive coronary studies and noninvasive measurement of peripheral artery function. Flow-mediated dilatation of the brachial artery measured after hyperemia has been considered the gold standard for noninvasive assessment of endothelial function. It is predictive of cardiovascular risk in asymptomatic populations and is a predictor of the presence of cardiovascular disease.[29, 30]

More recently, small artery elasticity (SAE) as measured via the radial artery, has been demonstrated to be a prognostic marker of endothelial function.[31] Diastolic pulse wave contour analysis provides information regarding elasticity of the small and medium arteries and is a surrogate for endothelial function and cardiac risk. Patients with coronary artery disease, hypertension, and diabetes, demonstrate a reduction in the oscillatory component of the diastolic waveform, reflecting capacitance abnormalities in the distal vessels, or reduced SAE. This reduction in SAE has also been detected in asymptomatic individuals at risk for cardiovascular disease and is associated with cardiac events. [32]

There is overwhelming evidence that endothelial dysfunction precedes CAV, however treatment strategies for CAV do not include screening for evidence of endothelial dysfunction, and noninvasive studies have not been assessed widely in this population. The question arises whether noninvasive measurements such as SAE can be expanded to heart transplant recipients to evaluate risk for CAV and to aid in the development of effective therapies.

Endothelial Cells and Cardiovascular Disease

Circulating endothelial cells and endothelial progenitor cells are in simplest terms, the repair cells of the human vasculature. When an artery is injured, mature endothelial cells (CEC) are shed from the endothelium, which is re-endothelialized by endothelial progenitor cells (EPC) that are released from the

bone marrow.(figure 1) These processes are mediated by a complex interaction of cytokines, adhesion molecules, growth factors, endothelial nitric oxide synthase (eNOS) and various signaling pathways, eg. PI3K/Akt. [33] [34] In addition to endogenous mediators, estrogen, statins and epogen increase mobilization of EPC. [35] [36] [34] Both EPC and CEC have been shown to be predictive of cardiovascular events, coronary artery disease, severity of coronary artery disease, and correlate with risk factors for coronary artery disease.[37, 38] [39] [40-43] Patients with CAD or at risk for CAD demonstrate **decreased EPC** and migratory activity of EPC and **increased CEC**.

In cardiac transplant, the endothelial layer is the first biological interface encountered by the recipient's immune system. [44-46] Endothelial cell apoptosis has been demonstrated in early CAV and may be related to early immunologic injury. [47] The relationship between endothelial cells and heart transplant outcomes has been less well characterized. In a study of EPC (CD 31+) and CEC outgrowth colonies, Simper and colleagues showed that EPC were significantly reduced in HTR with CAV but there was no difference in CEC between HTR with CAV and those without and between normal and HTR. [45] When EPC counts were determined using flow cytometry and seven phenotypes, no difference was found between EPC in patients with CAV and those without. [48]

In summary, transplant is characterized by multiple potential mediatiors of endothelial injury, which may contribute to the development of CAV. These complex interactions complicate early detection and the development of effective strategies. We have chosen to target endothelial function, the final common pathway of the various insults, as a potential marker of outcome, specifically CAV. The current study seeks to evaluate CEC, CEC activation, and SAE in HTR and to determine their relationships with CAV.

SPECIFIC AIMS AND HYPOTHESES

Specific Aim 1: To evaluate novel markers of endothelial function, SAE and CEC, in a heart transplant population.

Specific Aim 2: To determine the association between SAE, CEC and CAV, defined as greater than 25% stenosis in a major coronary artery on angiogram.

The ultimate goal of this project is to identify markers that can be utilized to noninvasively screen for CAV and to use as a therapeutic target. In this study, we tested the following hypotheses:

1. Heart transplantation is characterized by impaired endothelial function when compared to normal subjects
2. CAV is associated with a reduction in SAE and increased CEC when compared to HTR without CAV.

METHODS

Design

This is a cross-sectional design that compares SAE and CEC in HTR and healthy normal subjects and in patients with and without CAV. The outcome variables include CEC count, percent VCAM-1 positive cells (CEC activation), SAE, and CAV defined as angiographic evidence of greater than 25% stenosis in an epicardial artery. The data used in this study is a combination of data from two studies: the first study was designed to characterize endothelial function in heart transplant recipients, and data was collected prospectively as patients presented to clinic. The second study was a cross-sectional design to compare endothelial markers between patients with and without CAV and to normal patients. Patients in this study were matched based on age, gender, and transplant duration.

