

VASCULAR TISSUE PLASMINOGEN ACTIVATOR AND THE DEVELOPMENT OF CORONARY ARTERY DISEASE IN HEART-TRANSPLANT RECIPIENTS

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Abstract Background. An aggressive and potentially fatal form of coronary artery disease may develop after cardiac transplantation. We studied the role of vascular tissue plasminogen activator (t-PA), the primary mediator of fibrinolysis, in the development of this problem.

Methods. We studied 78 consecutive recipients of cardiac allografts over a five-year period, and we collected follow-up data over a mean (\pm SE) of 32.5 ± 2.0 months. The patients were studied with ventricular function tests, serial endomyocardial biopsies (16.6 ± 0.5 per patient), and annual coronary angiography. Measurements of t-PA and its inhibitor were performed immunocytochemically on unfixed cryostat sections of endomyocardial-biopsy specimens with the use of monoclonal antibodies to t-PA and its inhibitor.

Results. In biopsy specimens obtained during the first three months of follow-up, 38 allografts had a normal distribution of t-PA in arteriolar smooth-muscle cells, whereas in 40 allografts there was depletion of t-PA that persisted in subsequent follow-up. Coronary artery disease

developed during follow-up in 31 of 40 allografts (78 percent) with depletion of t-PA, but the disease developed in only 9 of the 38 allografts (24 percent) with normal t-PA levels ($P < 0.001$). Allografts with depletion of t-PA also had the t-PA inhibitor and were at greater risk for earlier and more severe disease than were allografts with normal arteriolar t-PA levels. Twelve patients whose allografts were depleted of t-PA either received a second transplant or died, whereas only one of the patients whose allografts had persistently normal t-PA levels died ($P < 0.001$).

Conclusions. These findings reveal an association between the depletion of t-PA from arteriolar smooth-muscle cells and the subsequent development of coronary artery disease and decreased graft survival. Although we cannot be certain about a cause-and-effect relation, our data suggest a possible role for deficient fibrinolysis in the development of coronary artery disease in transplanted human hearts. (N Engl J Med 1995;333:1111-6.)

THE development of coronary artery disease after cardiac transplantation is a leading cause of graft failure in recipients who survive the first year after operation.^{1,2} This transplantation-associated arteriopathy is characterized by rapid development,^{3,4} the concentric narrowing of smaller coronary arteries,^{5,6} and the lack of correlation with known atherogenic risk factors.¹⁻⁶

In normal hearts and stable cardiac allografts the distribution of tissue plasminogen activator (t-PA) has been found to be limited to the vascular smooth-muscle cells of arteries and arterioles.⁷⁻¹⁰ Biopsies of failing cardiac allografts have shown depletion of t-PA from vascular smooth-muscle cells,⁹⁻¹¹ and coronary artery disease develops in many of these grafts.¹¹⁻¹³

We studied biopsy specimens from cardiac allografts to determine whether the loss of arteriolar t-PA is associated with the development of coronary artery disease. We found that grafts with early depletion of t-PA showed subsequent angiographic evidence of coronary artery disease and that depletion of t-PA was associated with earlier and more severe disease as well as with decreased graft survival.

METHODS

Patients

We studied all 78 adult patients who received cardiac allografts between July 30, 1988, and July 30, 1993, at Methodist Hospital of Indiana and whose allografts had undergone endomyocardial biopsy before transplantation, who survived more than six months, and who had serial endomyocardial biopsies after transplantation. During the

study, 73 patients underwent catheterization one year postoperatively, 58 two years postoperatively, 39 three years postoperatively, 22 four years postoperatively, and 12 five years postoperatively. Five patients died between 6 and 12 months after transplantation, and their coronary arteries were evaluated histopathologically according to the method of Johnson et al.³ Immunosuppressive therapy consisted of prednisone at an initial dose of 1 mg per kilogram of body weight per day, with the dose tapered to 0.1 mg per kilogram per day; azathioprine at a dose of 1.5 to 2.0 mg per kilogram per day; and cyclosporine at an initial dose of 7 to 10 mg per kilogram per day, with the dose tapered to 3 to 5 mg per kilogram per day. Major episodes of rejection were treated by augmenting the immunosuppressive therapy with high-dose corticosteroids and by using rabbit antithymocyte globulin or mouse monoclonal antibody OKT3 to human lymphocytes.

