Tissue Plasminogen Activator, Plasminogen Activator Inhibitor-1, and Fibrin as Indexes of Clinical Course in Cardiac Allograft Recipients

An Immunocytochemical Study

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Background

Tissue-type plasminogen activator (TPA) is the principal activator of plasminogen. Since hemostasis in the microcirculation of allografts is a well-recognized complication of transplantation, we asked (1) whether the distribution and amount of cellular TPA in biopsies of transplanted human hearts are associated with fibrin deposits in and around the microcirculation, (2) whether similar changes involve the physiological inhibitors of TPA and plasmin, and (3) whether the presence of these activators and inhibitors of fibrinolysis in tissue is correlated with clinical outcome.

Methods and Results

We immunocytochemically quantified the presence of fibrin, plasmin, TPA, and the TPA inhibitor PAI-1 in 938 biopsies from 68 consecutive cardiac allografts over a 54-month period. The localization, distribution, and quantification of TPA in arteriolar smooth muscle cells revealed that 35 of the 68 allografts maintained vascular TPA reactivity consistent with time-zero biopsies of autologous donor hearts: this was designated as the normal TPA group. In contrast, 33 of the 68 allografts significantly lost vascular TPA reactivity compared with time-zero biopsies of autologous donor hearts: this was designated as the depleted TPA group. Analysis of sequential biopsies from both groups during 54 months revealed that the mean cumulative quantitative TPA value for the normal TPA group was 1.0±0.01, whereas the depleted TPA group value was 1.9±0.02 (P=.0001), and the mean cumulative quantitative fibrin value for the normal TPA group was 1.0±0.01, whereas the depleted TPA group value was 1.5±0.05 (P=.0001). Biopsies of allografts in the depleted TPA group contained endothelial reactivity for TPA–PAI-1 complexes, whereas biopsies from the normal TPA group did not. Plasmin-associated molecules were rarely identified in biopsies of the normal TPA group but were present in the depleted TPA group, and the fibrin-to-plasmin ratio in the normal TPA group always was less than the fibrin-to-plasmin ratio in biopsies from the depleted TPA group. Analysis of demographic and risk factors revealed no significant differences between patients in the normal and depleted TPA groups, but none of the 35 patients in the normal TPA group died or were retransplanted, and 13 of the 33 patients in the depleted TPA group died or required retransplantation (P=.0001).

Conclusions

Time-zero hearts (n=68) and 34 of 38 stable allografts contained immunocytochemically detectable TPA only in vascular smooth muscle cells. Twenty-nine of 30 patients with normal TPA in their time-zero biopsies who subsequently developed a poor clinical outcome were found to have depleted TPA in biopsies evaluated during their first postoperative month and remained depleted throughout the study. Of 33 patients with depleted TPA, 39% died or required retransplantation. Depleted arteriolar TPA associated significantly with vascular and interstitial deposits of fibrin, plasmin, and endothelial TPA–PAI-1 complexes. These findings indicate that hemostatic and fibrinolytic pathways are activated in failing allografts, and they reveal evidence of depleted TPA before clinical or histopathological signs of failure. Patients with such allografts were found to be at high risk of death independently of other widely used clinical/laboratory parameters of prediction. (Circulation. 1994;89:1599–1608.)

Key Words • plasminogen activators • microcirculation • endothelium • fibrinolysis • transplantation

There is compelling evidence that imbalances in the hemostatic, fibrinolytic, and natural anticoagulant pathways are integral components of the allogeneic rejection reaction to organ grafts.1-11 Exactly how these pathways are involved is a matter of continuing study, but recent investigations have focused on the role of endothelial cells.2-8,15 Studies of endothelial natural anticoagulant pathways have revealed a loss of both the thrombomodulin and the heparan sulfate proteoglycan–antithrombin pathways in failing cardiac2,4,5,8,9,11,15 and renal1,3,10,11 allografts. A common immunocytochemical finding in these solid organ grafts, as well as in the placental graft of human pregnancy,16-19 is the presence of fibrin deposits in the microcirculation.

Plasmin-mediated fibrinolysis is the physiological response to fibrin deposits,20 and the plasmin that mediates this reaction is derived from activated plasminogen.21 Tissue-type plasminogen activator (TPA) in endothelial22 and vascular smooth muscle cells7 is the principal activator of plasminogen in arteries and veins.23 In light of these observations, we have studied TPA in vessels of transplanted human hearts from time zero throughout a 54-month period. These experiments also involved qualitative and quantitative immunocytochemical determinations of fibrin and the physiological inhibitors of TPA and plasmin in the same biopsies. The results of this investigation show that diminished arte-
riolar smooth muscle cell TPA is associated with increased numbers of fibrin-reactive vessels, decreased fibrinolysis, and increased morbidity and mortality. The most striking clinical observation from this study was the finding that decreased vascular TPA was identified very early in biopsies of allografts that subsequently failed.