Participants

Heart transplant recipients undergoing routine annual evaluations at the University of Minnesota Medical Center were invited to participate in this study. Patients were eligible if they were age 18 years or older and within at least one year of transplant. Patients were excluded if they were re-transplant recipients, received multiple organs, had an active infection, were experiencing acute rejection (grade 3a or greater), or had chronic kidney disease stage 4, defined as GFR< 30ml/min/1.73m^2 or acute renal failure. Healthy adults, age 18 and greater, were also recruited from the University of Minnesota, Twin Cities campus, specifically, the clinic areas and the campus center. Subjects were

excluded for: Current tobacco use, history of chronic kidney disease, active infection, history of organ transplantation, diabetes mellitus, uncontrolled hypertension, known coronary artery disease, connective tissue disorder, and history of hyperlipidemia.

The study protocol was approved by the University of Minnesota institutional review board, and informed consent was obtained from all participants. From April 2008 to September 2010, 97 HTR and 22 healthy normal subjects were enrolled.

Coronary angiography

At our institution, surveillance coronary angiogram is performed at 6 weeks and yearly until the 4th transplant year, after which they are performed every other year. Coronary angiograms were read by two blinded interventional cardiologists. Cardiac allograft vasculopathy was defined as the presence of a lesion of 25% or greater in an epicardial vessel on angiogram.

Circulating Endothelial Cell (CEC) Isolation, quantitation, and phenotyping

Blood samples for CEC studies were collected from 63 patients in vacutainer tubes containing EDTA (BD Vacutainer Systems, Franklin Lakes , NJ) and were processed within 1 hour after collection. The procedure was performed as previously reported in detail.[49]. Briefly, for CEC quantitation we applied immunochemical staining of buffy coat smears prepared from 1ml of whole blood

by centrifugation on Histopaque 1077 (Sigma). Monoclonal endothelial-specific anti-CD146 antibody P1H12 and secondary anti-mouse alkaline phosphatase-labeled antibody (Jackson IRL, Westgrove, PA) with visualization using Fast Red substrate (Vector, Burlingame, CA) were used for staining the smears. P1H12-positive cells were counted under a light microscope and results were expressed as a number of CEC per 1 cc of peripheral whole blood.

For CEC characterization, we used a second method of CEC isolation applying goat anti-mouse immunomagnetic beads (Invitrogen Dynal, Oslo, Norway) coated with P1H12 antibody. After 40 min at 4^{o}C of incubation of whole blood with beads, beads with attached CEC were isolated in a magnetic separator and placed on a slide using a cytospin centrifuge. Slides were fixed with 4% paraformaldehyde for 10 minutes and double-stained with P1H12 antibody and rabbit polyclonal anti-VCAM-1 antibody (Santa Cruz, Biothechnology, Santa Cruz, CA) with appropriate anti-mouse FITC and anti-rabbit TRITC-conjugated antibodies (both from Jackson IRL, Westgrove, PA).

The results were expressed as the percentage of VCAM-1 positive (activated) CEC among the total population of CEC.

Small Artery Elasticity

Small Artery Elasticity measurements using the CR 2000 CV Profilor (HDI, Eagan, MN).

Small artery elasticity was measured using the HDI/Pulsewave™ CR-2000 Research CardioVascular Profiling System (HDI, Inc., Eagan, MN). Three measurements were recorded in all subjects. Tracings were reviewed by the principal investigator and co-investigator for accuracy. Inaccurate waveforms were excluded. The mean SAE was used for analysis.

Statistical Analysis

Demographics and transplant characteristics were summarized as means and standard deviations, medians and ranges or percents, as appropriate. Unadjusted comparisons of variables between groups were performed using t-tests for means, Wilcoxon rank test for medians, and chi-square tests for proportions. In performing t-tests, the Satterthwaite correction for the p-value was used when there was significant evidence of unequal variances in the comparison groups.

Within transplant recipients, stepwise selection was used to identify associations with circulating endothelial cells, percent VCAM and small artery elasticity using linear regression. Likewise, stepwise selection was used to identify risk factors for CAV using logistic regression. The p-value threshold for admitting a covariate to the model was 0.15, while the p-value for retaining a covariate in the model was 0.10.