Functional classification was determined with the use of New York Heart Association criteria,¹⁴ and the ejection fraction was measured by radionuclide ventriculography. The condition of patients who were in functional class III or IV, associated with decreasing ejection fractions, was designated as clinically unstable, and the condition of patients in functional class I or II, associated with stable ejection fractions, was designated as clinically stable. Cholesterol and triglycerides were measured by enzymatic methods, and measurement of donor-specific cytotoxic antibodies and typing of major histocompatibility complex class I (HLA-A and HLA-B) and class II (HLA-DR) antigens were performed with conventional microlymphocytotoxicity assays. Donor hearts were perfused with Stanford cardioplegia solution, and the mean (\pm SE) duration of ischemia was 132.2 ± 6.3 minutes. Endomyocardial biopsies (mean of 16.6 ± 0.5 per allograft) were performed at the time of transplantation; on postoperative day 10; every 2 weeks during the first 2 months; 3, 4.5, 6, 9, and 12 months postoperatively; and every 6 months thereafter. The biopsy specimens were evaluated histopathologically according to methods of the International Society for Heart Transplantation.¹⁵

Coronary Arteriography

Coronary angiography was performed according to the Judkins technique, usually after left ventriculography. Coronary artery disease was assessed by two angiographers who were unaware of the t-PA status of the biopsy specimens. In each case, the angiographers reached a consensus with respect to the severity of disease. The disease was considered to be mild if there were discrete luminal irregularities in the proximal and midportions of major coronary vessels that caused less than a 10 percent decrease in luminal diameter, moderate if there were tubular or multiple stenoses in proximal and midportions of major coronary arteries causing a 10 to 50 percent decrease in luminal

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diameter, and severe if there were diffuse concentric narrowing, tapering, or diffusely irregular vessels that ended abruptly and caused more than a 50 percent decrease in luminal diameter. Lesions classified as mild and moderate occur in large vessels, as observed in most coronary disease not involving transplantation, and lesions classified as severe occur in small vessels, as described in transplanted hearts.^{2,4} Thus, the angiographic classification of severity relates to vessel size (i.e., "mild" and "moderate" reflect large-vessel disease, and "severe" reflects small-vessel disease).

Immunocytochemical Analysis

Tissue manipulations, immunocytochemical reactions, and specificity studies of monoclonal antibodies to t-PA (ESP-4, American Diagnostica, New York) and complexes of t-PA with plasminogen-activator inhibitor type 1 (PAI-1 379, American Diagnostica) and the fluorochrome-labeled antibody to mouse immunoglobulins (Protos ImmunoResearch, San Francisco) have been published previously.⁷⁻¹⁰ All experiments included antibody and conjugate controls and isotype-matched irrelevant antibodies. The presence and extent of immunocytochemical reactivity to t-PA were evaluated from coded photomicrographs projected in a darkened room and evaluated by three investigators unaware of the origins of the biopsy specimens, as previously reported.¹⁰ The t-PA-reactive arteriolar smooth-muscle cells were identified by using monoclonal antibody to smooth-muscle-specific α -actin (1A4, Biomakor, Rehovot, Israel) and by staining with hematoxylin and eosin the same sections that reacted with monoclonal antibody to t-PA. Arterioles the size of one myocardial cell or smaller were evaluated for t-PA as described previously.^{7,10,12} If all arteriolar smooth-muscle cells reacted to antibody, t-PA levels were defined as normal, and if only isolated or no smooth-muscle cells reacted, t-PA levels were defined as depleted. All arterioles within the same biopsy specimens reacted similarly (i.e., either they all had normal reactivity or they all had subnormal reactivity).

Statistical Analysis

Patients were classified into two groups on the basis of the biopsy results during the first three months after transplantation: those with depletion of t-PA and those with normal levels of t-PA. The incidence of coronary artery disease and rates of graft survival during follow-up were compared in these two groups. Results in the two groups were compared with use of Wilcoxon rank-sum tests for numeric variables and Fisher's exact test for categorical variables. Demographic variables that differed significantly between the two groups were included in logistic-regression (presence and severity of coronary artery disease or graft failure) and Cox regression (interval to coronary artery disease or graft failure) analyses to determine the statistical significance of t-PA levels. Consequently, odds ratios (logistic regression) and relative risks (Cox regression) were adjusted for differences between groups. The interval from transplantation until the end of the follow-up period was used to adjust for differences in follow-up times. Kaplan-Meier estimates were used to illustrate the effect of t-PA levels on the time from transplantation to the detection of coronary artery disease and the time from transplantation to a second transplantation or death. All P values of less than 0.05 were considered to indicate statistical significance, and all data are reported as means \pm SE. Statistical analyses were performed with PC-SAS version 6.08.