Methods

Patients

The experimental design of this investigation was to integrate clinical and functional data with the results of qualitative and quantitative immunocytochemical determinations of components of the hemostasis and fibrinolytic pathways in all biopsies from consecutive cardiac allografts performed at Methodist Hospital between the first day of July, 1988, and the last day of June, 1992. During this 48-month period, 68 cardiac allograft procedures were done, and all 68 recipients were enrolled in this investigation. The study period extended from the first day of July, 1988, to the last day of December, 1992. The mean study time for these 68 patients during this 54-month period was 25.2±1.9 months. During the study, 938 biopsies were studied, and the mean number of biopsies evaluated per patient was 13.8±0.6.

Immunosuppressive treatment consisted of prednisone begun as 1 mg · kg⁻¹ · d⁻¹ and tapered to 0.1 mg · kg⁻¹ · d⁻¹, cyclosporine begun as 7 to 10 mg · kg⁻¹ · d⁻¹ and tapered to 3 to 5 mg · kg⁻¹ · d⁻¹, and azathioprine 1.5 to 2.0 mg · kg⁻¹ · d⁻¹. Major rejection episodes were treated by increasing immunosuppressive therapy with high-dose steroids, and refractory cases received rabbit antithymocyte globulin or OKT3.

The functional classification of patients was determined according to the New York Heart Association and by a specific activity scale, and allograft function was determined by radionuclide ventriculography. Patients with three or more functional classifications of class II or greater associated with decreasing ejection fractions were considered to be clinically unstable, and patients with consistent classifications of I and nondecreasing ejection fractions were considered to be clinically stable.

Cholesterol (normal, 165 to 240 mg/dL) and triglycerides (normal, 10 to 200 mg/dL) were measured by enzymatic methods in spectrophotometry. Donor-specific cytotoxic antibodies and major histocompatibility complex classes I (HLA-A and HLA-B) and II (HLA-DR) immunophenotyping were done by microlymphocytotoxicity assays.

Donor hearts were perfused with Stanford cardioplegia solution before transport. Mean ischemic time for the 68 donor hearts was 130.8±6.4 minutes. Patients were followed with serial biopsies performed during cardiac catheterization. Endomyocardial biopsies were obtained from the right ventricle of all hearts before and after transplantation. Time-zero control biopsies were obtained before perfusion with recipient blood. Biopsies were taken on the 10th postoperative day, every 2 weeks during the first 2 months, and at 3, 4.5, 6, 9, and 12 months and every 6 months thereafter. Cellular infiltrates were evaluated by conventional light microscopy according to the International Society for Heart Transplantation, and only major rejection episodes (grades 3 and 4) were considered rejections. Biopsies with cellular infiltrates during the follow-up of a rejection episode were considered part of the same rejection period.

Immunocytochemistry

Biopsies were snap frozen in liquid nitrogen and stored at −20°C. The interval between biopsy and snap-freezing was <30 minutes. Thin sections (4 μm) were prepared with a Tissue-Tek cryostat (Miles Laboratories, Elkhart, Ind.), removed from the cryostat blade by flash condensation onto glass microscope slides, and air dried without chemical fixation by an electric fan overnight at room temperature. Tissue sections were washed for 30 minutes in 0.01 mol/L pH 7.2 phosphate-buffered physiological saline (PBS), incubated for 15 minutes in a moisture box with 25 μL of primary antibody, rinsed in PBS, and washed three times for 10 minutes in a glass reservoir (7.5×10×12.5 cm) filled with PBS agitated with a magnetic stirrer. After the last wash, sections were incubated for 15 minutes with 25 μL of fluorescein isothiocyanate (FITC)- or rhodamine isothiocyanate (RITC)-labeled F(ab')₂ anti-species antibody to the isotype of the first antibody. Double-antibody experiments were done with primary antibodies from different species and appropriately matched anti-species FITC and RITC conjugates. After the final wash, tissue sections were coverslipped with pH 8.0 PBS-buffered glycerol.

Antibodies and Control Experiments

The sources, specificities, and dilutions of antibodies are detailed in Table 1. Each experiment included antibody, PBS, and conjugate controls. These were done at room temperature by exposure of cryostat sections to only PBS, isotype-matched irrelevant antibody, or conjugate. The irrelevant rabbit antibody was anti-human IgE (Dakopatts, Glostrup, Denmark), and mouse irrelevant monoclonals were IgG1 anti-cytomegalovirus early antigen (Chemicon, Temecula, Calif) and IgG2a anti-Epstein-Barr virus capsid antigen (Chemicon). The dilution for each antibody was two serial dilutions before end point titration. Antibodies were ultracentrifuged at 100 000g at 4°C for 1 hour to remove complexes and aggregates, and monthly titrations and ultracentrifugations were done for each antibody. The reactivity of anti-TPA was removed by absorption with recombinant TPA from Genentech (San Francisco, Calif), confirming specificity of the antibody.

