Tumor Necrosis Factor Production during Human Renal Allograft Rejection Is Associated with Depression of Plasma Protein C and Free Protein S Levels and Decreased Intragraft Thrombomodulin Expression

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Summary

Fibrin deposition is a common accompaniment of renal allograft rejection, indicating disruption of the normal physiologic balance between procoagulant and anticoagulant pathways. In vitro, tumor necrosis factor (TNF) induces endothelial expression of the procoagulant, tissue factor, and downregulation of thrombomodulin, a key component of the thrombomodulin/protein C (PC)/protein S (PS) pathway, which normally maintains an anticoagulant state by inactivating thrombin, preventing further thrombin formation by degrading factors Va and VIIIa, and decreasing plasminogen activator inhibitor activity. Raised levels of TNF were recently demonstrated within the blood of patients during episodes of renal allograft injection, and may be an early and discriminatory marker of rejection. This led us to investigate prospectively whether monitoring of serum TNF levels was of value clinically, and was associated with effects on circulating PC and PS levels, or alterations in intragraft thrombomodulin expression. Plasma samples (n = 454) were collected three times/week from all patients (n = 25) undergoing renal transplantation during a 9-month consecutive period, and assayed by ELISA and functional assays for TNF, PC, and free PS (FPS). Portions of renal biopsies, taken to evaluate episodes of acute deterioration of renal function, were evaluated by immunoperoxidase labeling for the presence and distribution of TNF, thrombomodulin, PC, PS, thrombin, fibrin, and factors V and VIII. Comparison of 78 plasma samples collected during 26 episodes of biopsy-proven acute cellular rejection with samples collected during periods of stable renal function (n = 349) showed that TNF levels rose significantly (390 ± 242 pg/ml, $p < 0.01$) above background levels 3 days before rising serum creatinine concentrations, and peaked (2,426 ± 978 pg/ml) on the day of clinical rejection. PC-antigen (Ag) concentrations also decreased 3 days before rejection (68 ± 13%, $p < 0.05$), and were maximally depressed (49% ± 16%, $p < 0.001$) on the day of rejection. FPS levels were normal until the day before rejection (63% ± 8%, $p < 0.01$) and, like PC, were maximally depressed (43 ± 10%) at rejection. Plasma TNF levels were significantly and inversely correlated with PC-Ag ($p < 0.001$) and FPS ($p < 0.005$) levels during rejection, regardless of whether such rejection episodes were steroid responsive or required OKT3 monoclonal antibody therapy. TNF, PC, and FPS levels were normal during episodes of cyclosporine toxicity and viral infection. Immunoperoxidase studies showed that rejection was associated with a intrarenal production of TNF, decreased microvascular labeling for thrombomodulin, and widespread endothelial and interstitial deposition of PC, PS, thrombin, fibrin, and factors V and VIII. These findings suggest that TNF is an important mediator of renal allograft rejection, causing depression of the intragraft thrombomodulin/PC/PS pathway and thereby contributing to intragraft fibrin deposition. Moreover, these results indicate that monitoring of circulating concentrations of TNF, PC, and FPS has relevance to the early detection and differential diagnosis of human acute renal allograft rejection.

Human acute interstitial renal allograft rejection is associated with progressive graft infiltration by mononuclear cells, particularly macrophages, $CD8^+$ T cells, and lesser numbers of $CD4^+$ T cells (1, 2). Some 10–20% of interstitial mononuclear cells present in renal biopsies taken at the time of rejection show features of immune activation

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such as expression of IL-2R (CD25) and the procoagulant molecule, tissue factor (3, 4). Intragraft macrophage and endothelial expression of tissue factor are associated with extensive local fibrin deposition, whereas CD25, tissue factor expression, and widespread fibrin deposition are not present in biopsies from patients with cyclosporin toxicity or normal renal function posttransplantation (3, 4). A pivotal role for these IL-2R$^+$ mononuclear cells in mediating kidney rejection is demonstrated by the successful treatment or, in some cases, prevention of allograft rejection in clinical (5) and experimental (6, 7) studies using CD25 mAbs.

How allograft rejection occurs, once immune activation has taken place, is still only understood in very broad terms. An increasing number of cytokines, whose production and actions are known largely through in vitro studies, are implicated by monitoring peripheral blood or urine samples from renal transplant recipients undergoing episodes of allograft rejection. These include TNF (8), IL-1 (9), IL-2 (10) and its corresponding IL-2R (11), and IL-6 (12). However, with the exception of IL-2, whose suppression of production by cyclosporin is considered central to current immunosuppressive therapy (13), the contribution and relative importance of such cytokines to the rejection process in vivo are unknown.

TNF, produced by activated macrophages and to a lesser extent, activated T and B cells, is a pleiotropic cytokine involved in host defenses against parasitic infections and tumors, and mediation of septic shock (14). We have focused on the potential role of TNF as an effector molecule in renal allograft rejection for several reasons. First, TNF, at least in vitro, profoundly alters the thrombogenicity of endothelial cells by inducing expression of procoagulant molecules (15) and downregulating expression of thrombomodulin (16), a key component of the physiologically potent thrombomodulin/protein C (PC)[1]/protein S (PS) anticoagulant system (17). In addition, TNF induces endothelial expression of leukocyte adhesion molecules (18, 19), production of IL-1 (20, 21) and IL-6 (22), and upregulation of the density of class I MHC antigens (23). Second, recent studies in rats have shown that intragraft mononuclear cells produce TNF in vivo during acute kidney rejection (7), and that anti-TNF antibodies prolong allograft survival (24). Last, immunohistologic and functional studies of cardiac allografts in untreated rats showed that TNF production by activated (IL-2R$^+$) intragraft mononuclear cells was associated with local downregulation of thrombomodulin expression, induction of tissue factor expression, and widespread intragraft fibrin deposition, whereas these features were absent in allografted rats that were treated with cyclosporin and showed long-term graft survival (25). Hence, many of the actions attributable to TNF in vitro appeared to be also demonstrable in vivo during allograft rejection.