Potential covariates for selection in the stepwise models included demographics (sex, age, body mass index), medical history (treatment for hypertension or hyperlipidemia, diabetes, history of ischemic cardiomyopathy), laboratory values (blood pressure, lipid panel, glucose, serum creatinine, BUN), and transplant-specific variables such as duration of transplant, history of rejection, history of cytomegalovirus infection and presence of CAV. Low-density lipoprotein (LDL) was calculated from high-density lipoprotein (HDL) and triglycerides in one participant to avoid a missing value.

Since the HTR group is combined from two studies, all variables were not present in all of the HTR. CEC were not collected in the HTR from the matched study using the same method as the initial study, so these results were not used in this group of patients. Due to a learning curve, SAE was not available in some of the initial participants. As a result, there was variation of the sample size between analyses to accommodate variables of interest. Measurements of CEC1cc and VCAM percentage were available in 63 HTR and measurements of small artery elasticity were available in 71 HTR. All three variables were available in 37 HTR.

Because the stepwise selection algorithm requires each observation to have complete data on the entire list of the pool of potential covariates, selection models for each outcome were fit with and without important covariates that had

high levels of missing data. For example, the presence of CAV was available in 94 of 97 HTR.

For this reason, logistic regression models for CAV were fit twice, once with CEC1cc and VCAM% included in the selection pool and once without. Since mean SAE was included in the pool of covariates for the selection process, the two models used a maximum sample size of 37 and 71 participants, respectively. In all cases, the final model resulting from a model selection process was then refit in the largest subset of participants having complete data for the selected covariates. This sometimes resulted in an increase in p-value for some selected covariates in the model.

The complete list of covariates in the model selection process included: age, female, body mass index, duration of transplant, history of ischemic cardiomyopathy, diabetes, history of rejection, rejection count, cytomegalovirus infection history, blood pressure (systolic and diastolic blood pressure), total cholesterol, LDL, HDL , triglycerides, triglyceride to HDL ratio, glucose, serum creatinine, glomerular filtration rate, BUN, medication use use (tacrolimus, cyclosporine, myophenolate mofetil, sirolimus, prednisone, azathioprine, statin, antihypertensives). In certain models, presence of CAV, mean SAE and mean LAE, square root (CEC/1cc) and VCAM % were also included.

RESULTS

Demographic information is displayed in table 1. To summarize, this was mostly a male population, consistent with other transplant studies and registries, the median age was 61 years at the time of enrollment, the median duration of transplant was 3 years, and 57% had cardiac allograft vasculopathy by angiogram. Fifty-six percent of patients were transplanted for nonischemic cardiomyopathy. Eighty-five percent of patients were on statins and the mean LDL was 86 mg/dL. The mean HBA_1C was 6.38%. Sixty-nine percent were taking therapy for hypertension.

Unadjusted comparisons were made between HTR and controls (table 3). There were more women in the control group (p=0.02), the BMI was lower (p=0.0003), and there was a nonsignificant trend towards younger participants (p=0.06). There were no significant differences in age, gender, BMI or SBP between patients with CAV and those without. (table 4) On average, the patients with CAV were transplanted longer (p=0.000).

Our current immunosuppressant protocol includes prednisone, a calcineurin inhibitor(CNI), either tacrolimus or cyclosporine, and mycophenolate mofetil. Some patients who develop CNI renal toxicity or CAV receive sirolimus as a substitute for CNI or mycophenolate mofetil. After 6 months, most patients are weaned off prednisone. Immunosuppressant therapies are listed in table 2.

Circulating Endothelial Cells Enumeration and Activation

In HTR, the mean CEC count was 0.7cells/1cc ± 1.72. The mean VCAM% was 67.5%. There was tendency towards lower CEC count in HTR($p=0.046$), while there was a significantly higher degree of CEC activation($p=0.0004$). Both the transplant and controls had normal CEC counts. (table 3) There was no difference in CEC count and activation between patients with CAV and those without. (table 4)

Small Artery Elasticity

The mean SAE was 4.43±2 ml/mmHgx100. The HTR demonstrated a significant reduction in SAE when compared to the controls. ($p<0.0001$). (Table 1) A stratified analysis was performed based on age less than or greater than 55 years and gender. Results are shown in tables 5a and 5b. SAE was significantly reduced in all groups when compared to the normal controls except for women 55 years and older. A similar comparison was performed in HTR with and without CAV. When stratified based on gender and age, there was no significant difference is SAE between HTR with CAV and those without CAV. (table 6a and 6b)