Microscopy

Tissue preparations were studied by epi-illumination in a Leitz Orthoplan microscope fitted with interference optics and an HBO-100 mercury-arc lamp. The epi-illuminator contained an I-3 type FITC filter complex consisting of a 450- to 490-nm excitation filter with a 510-nm dichroic mirror and a 515-nm barrier filter allowing wavelengths >515 nm to pass and an N2.1 type RITC filter complex consisting of a 515- to 560-nm excitation filter with a 580-nm dichroic mirror and a 580-nm barrier filter allowing wavelengths >580 nm to pass. A Leitz camera that contained ASA 200 daylight 35-mm Ektachrome film was used.

Quantification of Immunocytochemical Results

Immunocytochemical reactivities were graded for statistical purposes from coded photomicrographs of equivalent magnifications. These were projected from the same distance in a darkened room and scored by three investigators unaware of the origins of the biopsies, as previously reported. Donor hearts were perfused with Stanford cardioplegia solution before transport. Mean ischemic time for the 68 donor hearts was 130.8±6.4 minutes. Patients were followed with serial biopsies performed during cardiac catheterization. Endomyocardial biopsies were obtained from the right ventricle of all hearts before and after transplantation. Time-zero control biopsies were obtained before perfusion with recipient blood. Biopsies were taken on the 10th postoperative day, every 2 weeks during the first 2 months, and at 3, 4.5, 6, 9, and 12 months and every 6 months thereafter. Cellular infiltrates were evaluated by conventional light microscopy according to the International Society for Heart Transplantation, and only major rejection episodes (grades 3 and 4) were considered rejections. Biopsies with cellular infiltrates during the follow-up of a rejection episode were considered part of the same rejection period.

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TABLE 1. Definition of Antibodies Used in the Present Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Source</th>
<th>Species</th>
<th>Specificity Test</th>
<th>Dilution</th>
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<tr>
<td>ESP-4</td>
<td>Tissue plasminogen activator</td>
<td>American Diagnostica New York, NY</td>
<td>Mouse, IgG1</td>
<td>Inhibition of TPA activity, immunohistology</td>
<td>1:5</td>
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<td>PAI-1 379</td>
<td>Free PAI-1 and TPA-PAI-1 complexes</td>
<td>American Diagnostica New York, NY</td>
<td>Mouse, IgG1</td>
<td>Inhibition of PAI-1 activity, immunohistology, ELISA</td>
<td>1:5</td>
</tr>
<tr>
<td>PAI-1 380</td>
<td>Free PAI-1</td>
<td>American Diagnostica New York, NY</td>
<td>Mouse, IgG1</td>
<td>Western blot, immunohistology, ELISA</td>
<td>1:5</td>
</tr>
<tr>
<td>VW factor</td>
<td>Human von Willebrand factor</td>
<td>Serotec Oxford, England</td>
<td>Mouse, IgG1</td>
<td>Immunoelectrophoresis, immunodiffusion</td>
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<td>VW factor</td>
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<td>Dako Santa Barbara, Calif</td>
<td>Rabbit</td>
<td>Crossed IEP</td>
<td>1:80</td>
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<tr>
<td>Fibrin</td>
<td>Fibrin β-chain N-terminal neotope</td>
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<td>Mouse, IgG1</td>
<td>Western blot</td>
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<td>NeoAntigen</td>
<td>Neoantigen of human α2-antiplasmin-plasmin complex</td>
<td>Behring Diagnostics La Jolla, Calif</td>
<td>Rabbit</td>
<td>ELISA</td>
<td>1:100</td>
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<td>α2Plasmin inhibitor</td>
<td>Human α2-plasmin inhibitor</td>
<td>Dakopatts Santa Barbara, Calif</td>
<td>Rabbit</td>
<td>Crossed IEP</td>
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<td>1A4</td>
<td>α-Smooth muscle actin</td>
<td>Biomakor Rehovot, Israel</td>
<td>Mouse, IgG2a</td>
<td>Immunohistology, immunocytochemistry</td>
<td>1:50</td>
</tr>
<tr>
<td>FITC goat F(ab')2 anti-rabbit IgG</td>
<td>Rabbit IgG</td>
<td>Protos ImmunoDx San Francisco, Calif</td>
<td>Goat</td>
<td>Affinity purified</td>
<td>1:50</td>
</tr>
<tr>
<td>RITC goat F(ab')2 anti-rabbit IgG</td>
<td>Rabbit IgG</td>
<td>Protos ImmunoDx San Francisco, Calif</td>
<td>Goat</td>
<td>Affinity purified</td>
<td>1:50</td>
</tr>
<tr>
<td>FITC goat F(ab')2 anti-mouse IgG</td>
<td>Mouse IgG</td>
<td>Protos ImmunoDx San Francisco, Calif</td>
<td>Goat</td>
<td>Affinity purified</td>
<td>1:40</td>
</tr>
<tr>
<td>RITC goat F(ab')2 anti-mouse IgG</td>
<td>Mouse IgG</td>
<td>Protos ImmunoDx San Francisco, Calif</td>
<td>Goat</td>
<td>Affinity purified</td>
<td>1:40</td>
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ESP indicates epitope-specific plasminogen activator; PAI-1, plasminogen activator inhibitor-1; VW, von Willebrand; FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate; TPA, tissue-type plasminogen activator; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; and IEP, immunoelectrophoresis.