The current study determined the levels of TNF, PC, and PS within serial blood samples from renal transplant recipients during the first 2–3 wk posttransplantation. In addition, diagnostic renal biopsies taken during episodes of deterioration in renal function were evaluated by immunoperoxidase labeling for endothelial expression of thrombomodulin, and deposition of PC, PS, and fibrin. The results demonstrate the clinical relevance of TNF as a mediator of human renal allograft rejection.

Materials and Methods

Patients and Immunosuppressive Therapy. All adult patients undergoing renal transplantation at Prince Henry's Hospital over nine consecutive months were studied prospectively. Patients received 1 g of methylprednisolone at the time of surgery, and were initially immunosuppressed according to a standard "induction therapy" protocol consisting of prophylactic OKT3 mAb (5 mg/d i.v., for 7–10 d, beginning on the day of transplantation), plus azathioprine (0.5 mg/kg/d) and prednisolone (20 mg/d). Patients thereafter received triple therapy with cyclosporin, azathioprine, and prednisolone. Azathioprine was increased to 2 mg/kg/d upon cessation of OKT3 mAb treatment, steroid therapy was maintained indefinitely, and cyclosporin was introduced, once serum creatinine levels had fallen to <250 μM, initially at a dose of 8 mg/kg/d, and thereafter according to the results of cyclosporin trough whole-blood RIA. Rejection episodes were treated for 3 d with pulse doses of intravenous methylprednisolone (0.5 g/d), followed, if steroid unresponsive, by a 10–14-d course of OKT3 mAb (5 mg/d i.v.). Cyclosporin was discontinued during OKT3 mAb treatment and was recommenced 2 d before cessation of mAb therapy.

Blood Samples. Citrated plasma samples were collected three times/wk beginning at the time of transplantation, and continuing throughout the period of hospitalization, as well as at weekly outpatient follow-up sessions for 3 mo postdischarge. Plasma was isolated promptly by centrifugation, coded, and stored in aliquots at −80°C. A normal plasma pool was prepared by collection of citrated plasma from 10 healthy lab staff (six males, four females) not receiving anticoagulant or other medication..

Tissue Samples. Episodes of deterioration of renal function, in conjunction with clinical suspicion of rejection, were evaluated by renal biopsy. Biopsy cores were divided into portions that were: (*a*) formalin fixed and paraffin embedded, for standard light microscopy and immunoperoxidase analysis of immune reactants (C1q, C3, IgA, IgG, IgM, fibrinogen), or (*b*) fixed at 4°C in paraformaldehyde-lysine-periodate (26), washed overnight in PBS/7% sucrose, and quick frozen in liquid nitrogen, with storage at −80°C, before immunoperoxidase studies on cryostat sections, as detailed below. Control normal tissues (n = 4) were obtained from unused donor kidneys or kidneys resected for localized tumors. Portions of kidneys showing severe interstitial and/or vascular rejection (n = 4), obtained after transplant nephrectomy for endstage rejection, were used as positive control tissues.

Antibodies. Peroxidase-conjugated and unconjugated rabbit anti-human PC and PS antibodies, plus peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig, rabbit anti-human von Willebrand factor, and rabbit anti-human fibrinogen/fibrin were obtained from Dako (Sydney, Australia). Rabbit anti-human TNF was obtained from Genzyme (Boston, MA). mAbs and rabbit polyclonal antibodies to human PC, PS, thrombomodulin, factor V, and thrombin were prepared by Prof. H. Salem (Monash University, Melbourne, Australia). Additional gifts of mAbs included mAb to the activation peptide of PC (PC-121) (27), from Prof. F. Walker (American Red Cross, Farmington, CT); mAb to crosslinked fibrin (T2G1), from Dr. B. Kudryk (The New York Blood Center, New

[1] *Abbreviations used in this paper:* Ag, antigen; FPS, free protein S; PC, protein C; PS, protein S.

York); and mAb to TNF (J2D10) (28), from Prof. I. McKenzie (Melbourne University, Parkville, Australia). Production and use of PHM5, a mAb that gives dense labeling of all renal endothelium, as well as glomerular podocytes, in normal and rejecting kidneys, were described previously (29).

TNF Bioassay and ELISA. TNF levels in patient plasma samples were measured using the L929 fibroblast cytotoxicity bioassay (30), in the presence of actinomycin-D (Sigma Chemical Co., St. Louis, MO), and plates were stained with coomassie blue and their absorbance read at 540 nm. Samples containing significant bioactivity (> 100 pg/ml) were evaluated using a capture ELISA. Briefly, microtiter trays (Nunc, Sydney, Australia) were coated overnight with anti-TNF mAb (10 μg/ml), blocked with 5% powdered milk, and incubated successively with patient samples, rabbit anti-human TNF, swine anti-rabbit Ig-peroxidase conjugate, and *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma Chemical Co.); absorbances were read at 490 nm. Results in both assays were expressed as picograms per milliliter, calculated by the ELISA reader (MR-5000; Dynatech, Sydney, Australia) from a standard curve generated using serial dilutions of human r-TNF (Genzyme). The sensitivity of each method was 20 pg/ml, and the results achieved by bioassay and ELISA were highly correlated ($r = 0.83$, $n = 126$).

Protein C ELISA and Amidolytic Assay. PC antigen was measured using a capture ELISA, whereby microtiter trays were coated overnight with rabbit anti-human PC antibody, blocked with 5% powdered milk, and incubated successively with patient or normal pooled plasma (diluted 1:100 in PBS/0.1% Tween-20), peroxidase-conjugated rabbit anti-human PC, and OPD substrate. To assess whether alterations in PC antigen levels were associated with decreased PC function, PC amidolytic activity was determined by chromogenic assay (31), using an Actichrome PC kit (American Diagnostica, Sydney, Australia). Briefly, this involved activation of plasma PC by Protac, derived from the venom of *Agkistrodon contortrix*, and measurement of cleavage (absorbance at 405 nm) of the activated PC-specific substrate, Spectrozyme, with or without addition of a neutralizing antibody to PC. Both assays were performed in triplicate and their results, expressed as a percent of normal pooled plasma, were highly correlated ($r = 0.90$, $n = 48$).