RESULTS

Studies of Donor Hearts before Transplantation

Monoclonal antibody to t-PA was found to react in all biopsy specimens obtained from the 78 donor hearts before transplantation, but only with vascular smooth-muscle cells of arteries and arterioles (Fig. 1A). All negative-control experiments were negative, and all positive-control experiments with antibody to smooth-muscle-specific α -actin were positive.

Studies of Allografts after Transplantation

The transplanted hearts were evaluated for t-PA reactivity with serial endomyocardial biopsies, and

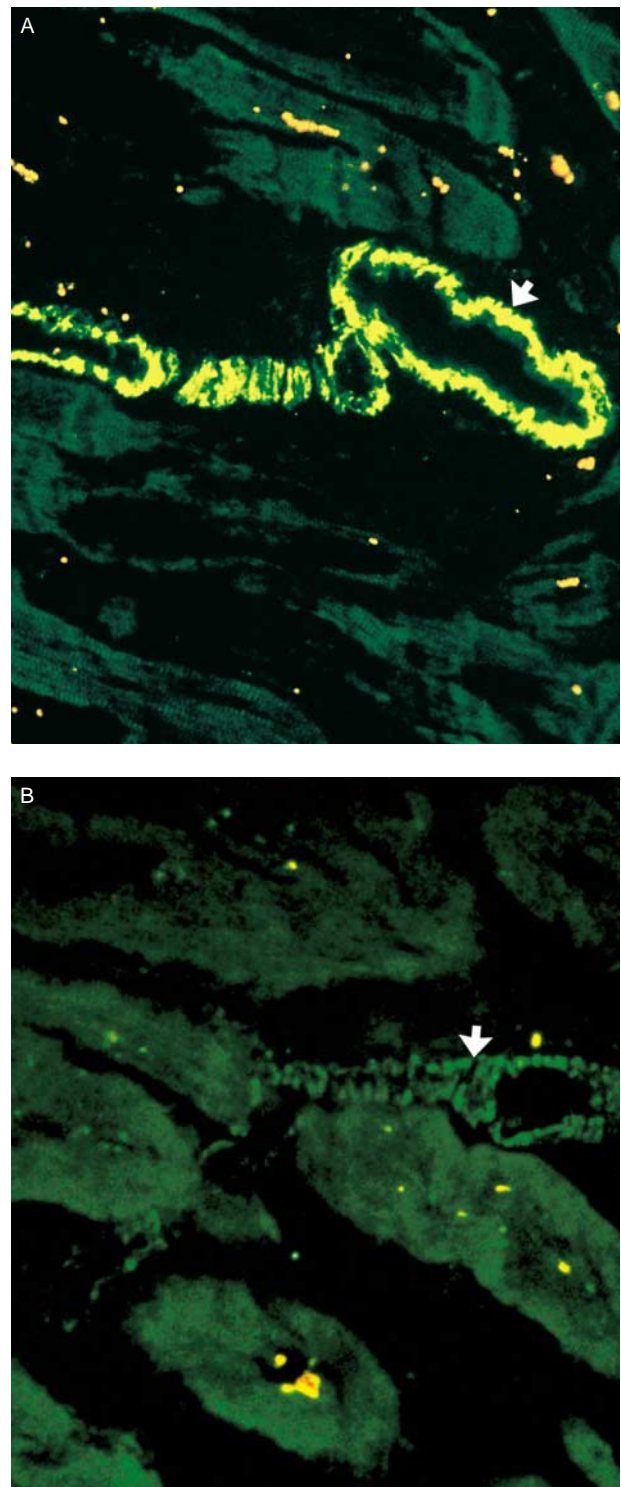


Figure 1. Reactivity of Heart-Biopsy Specimens to Antibody to t-PA before and after Transplantation ($\times 400$).