Associations with Endothelial Markers and CAV

Stepwise regression was performed to evaluate associations with CEC, CEC activation, SAE, and CAV in HTR. Serum creatinine, BUN, female gender, and presence of CAV were significantly associated with CEC. Increasing BUN, and presence of CAV were associated with a reduction in CEC while increasing serum creatinine and female gender were associated with an increase in CEC. (table 7) Increases in square root of CEC, age, and presence of CAV were found to be significantly associated with a reduction in SAE, and transplant duration was found to be positively associated with SAE. (table 8) Only prednisone and cyclosporine were found to be associated with CEC activation (VCAM percent). Cyclosporine was significantly associated with increased activation ($p=0.02$) while prednisone was associated with reduced activation ($p=0.05$).

Stepwise regression was performed using 2 models to evaluate associations with CAV. No endothelial markers were found to be associated with CAV. In the model that included all endothelial markers, i.e., SAE, CEC, and VCAM%, only anti-hypertensive therapy were associated with CAV($p=0.015$). (table 8) In the model that included only SAE, transplant duration and anti-hypertensive therapy were associated with CAV, $p =0.008$ and 0.005, respectively.

In summary, we found that SAE was reduced in HTR when compared to healthy controls. CEC count was normal but reduced in HTR, and CEC activation was significantly elevated in the HTR. On stepwise analysis, the presence of CAV resulted in a reduction in SAE and a reduction in CEC, however CAV was not

associated with CEC activation. Neither SAE, CEC, nor VCAM percent were associated with CAV.

DISCUSSION

In this study, we have demonstrated that HT is associated with a reduction in SAE and CEC and an increase in CEC activation. In stepwise regression, CAV was significantly associated with a reduction in SAE. Not surprisingly, age and CEC were also associated with reductions in SAE. Functional and structural changes in the arterial wall precede the development of atherosclerosis and associated events. Decreased elasticity of the resistance vessels manifest as alterations of the pulse wave contour in late systole and may augment pressure in late systole.[32] As vascular tone is modulated in part by nitric oxide produced by an intact endothelium, these alterations in vascular tone can be considered evidence of endothelial dysfunction. SAE can be measured noninvasively using tonometry of the radial artery. Abnormal SAE is manifested as pressure augmentation in late systole. Small artery elasticity has been demonstrated to be a marker of endothelial function and is associated with atherosclerotic risk. In an asymptomatic population of 6523 women and men of various ethnicities without overt cardiac disease, SAE added prognostic information for cardiovascular events beyond blood pressure, BMI, tobacco use, diabetes, cholesterol, and triglycerides.[50-52]

Endothelial dysfunction is present after heart transplantation due to a variety of insults. The presence of endothelial dysfunction after heart transplantation has been documented by a variety of techniques. While Pierce and colleagues demonstrated that persistent endothelial dysfunction, as measured by aortic augmentation index, was associated with etiology of heart failure and improved after transplantation, Holm demonstrated persistent peripheral endothelial dysfunction, evaluated by skin laser-Doppler perfusion and presence of proinflammatory cytokines, up to thirteen years after transplant. [53, 54] We have demonstrated persistent reduction in small artery elasticity up to 23 years after transplantation. While in the general population, aging may affect the development of arterial stiffness, the presence of reduction in SAE despite age and gender suggests that factors associated with the transplanted state, such as immunosuppressant therapy and endothelial injury at the time of transplant, are also contributory. The unexpected relationship between duration of transplant and SAE suggests that increasing duration of transplant is associated with better endothelial function. There are several potential explanations. There may be hemodynamic stabilization and improvement in endothelial function after transplant, which has been described. An interaction between age and transplant duration may be present, but was not assessed in this study. Finally, and more likely, the patients who have survived transplant longer have a better

risk profile and prognosis; thus, it is possible that those who have a better SAE live longer, although this conclusion cannot be drawn from these analyses.