Protein S ELISA. Unseparated plasma samples were assayed for total PS, and supernatants remaining after precipitation with polyethylene glycol and centrifugation were assayed for free protein S (FPS) using a capture ELISA (32). Briefly, microtiter trays were coated overnight with rabbit anti-human PC antibody, blocked with powdered milk, and incubated with plasma (diluted 1:100 in PBS/0.1% Tween-20), peroxidase-conjugated rabbit anti-serum PC, and OPD substrate. Assays were performed in triplicate, and results were expressed as a percent of normal pooled plasma.

Immunoperoxidase Studies. Cryostat sections of PLP-fixed renal biopsies and control kidney specimens were evaluated by immunoperoxidase labeling with mAbs and rabbit polyclonal antibodies to thrombomodulin, PC, PC activation peptide, PS, thrombin, crosslinked fibrin, factor V, and von Willebrand factor. Antibodies were localized using a two-layer indirect immunoperoxidase method, followed by incubation with the substrate, diaminobenzidine, counterstaining with hematoxylin, and mounting (26). Our previous studies have shown that cytokines such as TNF are poorly preserved in PLP-fixed tissues, presumably through failure of adequate crosslinking and immobilization of these small molecular weight glycoproteins, whereas they can be detected in corresponding acetone-fixed sections prepared from the same tissues (6, 25). Hence, cryostat sections of the unfixed portions of diagnostic renal biopsies that remained after immunofluorescent examination in six patients were fixed in acetone and evaluated using an anti-TNF mAb and a four-layer immunoperoxidase technique (26).

Analysis of Results. Plasma assays and immunoperoxidase labeling were performed and evaluated routinely without knowledge of the patient's clinical course. Abnormal plasma results were confirmed by re-assay on at least one later occasion; interassay variation was consistently <10%. After decoding, test results were compared with clinical and histopathologic data. Analysis was performed using the Statfast statistics program (Statsoft, Tulsa, OK). *t* test analysis of TNF results was determined using $\log_{10}$-transformed data, or untransformed results in the case of PC and PS data; similar results were found by nonparametric analysis using the Mann-Whitney U test.

Particular attention was focused on: (*a*) the results of assays of samples taken around the time of proven rejection episodes, and conversely, (*b*) whether depressed plasma PC or FPS results, or raised plasma TNF levels, were demonstrable in the absence of clinically detected rejection episodes. Rejection episodes were determined by review of the medical records and the results of renal biopsy. A rejection episode was defined as a sudden (>40 μM/liter) and progressive rise in serum creatinine which, on renal biopsy, was associated with a standard histologic picture of acute interstitial and/or vascular rejection (1), and which responded to increased immunosuppression.

The intensity of immunoperoxidase labeling was graded semiquantitatively as trace (±), weak (+), moderate (2+), or strong (3+), focusing especially on the extent of residual labeling of endothelial cells using antibodies to thrombomodulin, as well as the distribution of TNF, PC, PS, and crosslinked fibrin. Intertubular capillaries, which are readily collapsed and hence difficult to recognize in cryostat sections even in normal biopsies, and which may be depleted during severe rejection (33), were identified by staining of adjacent sections with PHM5 mAb.

Results

Patient Data. The study group consisted of 25 consecutive adult renal transplant recipients, who received kidneys from 21 cadaveric and four living related donors. Recipients consisted of 14 males and 11 females, with a mean age of 39 yr, (range 22–56 yr). Serial blood collection during the period of hospitalization, plus at regular follow up for 3 mo postdischarge, yielded 454 plasma samples; an average of 18 ± 6 samples/patient were analyzed.

Normal Plasma Data. TNF plasma concentrations in normal laboratory workers ($n = 10$) were consistently low by both bioassay (43 ± 25 pg/ml) and ELISA (28 ± 21 pg/ml) methods, consistent with data from normal subjects reported by others (29). Hence, a cutoff was established whereby TNF blood concentrations of <100 pg/ml were considered within the normal range (mean ± 2 SD). Similar results were detected using serum samples from the same patients, or when plasma samples were defibrinated by heating to 56°C followed by rapid cooling on ice and centrifugation. Analysis of individual normal plasma samples (mean ± SD) showed: PC antigen (PC-Ag), 104 ± 14%; PC amidolytic activity, 105 ± 9%; total PS, 99 ± 10%; and FPS, 103 ± 14%.

Plasma Data during Acute Rejection. TNF (mean ± SD) and creatinine data from 78 plasma samples collected during the course of 26 episodes of biopsy-confirmed acute cellular

