0041-1337/03/7509-1565/0
TRANSPLANTATION
Vol. 75, 1565–1570, No. 9, May 15, 2003
Printed in U.S.A.

SERINE PROTEINASE INHIBITOR-9, AN ENDOGENOUS BLOCKER OF GRANZYME B/PERFORIN LYTIC PATHWAY, IS HYPEREXPRESSED DURING ACUTE REJECTION OF RENAL ALLOGRAFTS

THANGAMANI MUTHUKUMAR,[1] RUCHUANG DING,[1] DARSHANA DADHANIA,[1] MARA MEDEIROS,[1] BAOGUI LI,[1] VIJAY K. SHARMA,[1] CHOLI HARTONO,[1,2] DAVID SERUR,[1,2] SURYA V. SESHAN,[3] HANS-DIETER VOLK,[4] PETRA REINKE,[5] SANDIP KAPUR,[6] AND MANIKKAM SUTHANTHIRAN[1,7]

Background. **Serine proteinase inhibitor (PI)-9 with a reactive center P1 (Glu)-P1' is a natural antagonist of granzyme B and is expressed in high levels in cytotoxic T lymphocytes (CTL). In view of the role of CTL in acute rejection, we explored the hypothesis that PI-9 would be hyperexpressed during acute rejection. Because PI-9 can protect CTL from its own fatal arsenal and potentially enhance the vitality of CTL, we examined whether PI-9 levels correlate with the severity of rejection as well as predict subsequent graft function.**

Methods. **We obtained 95 urine specimens from 87 renal allograft recipients. RNA was isolated from the urinary cells and mRNA encoding PI-9, granzyme B, or perforin and a constitutively expressed 18S rRNA was measured with the use of real-time quantitative polymerase chain reaction assay, and the level of expression was correlated with allograft status.**

Results. **The levels of PI-9 ($P=0.001$), granzyme B ($P<0.0001$), and perforin mRNAs ($P<0.0001$), but not the levels of 18S rRNA ($P=0.54$), were higher in the urinary cells from the 29 patients with a biopsy-confirmed acute rejection than in the 58 recipients without acute rejection. PI-9 levels were significantly higher in patients with type II or higher acute rejection changes compared with those with less than type II changes ($P=0.01$). Furthermore, PI-9 levels predicted subsequent graft function ($r=0.43$, $P=0.01$).**

Conclusions. **PI-9 mRNA levels in urinary cells are diagnostic of acute rejection, predict renal allograft histology grade, and predict functional outcome following an acute rejection episode.**

Cytotoxic T lymphocytes (CTLs) induce target cell death by at least two effector pathways: the granule exocytosis pathway in which the granzyme B/perforin collaborate to induce target cell apoptosis and the Fas-Fas ligand pathway in which the cross linking of Fas by the Fas ligand displayed by CTLs results in target-cell demise (*1*).

A major biological puzzle is how the CTLs protect themselves from their own fatal arsenal. One potential explanation is that there are endogenous protective mechanisms that are up-regulated, pari passu, with cytotoxic effector mechanisms, and these endogenous blockers serve to limit the lytic activity. In this regard, Fas-Fas ligand associated apoptosis is constrained by a cellular protein FLIP (c-FLIP), and hyperexpression of c-FLIP has been hypothesized as a mechanism for the escape of tumors from CTL attack (*2*, *3*). With respect to granzyme B/perforin lytic pathway, a serine proteinase inhibitor (PI)-9 has been shown to irreversibly inactivate granzyme B and block CTL killing by way of the pathway of granzyme B/perforin (*4–6*).

PI-9 is expressed in high levels in CTLs (*5*). In view of the role of CTL in acute rejection (*7*), we explored the hypothesis that PI-9 would be hyperexpressed during acute rejection. Because PI-9 can protect CTL from its own fatal arsenal and potentially enhance the vitality of CTL, we examined whether PI-9 levels correlate with the severity of acute rejection as well as predict subsequent graft function.

METHODS

Collection of Urine Samples and Kidney Biopsy Specimens

We collected 95 urine specimens from 87 kidney transplant recipients (Table 1). Sixty-two biopsies were obtained from 58 patients to determine the cause of renal-graft dysfunction. In these patients, urine was collected within 24 hours of the core needle biopsy of the allograft. All biopsy specimens were fixed in formalin, embedded in paraffin, stained with hematoxylin-eosin, periodic acid-Schiff, and Masson's trichrome and studied by a single pathologist who was unaware of the results of molecular studies. On the basis of 97 Banff classification (*8*), 33 biopsy specimens were classified as acute rejection, 14 specimens as other histologic findings (other), and 15 biopsies as chronic allograft nephropathy. The remaining 33 urine samples were from 29 patients who were classified as having stable allograft function after transplantation. In these patients, the serum creatinine levels either had decreased or had not increased by more than 0.2 mg/dL during the 7 days before and 7 days after urine collection.

This work was supported in part by a National Institutes of Health NIAID award (RO1 AI51652) and by an award from Satellite Healthcare, Inc. TM was a recipient of a fellowship award from the International Society of Nephrology. DD was a recipient of the American Society of Transplantation Fujisawa Clinical Fellowship Award. MM is supported by the Patronato del Hospital Infantil de México "Federico Gómez" and CONACYT, Mexico.

[1] Division of Nephrology, Departments of Medicine and Transplantation Medicine, Weill Medical College of Cornell University, New York-Presbyterian Hospital, New York, NY.

[2] The Rogosin Institute, New York-Presbyterian Hospital, New York, NY.

[3] Department of Pathology, Weill Medical College of Cornell University, New York-Presbyterian Hospital, New York, NY.