CECs are rare in the blood of healthy individuals, but increased numbers are found in a number of pathologic states such as inflammatory, immune, and cardiovascular diseases and appear to be evidence of vascular damage. In normal health, VCAM-1 expression is found in only a small percentage of CECs, In certain states, such as sickle cell, VCAM-1 expression is increased, suggesting systemic vascular activation and inflammation.[55]

In our series of heart transplant recipients, we did not find an increase in the CEC count as expected, but there was a substantial level of cellular activation as evidenced by most patients having VCAM positive cells. In this series, transplant recipients had lower CEC than healthy normals, though both groups had normal levels. CAV was also found to be associated with a reduction in CEC. CAV is felt to be a form of chronic rejection, and anti-endothelial antibodies have been implicated in antibody-mediated rejection and the development of CAV.[56] Interactions with immunosuppressive therapy are also a consideration. Finally, there is data suggesting that CEC shedding is associated with apoptosis. [57] The current findings raise the question of whether there is less shedding of CEC or enhanced apoptosis and clearance of CEC in HT and CAV, however this is not supported by the literature. A greater degree of apoptosis has been seen in

normal subjects when compared with sickle cell patients suggesting that a state in which the endothelium is activated does not necessarily result in more apoptosis. [55]

Female gender was found to be associated with an increase in CEC in HTR. The mean age of women in this group was 57 years. Sixty-four percent were over the age of 50, suggesting that most of the women were post-menopausal. Estrogen has long been known to be cardioprotective. Estradiol is an activator of eNOS and has been shown to accelerate re-endothelialization and to attenuate medial thickening after carotid artery injury in mice by enhancing mobilization and proliferation of bone-marrow derived EPCs. [35] Although this study assesses mature CEC that are increased during vascular injury, we are indirectly assessing the balance of the repair process. Thus, the effect of female gender on CEC may be partly due to estrogen withdrawal and loss of the protective effects on the endothelium.

We did not find significant associations of CEC activation on stepwise regression. Prednisone appeared to reduce CEC activation while cyclosporine increased activation. While these findings were statistically significantly, caution should be used in the interpretation due to the extremely small numbers of patients on these therapies.

Finally, the only significant associations with CAV in this study were the use of anti-hypertensive therapy and transplant duration. The association of anti-hypertensive therapy with CAV is confounded by the fact that these therapies may have been started to manage the hemodynamic effects of CAV. It is notable that transplant duration was associated with both the presence of CAV and increased SAE. In this study, we did not quantify the severity of CAV or evaluate the type of vascular remodeling present, which may have been useful in interpreting these findings. The lack of other associations, specifically endothelial markers, may be a function of the small sample size as well as the large number of variables that were studied. Employing a more stringent definition of CAV, as has been proposed by the International Society of Heart and Lung Transplant, may have resulted in determination of prognostically significant markers.

LIMITATIONS

This study was a cross-sectional design to characterize markers of endothelial function in a heart transplant population. While we were able to use regression models to evaluate for associations, prospective studies are needed to assess predictive relationships between markers and outcome.

To evaluate for cardiac allograft vasculopathy, we used coronary angiogram which is currently the clinical gold standard. Coronary angiogram is not as

sensitive as intravascular ultrasound. This lack of sensitivity could have led to an underestimation of the degree of CAV and misclassification. Recognizing that the presence of CAV by angiogram is often under recognized, and that mild angiographic disease can be associated with significant disease on IVUS as well as hemodynamic changes, we included in our definition mild, diffuse CAV. By changing the definition of CAV to include more severe angiographic disease, we may improve our ability to find prognostic markers.

The transplant population in this study was a combination of participants from two studies of endothelial function. One population was collected prospectively and randomly, and the second population was part of a study that was matched based on gender, age, and duration of transplant. Including the group that was selected based on pre-specified parameters may have introduced selection bias. Furthermore, CEC were not measured in the matched group using the same methodology, and those results were not part of the models, thereby reducing the sample size for the models.

Finally, the sample size was a limitation in this study. Although 97 heart transplant recipients is a substantial population for a heart transplant study, data sets were not complete in all patients, resulting in reductions in sample size for the regression models. This was further complicated by the large number of variables studied and the complex and heterogeneous mechanisms underlying

CAV. We did find that CAV was associated with a reduction in SAE, but SAE was not associated with CAV when CAV was the outcome variable. These apparent discrepancies in associations are likely due to changes in sample size and in numbers of variables during the stepwise modeling.

Future studies should be designed as longitudinal trials which focus on evaluating changes in SAE as a predictor of outcomes in heart transplant recipients. Consideration should be given to limiting analyses of mechanistic variables in these studies, as the potential mechanisms for CAV are numerous. Focusing on multiple mechanistic variables may prevent finding meaningful markers of outcomes.