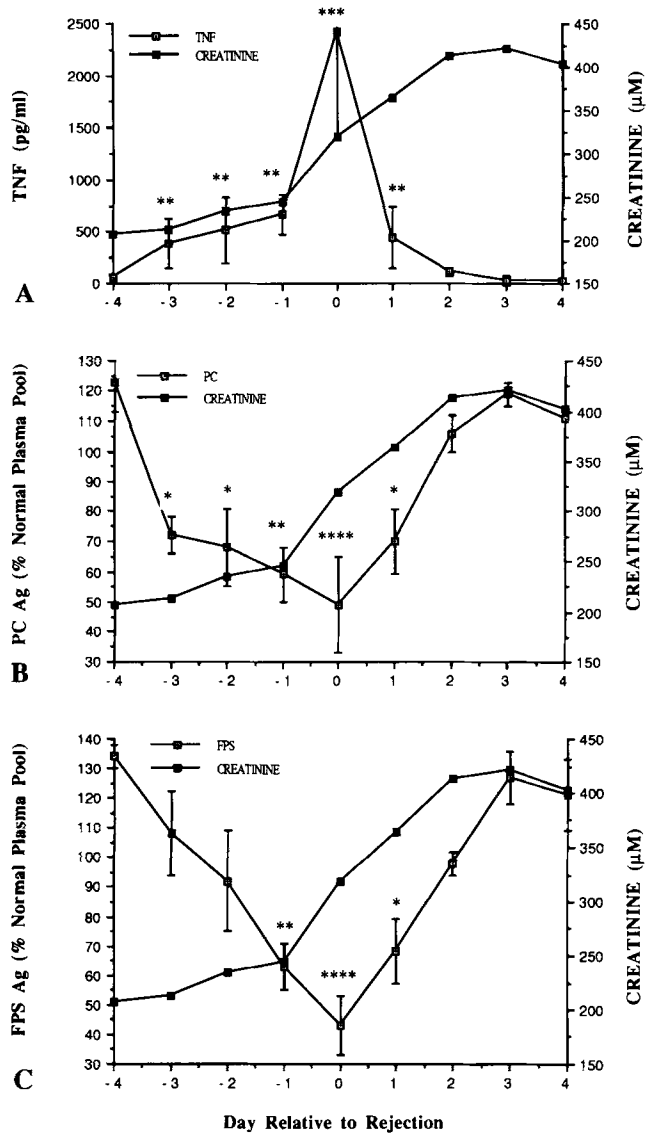

Figure 1. Cumulative serial data (mean ± SD) from blood specimens (n = 76) showing alterations in (A) TNF, (B) PC, and (C) FPS levels during 26 episodes of acute renal allograft rejection. The day of rejection was defined as described in Materials and Methods; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

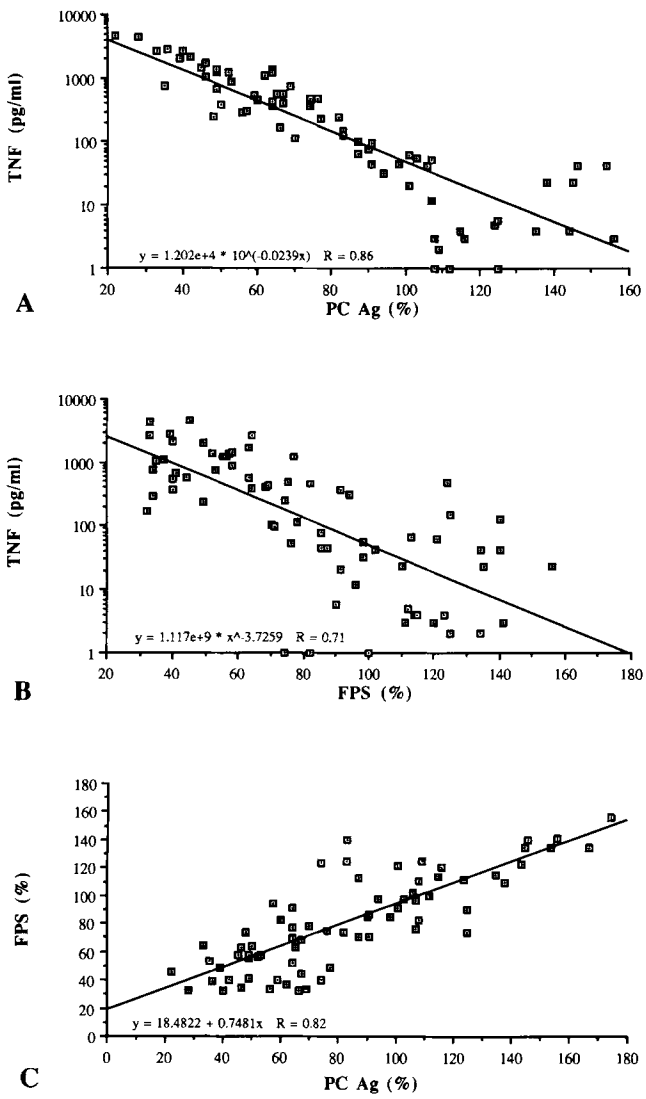

Figure 2. Correlations between (A) TNF and PC antigens, (B) TNF and FPS antigens, and (C) FPS and PC antigens in 76 samples collected at the time of renal allograft rejection.

rejection are summarized in Fig. 1 A. TNF concentrations began to rise (390 ± 242 pg/ml, $p < 0.01$) 3 d before any substantial increase in serum creatinine levels, and peaked (2,426 ± 978 pg/ml) on the day of rejection. Plasma TNF concentrations typically returned to basal levels within 2–3 d of this peak, in conjunction with high-dose, pulse steroids (n = 20) or OKT3 mAb (n = 6) administration, whereas creatinine concentrations often took 7–10 d to approximate pre-rejection values.

Rejection episodes were also associated with decreased levels of plasma PC (Fig. 1 B) and FPS (Fig. 1 C). PC-Ag concentrations were mildly depressed 2–3 d before rejection (~70%, $p < 0.05$), and reached a nadir of 49 ± 16% ($p < 0.001$) on the day of rejection (Fig. 1 B). Corresponding PC amidolytic activity estimations showed an almost identical pattern to PC-Ag results during acute rejection (results not shown). FPS levels dropped to 63 ± 8% ($p < 0.01$) 1 d before rejection, and were maximally depressed (43 ± 10%) at the time of rejection (Fig. 1 C). Plasma TNF levels were significantly and inversely correlated with PC-Ag (r = 0.86, $p < 0.001$; Fig. 2 A) and FPS (r = 0.71, $p < 0.005$; Fig. 2 B) levels during rejection episodes. PC-Ag and FPS results were directly correlated with each other (r = 0.82, $p < 0.001$) (Fig. 2 C).