brand factor and monoclonal antibody to fibrin. Fibrin deposits in ≥10% of the capillaries with interstitial fibrin extending around and often within myocardial cells were assigned a grade of 2. (3) Plasmin was identified by use of antibodies to the α2-antiplasmin inhibitor (α2PI) as well as to a neotope on α2PI-plasmin complexes. The grading of plasmin deposits was done with antibody to α2PI, and grades were assigned with fibrin. The grading scheme for TPA, fibrin, and plasmin considers immunocytochemical changes compared with a time-zero (score of 1) biopsy and assigns a score of 1 to biopsies with normal characteristics (ie, TPA in arteriolar SMCs and absence of fibrin and plasmin) and a score of 2 to biopsies with abnormal immunocytochemical changes such as loss of arteriolar SMC TPA and presence of fibrin and plasmin.

Statistical Analysis

Data are expressed as mean±SEM. Statistical differences were calculated by Fisher’s exact test and Goodman-Kruskal’s G statistic for ordered contingency tables for categorical variables or Student’s t test (unpaired and two-tailed) and Wilcoxon (rank sums) for continuous data. The criterion for statistical significance was P<.05.

Results

Data From Negative and Positive Control Experiments

No immunofluorescence was obtained from control experiments performed by exposing tissue sections to PBS, unlabeled primary antibodies, or fluorochrome-labeled second antibodies only. Negative results also were obtained when tissue sections were exposed to nonimmune rabbit or mouse immunoglobulins or to irrelevant isotype-matched first antibodies followed by fluorochrome conjugated F(ab')2 anti-isotype to the species-matched isotype of the first antibody. Results of positive control experiments done with mouse monoclonal or rabbit antibodies to von Willebrand factor reacted with vascular endothelium, as expected.

Data From Time-Zero Biopsies

Cells in the morphological position of arterial and arteriolar SMCs in all 68 time-zero biopsies reacted with Mab to TPA. This was confirmed immunocytochemically by the observation that SMCs in an adjacent serial section of the same vessel reacted with Mab to α-smooth muscle actin. The sections then were washed and stained with hematoxylin and eosin, and examination of these sections by light microscopy confirmed that only SMCs had reacted with Mab to actin or TPA, confirming recent reports that vascular SMCs contain TPA. Capillaries and veins were not reactive with Mab to TPA.

None of the time-zero biopsies were reactive with Mab to fibrin or with polyclonal antibodies to α2PI or the neoantigen of α2PI-plasmin complexes. The time-zero biopsies also did not react with Mab to either complexed or uncomplexed plasminogen activator inhibitor-1 (PAI-1). Since many publications have re-
ported TPA in cultured endothelial cells (see Reference 23 for review), it is necessary to stress that none of the 68 time-zero biopsies studied in this investigation contained TPA-reactive endothelial cells. Thus, time-zero hearts were found not to manifest immunocytochemical evidence of activated hemostasis or fibrinolysis, and TPA was identified only in arteriolar SMCs.

Data From Allograft Biopsies

All biopsies (n=938) were prospectively studied for TPA, fibrin, and the physiological inhibitors of TPA and plasmin. Evaluation of biopsies for TPA revealed two types of distribution within SMCs: One was indistinguishable from time-zero hearts (Fig 1a) and was designated as the normal TPA type. The second was significantly depleted of TPA compared with time-zero hearts (Fig 1b) and was designated as the depleted TPA type. Serial sections using Mab to α-SMC actin showed that there was no depletion of arteriolar SMCs. These distributional types were identified in early biopsies and persisted. Analysis of the distribution, localization, and quantification of TPA in all 938 biopsies revealed that 35 of the 68 patients maintained normal TPA throughout the duration of the study, and 33 of the 68 allografted hearts developed depleted TPA within the first month after transplantation (Table 2), although the time-zero biopsies from these hearts were normal.