Before transplantation (Panel A) the distribution of t-PA in an arteriole is normal (arrow). After transplantation (Panel B) the arteriole is depleted of t-PA (arrow).

analysis of the data revealed that 38 of the 78 allografts (49 percent) maintained a normal distribution of t-PA in arteriolar smooth-muscle cells, whereas 40 (51 percent) showed immunocytochemical evidence of depletion of t-PA from arteriolar smooth-muscle cells

within the first three months after transplantation (Fig. 1B).

Relation between Arteriolar t-PA Levels and Clinical Laboratory Data

Analysis of clinical laboratory data for the two groups revealed no significant differences in age, type of cardiac disease originally present, duration of ischemia, percentage of smokers, blood pressure, serum lipid levels, left ventricular ejection fractions, history of cytomegalovirus infections, presence of donor-specific cytotoxic antibodies, or HLA mismatches (Table 1), but the cohorts differed significantly with respect to sex, the number of histologically diagnosed episodes of rejection, and the length of follow-up (Table 1). The shorter follow-ups for patients whose allografts were depleted of t-PA were due to an increased rate of second transplantation or death. Although the patients' sex and the number of rejection episodes were related significantly to t-PA status, subsequent analysis revealed that neither of these variables was related significantly to either the occurrence (determined by logistic regression) or the timing (determined by Cox regression) of coronary artery disease or graft failure. In addition, the condition of patients with depletion of t-PA was less stable after transplantation than that of patients with normal levels of t-PA (P<0.001), as shown in Table 1.

Relation between t-PA Levels and the Development of Coronary Artery Disease

When the 78 transplanted hearts were evaluated, coronary artery disease had developed in 9 of the 38 allografts with a normal distribution of t-PA (24 percent). In contrast, angiographically detectable coronary artery disease developed in 31 of the 40 allografts with depletion of arteriolar t-PA (78 percent). Thus, coronary artery disease was significantly more likely to develop in allografts with depleted t-PA (P<0.001). Even after adjustment for the demographic variables that were significantly different between the two groups, the odds of coronary artery disease were significantly increased in the group with depletion of t-PA (odds ratio, 23.9; 95 percent confidence interval, 5.4 to 105.7).

When the interval from transplantation to the development of coronary artery disease was compared in the two groups, it was found that the disease developed significantly earlier after transplantation in allografts depleted of t-PA (Fig. 2). The mean (±SE) interval from transplantation to angiographic detection of disease was 16.3±2.2 months in the group with depletion of t-PA and 38.5±4.4 months in the group with normal t-PA levels (P<0.001). Kaplan–Meier estimates indicated that coronary artery disease developed within 15 months after transplantation in more than half (59 percent) of the group with depletion of t-PA, whereas the majority (65 percent) of the group with normal t-PA levels remained free of disease five years after transplantation.

Relation between t-PA Levels and Severity of Coronary Artery Disease

To determine whether the severity of coronary artery disease differed between allografts with normal levels

Table 1. Clinical-Laboratory Data on Patients Whose Allografts Had Normal Levels of t-PA or Depletion of t-PA within Three Months after Transplantation.*

CHARACTERISTIC	NORMAL t-PA LEVELS (N = 38)	DEPLETION OF t-PA (N = 40)	P VALUE
Age of recipient (yr)	49.8±1.3	49.0±1.8	NS
Age of donor (yr)	28.3±1.4	29.5±1.7	NS
Sex of recipient (M/F)	31/7	24/16	0.048
Sex of donor (M/F)	33/5	29/11	NS
Original cardiac disease (no. of patients)			NS
Coronary artery disease	21	15	
Cardiomyopathy	14	18	
Other	3	7	
Duration of ischemia (min)	124.0±8.2	140.0±9.5	NS
Length of follow-up (mo)	41.1±2.7	24.4±2.4	<0.001
Smoker (%)	2.6	2.5	NS
Systolic blood pressure (mm Hg)	138.4±1.5	139.9±1.7	NS
Diastolic blood pressure (mm Hg)	93.5±1.2	93.2±1.2	NS
Serum cholesterol (mg/dl)†	222.8±6.0	244.0±8.3	NS
Serum triglycerides (mg/dl)‡	203.6±12.7	236.0±18.4	NS
Left ventricular ejection fraction	0.59±0.02	0.55±0.02	NS
No. of HLA mismatches			
A or B	2.8±0.1	2.8±0.1	NS
DR	1.3±0.1	1.3±0.1	NS
Cytotoxic antibodies (no. of patients)	0	0	NS
No. of rejection episodes (grades 3 and 4)	0.3±0.1	0.6±0.1	0.01
Cytomegalovirus infection (no. of patients)	7	5	NS
Clinical condition after transplantation (no. of patients)			
Stable	37	14	<0.001
Unstable	1	26	

*Plus–minus values are means ±SE. NS denotes not significant.