Examples of serial plasma TNF, PC, and FPS data from patients with steroid-responsive or unresponsive acute rejection are shown in Fig. 3, A and B. The first patient, a 50-yr-old female who received a transplant from a living related donor, showed excellent renal function postoperatively. However, at 3 wk posttransplantation, her serum creatinine jumped 80 μM/liter (day 0; Fig. 3 A), in conjunction with clinical features of rejection. Renal biopsy on day 0 showed focal,

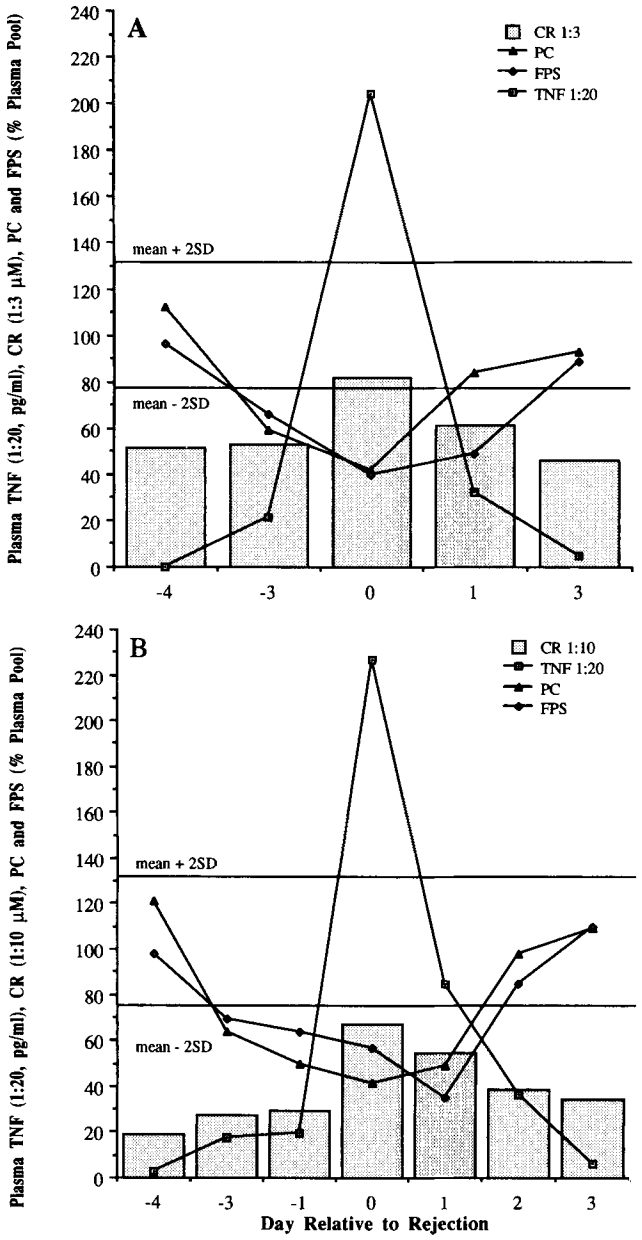

Figure 3. Representative examples of serial TNF, PC, and FPS levels during renal allograft rejection that was either (*A*) steroid responsive; or (*B*) steroid unresponsive, necessitating treatment with OKT3 mAb for 10 d. Composite graphs show TNF and creatinine data after proportionate reduction, as indicated in each panel. Mean ± 2 SD indicates normal ranges for PC and FPS antigens.

intense mononuclear cell infiltration around vessels and within interstitial areas, with intense tubular infiltration but only mild interstitial edema, no hemorrhages or thrombosis, and no evidence of vascular rejection; this pattern was reported as moderate interstitial rejection. The patient was treated for three consecutive days with intravenous steroids, and her renal function returned to normal. As seen in Fig. 3 *A*, TNF was detected 3 d before rejection (428 pg/ml), peaked on the day of rejection (4,089 pg/ml), and rapidly fell in association with steroid administration. Conversely, PC and FPS levels were 59% and 66%, respectively, 3 d before rejection, fell to 42% and 40%, respectively, on day 0, but rose to within the normal ranges by day 3.

The second patient, a 45-yr-old male recipient of a cadaveric donor kidney, demonstrates comparable results during steroid-unresponsive acute rejection (n = 6). This patient did well for the first postoperative week but then, as cyclosporin was introduced, experienced gradually decreasing renal function over several days. A renal biopsy, performed to try and differentiate between possible rejection, cyclosporin toxicity, and acute tubular necrosis, showed a mixed picture of a very light accumulation of mononuclear cells at the corticomedullary junction, plus tubular dilatation and degeneration, consistent with probably very mild interstitial rejection and resolving acute tubular necrosis. However, 3 d later, despite intervening administration of three bolus doses of intravenous steroids (days −3, −2, and −1; Fig. 3 *B*), the patient's serum creatinine rose dramatically. A biopsy on day 0 showed mild interstitial rejection, with interstitial edema and a mixed mononuclear cell infiltrate that encroached upon tubular epithelium. OKT3 mAb therapy was undertaken for 10 d, beginning on day 0, and the patient's clinical condition and renal function gradually returned to pre-rejection levels. In this patient, TNF levels remained at low, but significant, levels during the period of steroid administration (350–400 pg/ml), rose to 4,539 pg/ml on day 0, and then returned to normal by day 3, in association with OKT3 mAb administration. PC and FPS levels were maximally depressed to 41% and 35% on days 0 and 1, respectively.

A case of severe accelerated rejection occurred in a 35-yr-old male who received a transplant from a living related donor after a negative crossmatch. On the day after transplantation, this patient developed a fever of 38.7°C, and a postoperative renal scan showed greatly decreased graft perfusion. The patient's serum creatinine levels began to rise markedly (>100 μM) each day, and a renal biopsy was performed on day 4 posttransplantation. The biopsy showed severe vascular and interstitial rejection, with glomerular microthrombi, marked interstitial hemorrhages and mononuclear cell infiltration, extensive vascular wall infiltration, and widespread vascular IgM and C3 deposition. The patient, who had not received OKT3 prophylactic therapy, was begun immediately on a 14-d course of OKT3 mAb, and renal function and clinical parameters returned to normal by day 14. Study results in this patient were preoperative: TNF (109 pg/ml), PC (100%), and FPS (105%); day 1 posttransplantation: TNF (547 pg/ml), PC (92%), and FPS (103%); and day 4 (as OKT3 mAb was commenced): TNF (718 pg/ml), PC (61%), and FPS (63%); TNF, PC, and FPS values returned to normal by day 7, and subsequent days, posttransplantation.