Evaluation of the distribution, localization, and quantification of fibrin in the 938 biopsies revealed that samples from allografts with normal TPA contained little or no fibrin (Fig 2a), whereas fibrin was identified in samples from allografts with depleted TPA (Fig 2b). These deposits were localized to the microcirculation, often extending into interstitial spaces (Fig 2b). Quantification of these deposits revealed that the 35 allografts with consistently normal TPA had a mean cumulative fibrin value of 1.0±0.01, and the 33 allografts with consistently depleted TPA had a mean cumulative fibrin value of 1.5±0.05 (P=.0001), as seen in Table 2. When the TPA and fibrin data from the normal and depleted groups were examined mathematically for temporal associations with fibrin deposits, it was found that allografts with normal TPA were consistently associated with less fibrin deposition than were allografts with depleted TPA (Fig 3). Patients with depleted TPA and fibrin within the microcirculation who managed to survive showed a recovery of the arteriolar TPA associated with an absence of fibrin (Fig 3).

Evaluation of the distribution, localization, and quantification of plasmin in the 938 biopsies was done with an antibody to the α₂PI that binds to the enzyme active site and an antibody to the neotope formed by the plasmin-α₂PI complex. Vascular lesions that contained fibrin deposits also reacted with antibody to α₂PI, but fibrin-reactive areas in biopsies with depleted TPA did not contain α₂PI (Fig 4). Fibrin-to-α₂PI ratios revealed that the 35 patients with normal TPA had a mean cumulative fibrin-to-α₂PI ratio of 1.06±0.03, and the 33 patients with depleted TPA had a mean cumulative ratio of 1.25±0.03 (P=.0001), as shown in Table 2.
TABLE 2. Immunocytochemical Grades of Allografts With Normal and Depleted TPA*

<table>
<thead>
<tr>
<th>Molecules Studied</th>
<th>Normal TPA (n=35)</th>
<th>Depleted TPA (n=33)</th>
<th>P</th>
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<tr>
<td>TPA</td>
<td>1.0±0.01</td>
<td>1.9±0.02</td>
<td>.0001</td>
</tr>
<tr>
<td>Fibrin</td>
<td>1.0±0.01</td>
<td>1.5±0.05</td>
<td>.0001</td>
</tr>
<tr>
<td>Fibrin-to-a2PI ratio</td>
<td>1.06±0.03</td>
<td>1.25±0.03</td>
<td>.0001</td>
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</table>

*1.00 is best possible grade; 2.00 is worst possible grade.

To determine the functional status of TPA in allografts, biopsies were studied with Mab to the principal inhibitor of TPA, PAI-1. Two different Mabs to PAI-1 were used. One of these reacted with a neoptope of PAI-1 that is exposed when PAI-1 is complexed with TPA, thus allowing the identification of nonfunctional TPA molecules. The other Mab reacted with uncomplexed PAI-1. Antibody to the neoptope of TPA-complexed PAI-1 did not react with biopsies from the 35 allografts that contained normal TPA in arteriolar SMCs and no fibrin (Fig 5a). As stated earlier, none of these allografts had TPA-reactive endothelium, an example of which is shown in Fig 1a. However, the 33 allografts that manifested microvascular fibrin and depleted arteriolar SMC TPA frequently contained endothelium that reacted with antibody to TPA and to the neoptope of TPA-complexed PAI-1, allowing the in situ identification of nonfunctional TPA molecules (Fig 5b). In contrast, the Mab to uncomplexed PAI-1 did not react with any vascular tissues in either the normal or depleted TPA group of allograft biopsies. To test for depletion of endothelial cells, the sections were studied with antibodies to von Willebrand factor, and no differences were found between time-zero biopsies and allografts with normal or depleted arteriolar SMC TPA.

Integration of Biopsy and Clinical Data

In the normal TPA group there were no deaths and none of the patients required retransplantation, but in the depleted TPA group there were 11 deaths and two patients were retransplanted. This striking difference was highly significant (P=.0001). A similar order-of-magnitude difference was found when the two groups of patients were evaluated for their clinically assessable functional differences. These results showed that 34 of the 35 allografts with normal TPA were stable, but of the 33 allografts with depleted TPA, only 4 were stable and 29 were unstable (Table 3). Clinical demographics revealed no significant differences between normal and depleted TPA groups for age, smoking, or blood pressure, but there were significant differences for sex ratios and periods of follow-up times (Table 3). The follow-up times for the two groups differed significantly (P=.0001) because of high mortality and retransplantation of patients in the depleted TPA group. The sex ratio differences (ie, 30 men and 5 women in the normal TPA group and 17 men and 16 women in the depleted TPA group) reached a significance level of P=.004, which is consistent with poorer outcomes for female cardiac allograft recipients, as reported by the International Registry of Heart Transplantation.

Laboratory data for 35 patients with normal TPA differed very little from the 33 patients with depleted TPA; no significant differences were found for serum cholesterol or triglycerides, number of individual donor recipient HLA shared antigens for either class I or class II major histocompatibility antigens, or donor-specific cytotoxic antibodies. There was a tendency, although not statistically significant, for patients with depleted TPA to have more serious rejection episodes as measured by conventional histological examination of biopsies, and left ventricular ejection fractions between the two groups were only marginally significant (Table 3).