†To convert values for serum cholesterol to millimoles per liter, multiply by 0.02586.

‡To convert values for serum triglycerides to millimoles per liter, multiply by 0.01129.

of t-PA and allografts with depletion of t-PA, we calculated the percentages of angiographically defined mild, moderate, or severe coronary artery disease for each group (Table 2). Logistic-regression analysis of these data revealed that the status of t-PA in arterioles was significantly related to the severity of coronary artery disease (P<0.001). Indeed, among the allografts with coronary artery disease, those with normal t-PA levels had predominantly mild (large vessel) disease (78 percent), whereas those with depletion of t-PA had predominantly severe (small vessel) disease (48 percent) (Table 2). The relation between the depletion of t-PA and the severity of coronary artery disease is further illustrated by the finding that 15 of the 40 allografts with depletion of arteriolar t-PA (38 percent) during the first three months after transplantation had severe disease, whereas this occurred in only 1 of the 38 allografts with normal levels of arteriolar t-PA (2.6 percent).

Relation between t-PA Levels and Graft Survival

Analysis of the biopsy results of the two groups revealed a striking association between t-PA levels and graft survival (Table 3). Twelve of the 40 patients (30 percent) whose grafts had immunocytochemical evi-

dence of depletion of t-PA from arteriolar smooth-muscle cells within the first three months after transplantation underwent a second transplantation or died, whereas only 1 of the 38 patients whose allografts had normal t-PA levels died (2.6 percent, $P < 0.001$). Kaplan–Meier estimates and Cox regression analysis comparing the two groups indicated that graft survival was significantly decreased ($P = 0.003$) in the group with depletion of t-PA (Fig. 3). The mean interval from transplantation to death or a second transplantation was 16.0 ± 3.2 months in the group with depletion of t-PA (12 such events), whereas in the group with normal t-PA levels the only death occurred 29.9 months after transplantation. Angiographic and histopathological examination of the allografts that failed showed that 11 of the 12 allografts with depletion of t-PA (92 percent) had severe coronary artery disease a mean of 12.9 ± 2.3 months after transplantation, whereas in the single allograft that failed in the group with normal t-PA levels mild disease developed 24.1 months after transplantation.

Relation between t-PA and Plasminogen-Activator Inhibitor Type 1

There was no immunocytochemical evidence of complexes composed of t-PA bound by plasminogen-activator inhibitor type 1 in any of the 78 donor hearts before transplantation or in any of the 38 allografts that retained normal t-PA levels after transplantation. However, all 40 allografts with depletion of t-PA during the first three months after transplantation had immunocytochemical evidence of complexes of t-PA and its inhibitor on the endothelium of arterioles, capillaries, and venules, regardless of whether coronary artery disease or graft failure subsequently developed.

Summary of Findings

Allografts with depletion of t-PA were at significantly greater risk of coronary artery disease (relative risk, 8.3; 95 percent confidence interval, 3.6 to 19.4) and

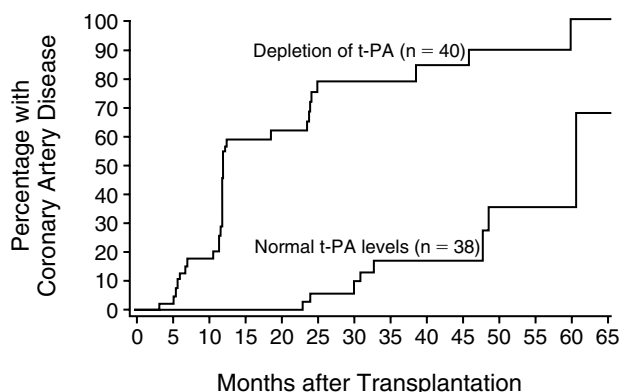


Figure 2. Kaplan–Meier Estimates of the Likelihood of Coronary Artery Disease According to Whether t-PA Levels Were Normal or Depleted within Three Months after Transplantation.