CONCLUSION

In this study, we demonstrated that heart transplantation is associated with reduced small artery elasticity, reduced circulating endothelial cells, and chronic endothelial cell activation. Furthermore, we have ascertained that the presence of CAV is associated with a reduction in SAE after adjustment for other factors. We believe that SAE can be used to noninvasively evaluate for and to predict CAV, although this was not seen in our analysis, possibly due to a small sample size. Identifying disease and risk of disease early may ultimately guide therapy and improve outcomes after heart transplant. Longitudinal and adequately powered studies are needed to determine the predictive value of SAE.

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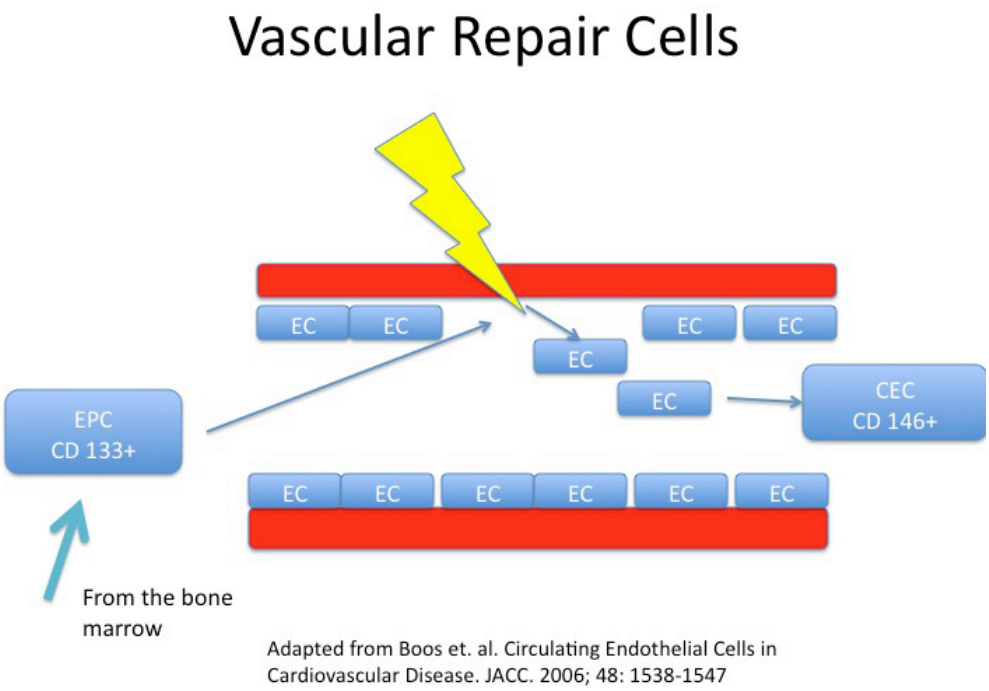
Figures

Figure 1 Interaction of Circulating Endothelial Cells and Endothelial Progenitor Cells in Vascular Repair



Tables

Table 1 Demographics of Heart Transplant Recipients

Demographics of Heart Transplant Recipients		
Variable	**N=97**	**Result**
Age (Median, years, range)		61, [18,76]
Gender (% Female)		23
Duration of Transplant (Median, years, range)		3, [1,23]
CAV Present (%)*		57
Mean SAE (Mean ml/mmHg x 100 ± SD)		4.43±2.02
History of Ischemic CM (%)		44
Statin Therapy (%)		85
LDL (Mean mg/dL± SD)		86.2±28.9
Anti-Hypertensive Therapy (%)		69
* n=94 ** n=71		

Table 2 Distribution of Immunosuppressant Therapy

Drug	N=97	All Transplants	CAV n	NonCAV
Azathioprine		2 (2%)	2	0
Cyclosporine		5 (5%)	4	1
Mycophenolate mofetil		73 (78%)	38	35
Prednisone		5 (5%)	4	1
Sirolimus		27 (29%)	20	7
Tacrolimus		74 (79%)	38	36

Table 3 Unadjusted Comparisons between Heart Transplant Recipients and Controls

Variable	Transplant (N=97)		Healthy (N=21)		P-Value
	Value (mean)	SD	Value (mean)	SD	
Age (years)	58	13	48	18	0.0631
Gender (% Female)	23		48%		0.0198
BMI ($lb/in^{2)}$	28.7	5.2	24.2	4.5	0.0003
Systolic Blood Pressure (mmHg)	123	34	122	11	0.82
VCAM%	67.5	41	42.3	19	0.0004
CEC count (per 1cc)	0.69	1.7	9.8	19	0.046
Square Root CEC	0.43	0.7	2.04	2.4	0.0083
SAE (ml/mmHgx100)	4.43	2.02	7.22	1.99	<0.0001