Evaluation of Failed Allografts

Both hearts removed for retransplantation and 8 of the 11 allografts from patients who died were studied. Immunocytochemical data from these organs were not different; thus, they are reported together as failed allografts. The arteriolar SMC TPA was restricted to sparse and isolated SMCs and usually was absent. Diffuse deposits of fibrin were found in the microcirculation, in the interstitium, and around and within myocardial cells (Fig 6). The microcirculation of these hearts did not react with antibodies to either α2PI or the
plasmin–α2PI neotope, suggesting depressed fibrinolysis. This was supported by calculations that showed maximum fibrin-to-α2PI ratios. The immunological identification of abundant fibrin and the absence of plasmin within the microcirculation of these failed allografts was associated with endothelial reactivity for TPA and TPA–PAI-1 complexes in the same vessels. Results of reacting adjacent serial sections with Mab to α-smooth muscle actin or staining adjacent serial sections with hematoxylin and eosin revealed that the reactive endothelial cells were associated with capillaries, venules, or veins and not with arteries or arterioles. A comparative analysis of these findings with data from time-zero biopsies and with data from clinically stable and clinically unstable allograft recipients is given in Table 4.

The failed allografts were studied by light microscopy. This revealed that four had acute ischemic changes compatible with recent myocardial infarctions, and one of these had a superimposed bacterial infection; three had moderate to severe lymphocytic infiltration with acute myocardial infarctions and severe coronary artery disease; one had severe coronary artery disease with interstitial fibrosis; and two had no histological abnormalities.

**Discussion**

The correlation of immunocytochemical findings with clinical outcome of the 68 patients in this study was telling. Most compelling was the finding that 39% of the 33 patients with consistently depleted arteriolar SMC TPA in their allograft biopsies died or required retransplantation, and none of the 35 patients with consistently normal arteriolar SMC TPA in their allograft biopsies died or required retransplantation. However, a more important finding would not have emerged if the patients had not been studied prospectively over the 54-month period. This is the observation that the status of arteriolar SMC TPA in biopsies obtained during the first postoperative month could be used to classify patients into normal and depleted TPA groups and that the long-term outcome for the depleted TPA group was poor. It is puzzling that such a significant finding declared itself so early in the course of transplantation, and it is unfortunate that no other laboratory measurement assisted in the identification of these high-risk patients.

Three observations in this study reached high levels of significance when biopsies with normal arteriolar SMC TPA were compared with biopsies depleted of TPA. First, time-zero biopsies did not contain immunologically detectable TPA in endothelium, and TPA was localized only on arteriolar smooth muscle cells, but venous and capillary endothelium commonly developed TPA reactivity in biopsies depleted of arteriolar SMC TPA. Earlier investigators have reported endothelial TPA in different organs as well as the presence of TPA mRNA

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**Fig 4.** Biopsy of allograft with depleted arteriolar tissue-type plasminogen activator. a. Section reacted with mouse monoclonal antibody to fibrin and rhodamine isothiocyanate (RITC)-labeled second antibody to mouse IgG. Note reactivity within the microcirculation (arrows). b. Same section reacted with rabbit antibody to α2-plasmin inhibitor and fluorescein isothiocyanate (FITC)-labeled second antibody to rabbit IgG. Note that many of the fibrin-reactive vessels are not plasmin reactive (arrows). Notice that the autofluorescence of lipofuscin is red when viewed through RITC filters (a) and that the same lipofuscin particles in panel b are orange when viewed through FITC filters. This also confirms that the same section is represented in both photomicrographs. Original magnification ×400.

**Fig 5.** Heart biopsies reacted with antibody to tissue-type plasminogen activator–plasminogen activator inhibitor-1 (TPA–PAI-1) complexes. a, Allograft with normal TPA shows an absence of TPA–PAI-1 complexes. b, Allograft with depleted TPA shows diffuse endothelial reactivity of TPA–PAI-1 complexes in the microcirculation (arrows). Original magnification ×400.
TABLE 3. Clinical/Laboratory Data for Allografts With Normal and Depleted TPA