Coronary artery disease was more likely to develop in patients whose allografts were depleted of t-PA, and the disease appeared much earlier ($P < 0.001$).

Table 2. Classification of Allografts with Normal Levels of t-PA and Allografts with Depletion of t-PA According to the Presence or Absence and the Type of Coronary Artery Disease.*

CORONARY ARTERY DISEASE STATUS	NORMAL t-PA LEVELS (N = 38)	DEPLETION OF t-PA (N = 40)
	<i>no. of patients (%)</i>	
No coronary artery disease	29 (76)	9 (22)
Coronary artery disease	9 (24)	31 (78)
Mild	7 (78)	11 (35)
Moderate	1 (11)	5 (16)
Severe	1 (11)	15 (48)

*The severity of coronary artery disease was determined angiographically on the basis of the size of involved vessels, as explained in the Methods section. Depletion of t-PA significantly affected the severity of coronary artery disease ($P < 0.001$ by logistic-regression analysis). After adjustment for the recipients' sex, the number of episodes of rejection, and length of follow-up, the percentage of patients with coronary artery disease was 17.9 percent (observed rate, 24 percent) in the group with normal t-PA levels and 83.9 percent (observed rate, 78 percent) in the group with depletion of t-PA.

graft failure (relative risk, 24.7; 95 percent confidence interval, 3.0 to 201.0) than allografts with normal t-PA levels. Although coronary artery disease did not develop in all the patients with depletion of t-PA during the study, according to Kaplan–Meier estimates the disease will occur in 89 percent of these patients within five years after transplantation. Although coronary artery disease did develop in some allografts with normal t-PA levels, according to Kaplan–Meier estimates all such allografts should remain free of coronary artery disease for the first 23 months after transplantation, as compared with 39 percent of allografts with depletion of t-PA. Finally, of the nine allografts with normal t-PA levels in which coronary artery disease developed, seven (78 percent) had mild coronary artery disease (Table 2), and of the nine allografts with depletion of t-PA in which coronary artery disease did not develop, five (56 percent) were found to have normal t-PA levels between 12 and 54 months after transplantation; these were the only allografts in which t-PA levels returned to normal during the study.

DISCUSSION

The central observation to emerge from our study is that before transplantation normal donor hearts have t-PA in arteriolar smooth-muscle cells and that allografts that become depleted of arteriolar t-PA soon after transplantation are at risk for coronary artery disease. The depletion of t-PA occurred within three months of transplantation, and coronary artery disease developed earlier and was more severe in allografts depleted of t-PA. The most compelling clinical correlate of these observations was that 12 of the 40 patients (30 percent) whose allografts were depleted of t-PA within the first three months after transplantation subsequently underwent a second transplantation or died, whereas only 1 of the 38 patients (2.6 percent) whose allografts retained normal t-PA levels died.

This study is limited by the fact that coronary artery

Table 3. Relation between t-PA Levels and Outcome.

DEATH OR SECOND TRANSPLANTATION	NORMAL t-PA LEVELS (N = 38)	DEPLETION OF t-PA (N = 40)	P VALUE
Observed rate — % (no.)	2.6 (1)	30.0 (12)	<0.001
Adjusted rate — %*	1.2	31.4	0.002

*Estimated by logistic-regression analysis after adjustment for the recipients' sex, the number of rejection episodes, and the length of follow-up; adjusted odds ratio, 37.8; 95 percent confidence interval, 3.6 to 392.3.

disease status was not monitored continuously and by contemporary findings that angiography is a less sensitive method of identifying coronary artery disease than intravascular ultrasonography. However, the measurement error associated with either the timing or the type of coronary-artery visualization would be the same in both study groups. We cannot state definitively that coronary artery disease did not precede the depletion of t-PA, but the inclusion criteria that we used reduce this possibility. On average, the depletion of t-PA was detected 0.6 month after transplantation. Thus, we can conservatively conclude that the depletion of t-PA is an early sign of the development of coronary artery disease. However, we do not mean to imply that there is a cause-and-effect relation between these two events.