Table 4 Unadjusted Comparison Between Patients with and without CAV

Variable	***CAV (n=54)***		***No CAV (n=40)***		***p***
	Value (Mean)	*SD*	*Value (Mean)*	*SD*	
Age (years)	59.4	1.6	56.5	2	0.28
Transplant Duration (years)	6.5	5.8	3.2	2.9	0.000
Gender (% Female)	28	---	19	---	0.3
BMI (lb/in^2)	28.8	5.4	28.6	5.1	0.9
Systolic Blood Pressure (mmHg)	126	35.9	118.9	32	0.3
VCAM %	68	44	65	39	0.79
CEC (per 1cc)	0.68	2	0.78	1.2	0.83
SAE (ml/mmHgx100)	4.39	1.6	4.47	2.4	0.87

Table 5a Stratified Comparison of Small Artery Elasticity in Males

	Males < 55 Transplant (N=14)		Normal (N=5)		P	Males ≥ 55 Transplant (N=44)		Normal (N=5)		P
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
SAE (ml/mmHgx100)	5.5	2.4	9.1	1.7	0.008	4.4	1.9	7.5	1.0	0.032

Table 5b Stratified Comparison of Small Artery Elasticity in Females

	Females < 55 Transplant (N=5)		Normal (N=7)		P	Females ≥ 55 Transplant (N=8)		Normal (N=3)		P
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
SAE (ml/mmHgx100)	4.4	1.4	7.1	1.2	0.044	2.9	0.6	6.0	2.3	*

***** The variances are unequal between groups (p=0.0056), hence the Satterthwaite p-value of 0.14 is more appropriate for the t-test. Without correction for unequal variances, the p-value is 0.0041. This may be due to an extreme value among the normal females over 55. The sample sizes here are small.

Table 6a Stratified Analysis of Arterial Elasticity in Males with and without CAV

	Males < 55 CAV (N=8)		No CAV (N=5)		P	Males ≥ 55 CAV (N=23)		No CAV (N=20)		P
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
SAE (ml/mmHgx100)	5.31	1.36	6.56	3.3	0.8	4.13	1.5	4.4	22	0.6

Table 6b Stratified Analysis of Arterial Elasticity in Females with and without CAV

	Females < 55 CAV (N=3)		No CAV (N=2)		P	Females ≥ 55 CAV (N=2)		No CAV (N=4)		P
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
SAE (ml/mmHgx100)	5.3	2.3	3.02	0.5	0.27	2.9	0.73	2.8	0.67	0.8

Table 7 Stepwise Regressions—Associations with Circulating Endothelial Cells in Heart Transplant Recipients

Associations with CEC (N=34)			
Variable	Regression Coefficient	P-value	Confidence Interval
Intercept	1.54	0.0153	[0.32,2.76]
Mean LAE	-0.06	0.0033	[-0.10,-0.02]
Serum Creatinine	0.83	0.0022	[0.33,1.34]
BUN	-0.04	0.0039	[-0.06,-0.01]
Female	0.77	0.0007	[0.35,1.18]
CAV Present	-0.41	0.0103	[-0.71,-0.10]
FK use	-0.28	0.1108	[-0.62,0.07]

Table 8 Stepwise Regression—Associations with Small Artery Elasticity in Heart Transplant Recipients

Associations with Small Artery Elasticity (N=37)			
Variable	Regression Coefficient	P-value	Confidence Interval
Intercept	10.3	<0.0001	[7.3,13.4]
Sqrt CEC1cc	-1.63	0.0008	[-2.5,-0.7]
Age (years)	-0.07	0.0013	[-0.11,-0.03]
Duration of Transplant (years)	0.11	0.0179	[0.02,0.21]
CAV Present	-1.17	0.0426	[-2.29,-0.041]
Glucose	-0.008	0.1370	[-0.018, 0.003]

Table 9 Stepwise Regression—Associations with Cardiac Allograft Vasculopathy

Associations with CAV (N=60)			
Variable	Odds Ratio	P-value	Confidence Interval
Antihypertension Treatment	4.4	0.0146	[1.3,14.3]
CEC1cc (square-root transformed)	0.60	0.1899	[0.275,1.292]
Female	0.83	0.7695	[0.229, 2.997]
Total Cholesterol	1.01	0.290	[0.992,1.026]