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal TPA (n=35)</th>
<th>Depleted TPA (n=33)</th>
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<tr>
<td>Age, y</td>
<td>50.6±1.3</td>
<td>48.7±2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>30/5</td>
<td>17/16</td>
<td>.004</td>
</tr>
<tr>
<td>Follow-up, mo</td>
<td>32.6±2.3</td>
<td>17.3±2.4</td>
<td>.0001</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>2.9</td>
<td>3.0</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>137.3±1.4</td>
<td>134.7±2.9</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>92.6±1.3</td>
<td>89.5±1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>205.3±12.6</td>
<td>240.7±16.4</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>218.5±6.1</td>
<td>233.1±10.7</td>
<td>NS</td>
</tr>
<tr>
<td>Ejection fraction, Lf</td>
<td>0.60±0.02</td>
<td>0.54±0.02</td>
<td>.03</td>
</tr>
<tr>
<td>Stable/unstable</td>
<td>34/1</td>
<td>4/29</td>
<td>.0001</td>
</tr>
<tr>
<td>Patients dead or retransplanted</td>
<td>0</td>
<td>13*</td>
<td>.0001</td>
</tr>
<tr>
<td><strong>Immunological</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-sharing, No.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHC class I</td>
<td>1.03±0.16</td>
<td>0.93±0.15</td>
<td>NS</td>
</tr>
<tr>
<td>MHC class II</td>
<td>0.34±0.09</td>
<td>0.57±0.17</td>
<td>NS</td>
</tr>
<tr>
<td>Donor-specific cytotoxic antibodies</td>
<td>0/35</td>
<td>0/33</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Histological</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of rejections (grades 3 and 4)</td>
<td>0.29±0.12</td>
<td>0.55±0.14</td>
<td>NS</td>
</tr>
</tbody>
</table>

TPA indicates tissue-type plasminogen activator; BP, blood pressure; and MHC, major histocompatibility complex.

*Eleven died, 2 were retransplanted.

in cultured endothelial cells. Thrombin has been shown to increase the mRNA for TPA in cultured venous endothelial cells, and α-thrombin thus could have caused the increase of endothelial TPA in allografts with depleted arteriolar SMC TPA. Second, time-zero biopsies did not contain immunologically detectable TPA–PAI-1 complexes, but venous and capillary endothelium commonly developed reactivity for these complexes in biopsies depleted of arteriolar SMC TPA. Endothelial cells exposed to α-thrombin increase PAI-1 synthesis, and PAI-1 synthesis also is increased by cytokines such as tumor necrosis factor and interleukin-1. In addition, PAI-1 and PAI-1 mRNA are increased by elevated plasma insulin associated with hypertriglyceridemia. Increased endothelial PAI-1 can bind either cellular or circulating TPA, thereby prohibiting TPA from converting plasminogen to plasmin. The TPA–PAI-1 complexes identified in this study were in areas of fibrin deposition, presumably because PAI-1–bound TPA cannot mobilize fibrin. This is supported by Hunt et al, who recently reported significantly depressed (P = .008) euglobulin clot lysis times in cardiac allograft recipients. Another possibility for the presence of TPA–PAI-1 complexes within the microcirculation could be receptors, distinct from PAI-1, for these complexes on endothelial cells. Third, time-zero biopsies did not contain plasmin inhibitors as measured by immunologically detectable αPI or the neotope of plasmin–αPI complexes, but venous and capillary endothelium commonly developed reactivity for these antigens in biopsies depleted of arteriolar TPA. Their presence indicates the presence of plasmin, and their absence signals an in situ absence of plasmin. The failed allografts did not contain plasmin-associated molecules, thus explaining the presence of abundant fibrin in these biopsies.

Results of this investigation show that depleted TPA from vascular SMCs in allografts is associated with graft failure, but it is not clear what causes the depletion of TPA. Cytokines such as interleukin-1 and tumor necrosis factor can downregulate TPA, and macrophages are a common source of cytokines, but our present and previous experience indicates that macrophages are not always increased in biopsies with immunohistochemical evidence of TPA depletion. Further evidence that

![Heart biopsy from a failing allograft reacted with antibody to fibrin. Note the presence of fibrin within myocardial cells in an area of myocardial infarction (arrows). Original magnification x400.](http://circ.ahajournals.org/lookup/fig/doi/10.1161/01.01.1605)
TABLE 4. Quantitative Comparative Analysis of Hemostatic and Fibrinolytic Components in Biopsies of Allografts of Differing Clinical Status

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>Arteriolar SMC TPA</th>
<th>Endothelial TPA-PAI-1</th>
<th>Endothelial Fibrin</th>
<th>Endothelial α2PI</th>
<th>Fibrin:α2PI Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-zero allografts</td>
<td>1.0±0.00</td>
<td>0%</td>
<td>1.0±0.00</td>
<td>1.0±0.00</td>
<td>1.0±0.00</td>
</tr>
<tr>
<td>(68 hearts)</td>
<td>(none of 68)</td>
<td>(68)</td>
<td>(68)</td>
<td>(68)</td>
<td>(68)</td>
</tr>
<tr>
<td>Stable allografts*</td>
<td>1.14±0.04</td>
<td>11%</td>
<td>1.08±0.03</td>
<td>1.04±0.04</td>
<td>1.07±0.03</td>
</tr>
<tr>
<td>(38 patients)</td>
<td>(4 of 38)</td>
<td>(38)</td>
<td>(38)</td>
<td>(38)</td>
<td>(38)</td>
</tr>
<tr>
<td>Unstable allografts*</td>
<td>1.85±0.04</td>
<td>97%</td>
<td>1.50±0.06</td>
<td>1.22±0.06</td>
<td>1.25±0.03</td>
</tr>
<tr>
<td>(30 patients)</td>
<td>(29 of 30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>Failing allografts</td>
<td>2.0±0.00</td>
<td>100%</td>
<td>2.0±0.00</td>
<td>1.0±0.00</td>
<td>2.0±0.00</td>
</tr>
<tr>
<td>(10 patients)</td>
<td>(10 of 10)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