Nonrejection Plasma Data. Results of assays of TNF, PC-Ag, and FPS-Ag in all patients and normal controls are summarized in Table 1. Rejection episodes were associated with significant elevation of plasma TNF and depression of PC and FPS, in contrast to samples (n = 349) collected during periods of stable renal function. Normal data were also found during episodes of cyclosporintoxicity, which was diagnosed in six patients based on elevated cyclosporin levels (> 800

Table 1. *Plasma TNF, PC, and FPS Levels in Renal Transplant Recipients or Normal Controls*

Group	No. samples (No. individuals)	TNF	PC antigen	FPS antigen
		pg/ml	*%*	*%*
Normal controls	35 (10)	43 ± 25	104 ± 14	103 ± 14
Stable renal function	349 (25)	23 ± 25	117 ± 21	109 ± 16
Acute rejection	78 (19)	1,128 ± 838*	69 ± 19‡	59 ± 18‡
CsA toxicity	16 (6)	76 ± 31	131 ± 32	115 ± 26
CMV infection	8 (2)	68 ± 71	135 ± 28	115 ± 20
Gram-negative sepsis	3 (1)	698 ± 269*	68 ± 24*	70 ± 41§

All data are mean ± SD. TNF bioassay results are shown; acute rejection data include all samples from day -3 to day 3 of rejection, as defined in Materials and Methods.
* $p < 0.01$ compared with normal controls or to transplant recipients with stable renal function.
‡ $p < 0.005$.
§ $p < 0.05$.

ng/ml), declining renal function, and response to reduction in the drug's dosage. Data were also normal in samples from two patients that were collected at the time of CMV infection, documented by the presence of intranuclear inclusion bodies on renal biopsy and serologic evidence. A single patient experienced an episode of Gram-negative septicemia, during which TNF, PC, and FPS data were abnormal and comparable with that seen in patients with acute rejection.

Immunoperoxidase Data. Histologic and immunofluorescent evaluation of 23 consecutive biopsies confirmed the presence of acute, predominantly interstitial, rejection with or without added vascular rejection. Unusual prominence of small vessel endothelial cells was remarked upon by the renal pathologist in the routine histologic reports of 18 of the biopsies, consistent with the concept of endothelial activation during rejection. The results of immunoperoxidase localization of thrombomodulin, PC, PS, and fibrin are summarized in Table 2; no staining was seen using isotype-matched control mAbs.

Rejection was associated with a marked decrease or absence of intertubular capillary thrombomodulin expression, in contrast to the dense labeling of all renal endothelium seen in kidneys collected for organ donation or removed for unrelated, therapeutic purposes. Such decreased staining was specific to the microvasculature, since thrombomodulin was generally expressed at normal levels by adjacent arterioles or larger vessels and by glomerular capillaries. In one-third of cases, thrombomodulin was also detected as interstitial labeling on and adjacent to the membranes of collections of mononuclear cells, especially macrophages, which were sites also rich in deposition of thrombin and crosslinked fibrin. Rejection was also associated with interstitial and capillary deposition of PC and PS (Table 2, Fig. 4), von Willebrand factor, and, in five cases, factor V. Areas of heavy PC deposition generally lacked corresponding labeling for PC activation peptide; the latter, when present, was usually only detected within tubular lumina or the cytoplasm of tubular cells. Examples of paired staining for thrombomodulin and PC and PS before and after treatment for acute rejection are shown in Fig. 4, which demonstrates the dynamic nature of alterations in

Table 2. *Distribution of Thrombomodulin, PC, PC Activation Peptide, PS, and Fibrin in Renal Biopsies From Patients with Acute Renal Allograft Rejection vs. Normal Controls*

Group	No.	TM	PC	PC activation peptide	PS	XL-fibrin
Normal controls	4	3+all endo	-	-	-	-
Mild rejection	7	± ITC	3+i/s and ITC	± i/s, 2+tubules	2-3+i/s and ITC	2+i/s, and ITC
Moderate rejection	13	± or -ITC	2-3+i/s and ITC	focal i/s, 2+ tubules	2+i/s and ITC	2-3+i/s and ITC
Severe rejection	3	-, except large arteries	2+i/s and ITC	3+tubules	2+i/s and ITC	2-3+i/s and ITC

Graded semiquantitatively as described in Materials and Methods. i/s, interstitial; ITC, intertubular capillaries; XL-fibrin, crosslinked fibrin.