It is intuitive to suggest that transplantation-induced coronary artery disease results from allogeneic recognition and rejection reactions, but the disease is not associated with the degree of histoincompatibility between pairs of donors and recipients¹⁶ or with the number of histologically defined episodes of rejection,¹⁷ and the incidence of transplantation-induced coronary artery disease is not affected by immunosuppressive drugs.¹⁸ Although allogeneic recognition reactions are initiated by allografting, many of the characteristic cells, effector molecules, and endothelial changes of allogeneic recognition have also been identified in spontaneous atherosclerotic lesions.¹⁹ Thus, it is not established that the underlying mechanisms differ in spontaneous and transplantation-induced coronary artery disease, notwithstanding the obvious differences in initiating factors and morphology.

Much of the experimental data on t-PA has come from in vitro studies of endothelial-cell cultures, in which t-PA messenger RNA has been found.²⁰⁻²² Although earlier investigators reported finding endothelial t-PA in organs other than the ones we studied by using different antibodies and immunocytochemical techniques under different conditions,^{23,24} we did not identify endothelial t-PA in biopsy specimens obtained just before transplantation or after transplantation from clinically stable allografts.^{7,10,12} However, using the techniques we used in the present study, we have identified endothelial t-PA bound to plasminogen-activator inhibitor type 1 in allografts from patients whose condition was unstable after transplantation or who subsequently died.¹⁰ In these cases the reactivity of en-

dothelial t-PA was associated with the presence of endothelial fibrin and lack of fibrinolytic activity.¹⁰

Although the causes of spontaneous and transplantation-induced coronary artery disease are not known, there is a growing awareness that activated hemostatic, fibrinolytic, and natural anticoagulant pathways are involved in both processes.²⁵⁻²⁹ Activated endothelial cells have been identified in lesions caused by both spontaneous and transplantation-induced coronary artery disease,^{19,30-32} and plasma membranes of activated endothelial cells are known to change from being thromboresistant to thrombogenic.^{33,34} A principal metabolic effect of this change is the generation of thrombin, which results in fibrin deposition^{27,28} and the secretion of t-PA from endothelial and smooth-muscle cells.³⁵ The secreted amounts of t-PA and its inhibitor are sufficiently large that immunoassay of these molecules in plasma has been used to identify patients at risk for myocardial infarction, coronary artery disease, or stroke.³⁶⁻³⁹ The decrease in arterial and arteriolar smooth-muscle cell t-PA in heart-graft failure⁷⁻¹² is associated with t-PA-reactive endothelial cells and endothelial complexes of t-PA with plasminogen-activator inhibitor type 1,¹⁰⁻¹² suggesting that endothelial secretion of these complexes could account for the increased concentration of reactive molecules in the blood of patients at risk for myocardial infarction, coronary artery disease, or stroke.³⁶⁻³⁹

Depletion of t-PA from vascular smooth-muscle cells is accompanied by the loss of the antithrombin natural anticoagulant pathway from arteries and arterioles.^{9,11,12,29,40,41} Since the process of t-PA depletion is associated with decreased binding of vascular antithrombin and increased deposition of fibrin, we have suggested that these events are all linked to the loss of antithrombin, which allows thrombin to deposit fibrin, activate endothelium, and consume t-PA.^{8,9,12,29} Therapies designed to impede the generation or activity of thrombin in the development of coronary artery disease should be extended in the light of our finding that the depletion of t-PA from arteries and arterioles can be

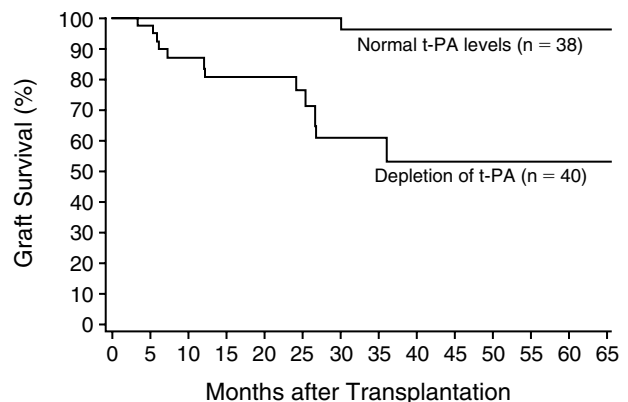


Figure 3. Kaplan-Meier Estimates of Graft Survival According to Whether t-PA Levels Were Normal or Depleted within Three Months after Transplantation.

Patients with depletion of t-PA were significantly more likely to need a second transplantation or die (or both) ($P = 0.003$).

used to predict the development and severity of transplantation-induced coronary artery disease.

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