SMC indicates arteriolar smooth muscle cells; TPA, tissue-type plasminogen activator; TPA-PAI-1, TPA-plasminogen activator inhibitor-1 complexes; α2PI, α2-plasmin inhibitor; and fibrin:α2PI ratio, fibrin-to-α2-plasmin inhibitor ratio, which is an in situ assessment of fibrinolysis. In this quantification scheme, 1.0 indicates a better prognosis than 2.0, as seen by comparing the time-zero with the failed allografts. The failed allografts consisted of eight deaths and two hearts removed for retransplantation.

*P=.0001 for comparison of hemostatic and fibrinolytic components in clinically stable and unstable allografts.

cytokines probably are not solely responsible for TPA depletion is the presence of endothelial thrombomodulin,2,8,11 because thrombomodulin also is downregulated by interleukin-1 and tumor necrosis factor.47 Our results clearly showed that TPA depletion was associated with fibrin deposits, and the most likely cause of fibrin deposition is thrombin-mediated conversion of fibrinogen to fibrin, raising the possibility that thrombin itself may interact directly with vascular SMCs. This is supported by the presence of thrombin receptors on SMCs55 and by the observation that thrombin regulates fibrinolysis in SMCs by affecting the mRNA for TPA and PAI-1.56 Indeed, thrombin in conditioned media of cultured human arteriolar SMCs can abolish TPA activity in the cell lysates,56 and thrombin incorporated into fibrin clots can be released in its active form when the clots are lysed.57 These observations plus reports that thrombin forms gaps between adjacent endothelial cells8,55 suggest that thrombin may participate in the depletion of TPA from vascular SMCs.

The majority of patients with depleted TPA during the first month after transplantation proved to sustain high morbidity and mortality, but a subgroup of this population subsequently developed normal TPA and became clinically stable, indicating that donor arteriolar SMCs have the capacity to regenerate TPA synthesis. The pathophysiology of accelerated hemostasis and TPA depletion in vessels of cardiac allografts remains to be elucidated, but the finding of normal TPA after many months of depletion suggests that this regenerative capacity may be amenable to therapeutic augmentation. Indeed, recent studies of experimental models of TPA synthesis have revealed that the production of this molecule is altered after specific manipulations of cells in vitro. For example, cells grown in the presence of gonadotropins, gonadotropin-releasing hormone, or certain growth factors produce TPA mRNA,60,61 but the effects are transient, they involve different intracellular pathways, and some of the same growth factors promote synthesis of PAI-1 mRNA.61 In addition, cells grown in the presence of retinoids also increase TPA mRNA synthesis and TPA secretion,2,62 and these reactions are not accompanied by increased PAI-1 synthesis.32 Alternatively, recent experimental63,64 and clinical65,66 data indicate that heparin augments the ability of TPA to exert its catalytic function in fibrinolysis. Heparin has the additional advantage of inhibiting the proliferation of SMCs,67 the migration of which accounts for some of the lesions found in transplant-induced vasculopathies.11,68

Thrombogenesis is controlled physiologically by the natural anticoagulant pathways.2,3,9,10 The reactions of these molecules normally range from direct inhibition of endothelial tissue factor by the tissue factor pathway inhibitor69 to the neutralization of thrombin by antithrombin III and the conversion of thrombin to an anticoagulant by thrombomodulin.70 There is very little information about the roles of most of the natural anticoagulant pathways in organ allografts,3,5,10,70,71 but it is known that the endothelial heparan sulfate proteoglycan–antithrombin III pathway is poorly represented in cardiac allografts with significant vascular disease compared with matched time-zero biopsies.2,8,9,11,15 In these cases, the loss of thromboreistance as measured by the appearance of fibrin was correlated with the loss of immunologically detectable antithrombin III from endothelial plasma membranes, and a survival advantage was reported in allograft recipients whose biopsies demonstrated the presence of antithrombin III on capillary endothelial cells.7 Inasmuch as these studies also reported no consistent correlation with cellular infiltrates,8,15 we propose that metabolically perturbed natural anticoagulant pathways allow thrombin to be generated and that the inhibited thrombin mediates fibrin deposition, which augments thrombin-driven secretion and subsequent depletion of TPA from its cellular reservoirs.

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