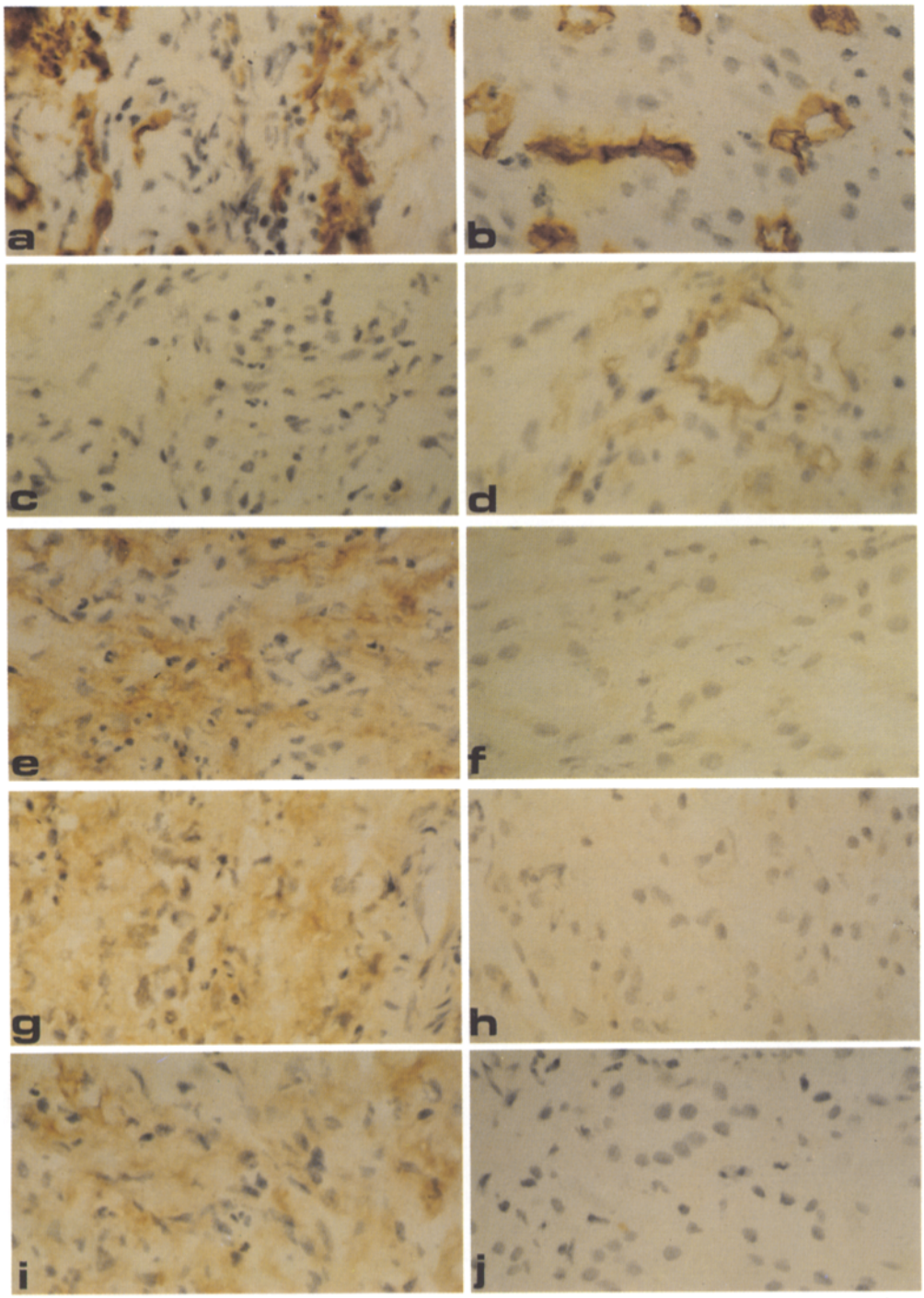

Figure 4. Immunoperoxidase localization of thrombomodulin, PC, PS, and crosslinked fibrin in sequential renal biopsies from a patient with acute rejection. Panels on the left are from a biopsy at day 14 posttransplantation that showed mild to moderate interstitial rejection; plasma TNF was 1,560 pg/ml, PC-Ag = 52%, and FPS = 44%. Panels on the right are from a biopsy taken 10 d later, after treatment of rejection with methylprednisolone, which showed resolving interstitial rejection; plasma TNF was <20 pg/ml, PC-Ag = 94%, and FPS = 89%. (*a* and *b*) Intertubular capillaries within each transplant biopsy are labeled with PHM5 mAb. (*c* and *d*) There is essentially no expression of thrombomodulin on intertubular capillaries during rejection, whereas after rejection therapy, the interstitial mononuclear cell infiltrate has decreased and intertubular capillaries again show labeling for thrombomodulin. (*e* and *f*) PC deposition is noted over capillaries and adjacent interstitial areas during rejection, as is (*g* and *h*) PS, whereas both are cleared on follow-up biopsy. (*i* and *j*) Widespread interstitial deposition of crosslinked fibrin is present during rejection, but not at resolution 10 d later. Hematoxylin counterstain, PLP fixation, indirect immunoperoxidase, ×300.

thrombomodulin expression and deposition of PC, PS, and fibrin.

All six patients with acute rejection in whom unfixed renal biopsy material was available showed staining for TNF. TNF was typically demonstrated as dense labeling of interstitial mononuclear cells and renal microvasculature (Fig. 5), whereas control sections from normal kidneys were unstained.

Discussion

This study was based on the concept that since rejection is an immunologic event, features of immune activation should not only be demonstrable, but indeed might be of use in the early detection and differential diagnosis of episodes of acute renal failure in the early posttransplant period. We have previously shown that human renal transplant rejection is as-

Figure 5. Immunoperoxidase detection of TNF within a renal biopsy of patient with acute cellular rejection showing dense labeling of large numbers of interstitial mononuclear cells and small vessel endothelium. Hematoxylin counterstain, acetone fixation, ×400.

sociated with intragraft mononuclear cell activation, as reflected by mononuclear cell expression of IL-2R and procoagulant antigens (4); these results were subsequently confirmed by Serón et al. (34). However, the development of faster, less invasive tests for detection of rejection would greatly facilitate the clinical management of patients who present such diagnostic dilemmas as the need to distinguish acute rejection from cyclosporin toxicity, viral infection or, on occasions, slowly resolving acute tubular necrosis. Careful studies by Colvin et al. (11) indicated that serial monitoring of peripheral blood IL-2R concentrations appeared to be of diagnostic value in the detection of kidney rejection, and this approach is under evaluation in many centers worldwide. Though obviously relevant to the practical diagnosis of rejection, such measurements do not directly improve our understanding of the precise molecular mediators of graft rejection. However, Maury and Teppo (8) reported increased serum levels of TNF during acute rejection, as well as during infection, indicating that TNF might be a clinically relevant mediator of rejection whose actions could be analyzed in vivo and in vitro. No data were presented in the latter study concerning TNF levels during episodes of cyclosporin toxicity, and reservations about the accuracy of diagnosis of rejection episodes arose since rejection was diagnosed only upon clinical criteria and limited fine needle aspiration studies. We therefore undertook: (*a*) a prospective, controlled study of the significance of TNF monitoring in renal transplant recipients, and (*b*) given the potent effects of TNF on endothelial cells in vitro, and previous evidence of macrophage and endothelial procoagulant expression during rejection (4), evaluation of whether TNF production during rejection was associated with effects on intragraft thrombomodulin/PC/PS anticoagulant system expression.

Plasma levels of TNF were reproducibly elevated in renal transplant recipients during episodes of biopsy-proven acute rejection, typically rising 2–3 d before elevation of serum creatinine data, and returning to normal with successful antirejection therapy. Such rejection-associated increases in plasma TNF were not simply attributable to OKT3 mAb–induced release (35), since plasma TNF levels rose before OKT3 mAb administration, and were also apparent in steroid-responsive cases not requiring OKT3 therapy. Conversely, TNF levels were not elevated during episodes of cyclosporin toxicity or CMV infection, but were elevated during the sole case of bacterial sepsis. Septicemic patients generally show marked hemodynamic alterations and positive blood cultures, and so are not usually difficult to discriminate from patients with acute rejection. Hence, given the good correlation observed between bioassay and ELISA methods, indicating the presence of both immunoreactive and biologically active TNF, these results show that monitoring of serial TNF levels in peripheral blood by ELISA is a rapid, useful, and practical aid to the early diagnosis of acute kidney rejection, and its discrimination from other causes of declining renal function and mononuclear cell infiltrates, such as episodes of cyclosporin toxicity or viral infection. Though the source of TNF was not identified in the current study, our studies in experimental rat renal (7) and cardiac (25) transplant models have shown that TNF is produced by graft infiltrating mononuclear cells, especially macrophages, during rejection, and spills over to be detectable within the peripheral blood, though at considerably lower concentrations than within corresponding graft homogenates.

PC and PS concentrations in plasma samples from renal transplant recipients were determined since we had previously shown that rat cardiac and renal transplant rejection was associated with intragraft production of TNF and widespread PC and PS deposition (25), resulting in low concentrations of PC and PS in corresponding blood samples. Moreover, preliminary screening of blood samples from patients with renal, cardiac, or liver transplant rejection showed depression of PC levels during rejection episodes, consistent with this concept of intragraft consumption of anticoagulant molecules during rejection. In the current study, we found that plasma PC and FPS concentrations in patients with stable renal function were comparable with concentrations in normal controls, but fell to <50% of normal during rejection, in inverse correlation to rising plasma TNF levels. Like TNF, PC and FPS data were also normal during episodes of cyclosporin toxicity or CMV infection, but fell significantly during bacterial sepsis, as previously reported (36). These findings indicate that acute rejection of human renal transplants is associated with an acquired deficiency of PC and FPS, which may contribute to the increased incidence of venous thromboembolism in renal transplant recipients (37, 38). Recently, Sorensen et

al. (39) also reported that in at least 7 of 11 renal transplant recipients, PC coagulant activity was lowered in parallel with increasing creatinine concentrations and development of clinical graft rejection; however, no data were presented on PS or TNF levels, nor were any renal biopsy data provided.

Immunohistologic studies showed that acute rejection was associated with local expression of TNF and marked reduction of endothelial labeling for thrombomodulin. Such reduction was not simply due to destruction of renal microvascular cells during severe rejection, as previously noted (33), since intertubular capillaries were still readily detected in sequential sections using PHM5 mAb (Fig. 4). Decreased thrombomodulin expression was largely confined to intertubular capillaries, arterioles, and venules, since endothelial cells of adjacent larger vessels continued to show strong labeling with antithrombomodulin antibodies during rejection episodes, and thrombomodulin microvascular expression rapidly returned to normal as antirejection immunosuppressive therapy was implemented. The association of rising TNF levels in vivo during rejection and decreased thrombomodulin expression within graft capillaries, followed by return of both thrombomodulin and TNF to normal prerejection concentrations in response to therapy, are consistent with, and reinforce the biologic relevance of, the effects of TNF on human endothelial cells previously documented only in vitro (16).

Several consequences of decreased intragraft thrombomodulin expression are likely. As reviewed by Esmon (17), thrombomodulin normally binds thrombin, blocking its ability to clot fibrinogen. In addition, thrombin bound to thrombomodulin functions as an anticoagulant by catalyzing the conversion of PC to activated PC, a step involving proteolytic release of a low molecular activation peptide from the light chain of PC. Activated PC, in the presence of its cofactor PS, inactivates factors Va and VIIIa, and promotes fibrinolysis by decreasing endothelial plasminogen activator inhibitor activity. The current findings are consistent with both local activation and swamping of this thrombomodulin/PC/PS pathway during rejection. Considerable intragraft PC had undergone activation, as suggested by the differences in labeling using antibodies reactive with both PC and activated PC versus that of a mAb specific to the low molecular weight PC activation peptide. Moreover, widespread PC and PS deposition were noted, to the extent that circulating levels of these molecules were temporarily deficient during rejection episodes. Hence, the widespread, dense endothelial and interstitial depositions of thrombin and fibrin detected during rejection are likely to reflect both the increased intragraft procoagulant activity, as previously documented (4), and the currently observed failure of the thrombomodulin anticoagulant pathway; both of which can be mediated, at least in part, by TNF.

By extrapolation from its known actions in vitro (14), local production of TNF has a number of potential consequences for the immunobiology of rejection. TNF may cause direct injury to adjacent cells, facilitate mononuclear activation and production of cytokines such as IL-1 and IL-6, and upregulate endothelial expression of leukocyte adhesion molecules and MHC class I target antigen expression. However, the current results implicate TNF in disruption of the normal anticoagulant state of intragraft endothelium, resulting in the widespread intragraft fibrin deposition, which is one of the hallmarks of kidney rejection (37, 40). Such fibrin deposition and associated production of fibrin degradation products can facilitate attraction and activation of macrophages, retard interstitial microcirculatory blood flow, and thereby directly impair renal function. As a corollary, allograft rejection is also associated with an acquired deficiency of PC and FPS in peripheral blood samples, which may contribute to the increased rate of thromboembolism found in renal transplant recipients (37, 38). Together, these findings provide a rationale for the monitoring of blood TNF, PC, and FPS levels as guides to the early detection and differential diagnosis of human renal allograft rejection.

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