[4] Institute of Medical Immunology, Charité University Hospital, Berlin, Germany.

[5] Department of Nephrology and Internal Intensive Care, Charité University Hospital, Berlin, Germany.

[6] Department of Surgery, Weill Medical College of Cornell University, New York-Presbyterian Hospital, New York, NY.

[7] Address correspondence to: Dr. M. Suthanthiran, 525 East 68th Street, Box 3, New York, NY 10021. E-mail: msuthan@med.cornell.edu.

Received 1 October 2002. Accepted 14 November 2002.

DOI: 10.1097/01.TP.0000058230.91518.2F

TABLE 1. Study cohorts for the measurement of mRNAs in urinary cells

	Acute rejection	Other findings	Chronic allograft nephropathy	Stable graft function
Number of renal graft recipients (n=87)	29	14	15	29
Age in years (mean±SD)	40±13	47±10	50±11	45±13
Gender (male/female)	15/14	9/5	8/7	16/13
Graft donor source (living/cadaver)	17/12	7/7	5/10	13/16
Number of urine samples	33	14	15	33

Immunosuppression consisted of either a cyclosporine-based or tacrolimus-based regimen. All acute rejection episodes were treated with a course of intravenous methylprednisolone. Antilymphocyte antibodies (muromonab-CD3 [OKT3] or antithymocyte globulin) were given to patients who had an episode of glucocorticoid-resistant acute rejection.

Isolation of RNA

Urine was centrifuged at 10,000*g* for 30 minutes at 4°C. RNA was isolated from the cell pellet using RNeasy minikit (Qiagen, Chatsworth, CA), quantified, and reverse transcribed to complementary DNA (cDNA) as previously described (*9*).

Design of Primers and Probes for the Measurement of mRNA with the use of Real-Time Quantitative Polymerase Chain Reaction Assay

We designed and synthesized oligonucleotide primers and fluorogenic probes for the measurement of PI-9 with the use of real-time quantitative polymerase chain reaction (PCR) assay (Table 2). We also designed and developed primers and probes for the measurement of mRNA for granzyme B/perforin and for the quantification of 18S ribosomal RNA (rRNA). We measured 18S rRNA as a measure of RNA yield from a given urine specimen.

Quantification of mRNAs by Real-Time Quantitative PCR Assay

mRNA levels of PI-9, granzyme B, and perforin and the levels of 18S rRNA were measured with the use of ABI Prism 7700 sequence detection system (PE Biosystems, Foster City, CA). PCR reaction for each sample was set up in duplicate as a 25-μL reaction volume using 12.5 μL TaqMan Universal PCR Master Mix, 2.5 μL of 1:10 diluted (1:1000 for 18S RNA measurement) template cDNA, 300 nM of primers, and 200 nM of probe. PCR amplification included an initial incubation at 50°C for 2 minutes and denaturation at 95°C for 10 minutes. This was followed by heating at 95°C for 15 seconds and 60°C for 60 seconds repeated for 40 cycles. The PCR amplicon for 18S rRNA was prepared, quantified, and used for developing standard curves. The standard curves were based on the principle that a plot of the log of the initial target copy number of a standard versus threshold cycle results in a straight line. mRNA levels in the samples were expressed as number of copies per microgram of total RNA isolated from the urinary cells.

Statistical Analysis

We used GraphPad Prism 3.02 statistical software for Windows (GraphPad Software, San Diego, CA). The levels of mRNA for PI-9, granzyme B, and perforin deviated significantly from a normal distribution ($P<0.0001$), which was substantially reduced by use of a log transformation. The natural log mRNA levels were used as the dependent variable in a one-way analysis of variance to test for any differences among the four diagnostic groups. Dunnett's test for multiple comparisons was then used to control the risk of a type I error while comparing the mRNA levels in the acute rejection group with those in the other, chronic allograft nephropathy, and stable posttransplant groups. The relation between the levels of mRNA for PI-9, granzyme B, and perforin was estimated with Pearson's correlation. Conventional receiver operating characteristic curve analysis of mRNA levels was used to determine cut points that maximized the combined sensitivity and specificity for distinguishing patients with acute rejection from those without acute rejection. Area under the curve was calculated, and sensitivity and specificity at the selected cut point were determined.

RESULTS

mRNA Levels in Urinary Cells

The level of PI-9 mRNA was higher in urinary cells from patients with acute rejection compared with those without acute rejection (Fig. 1) (Table 3). The natural log-transformed PI-9 mRNA levels were used as the dependent variable in a one-way analysis of variance to test for any differences among the four diagnostic groups. PI-9 mRNA levels, measured during an episode of acute rejection, were significantly different from the other three diagnostic groups ($P=0.001$). Dunnett's test for multiple comparisons revealed that PI-9 mRNA levels in urinary cells obtained during acute

TABLE 2. Sequence and location of oligonucleotide primers and fluorogenic probes

Gene	Accession no.		Sequence (location)
Proteinase inhibitor-9	NM004155	Sense	5′ TCAACACCTGGGTCTCAAAAAA 3′ (508–529)
		Antisense	5′ CAGCCTGGTTTCTGCATCAA 3′ (590–571)
		Probe	5′ FAM-AGCTACCCGGCAACAACTCTTCAATTTTACCT-TAMRA 3′ (536–567)
Granzyme B	J04071	Sense	5′ GCGAATCTGACTTACGCCATTATT 3′ (534–557)
		Antisense	5′ CAAGAGGGCCTCCAGAGTCC 3′ (638–619)
		Probe	5′ FAM-CCCACGCACAACTCAATGGTACTGTCG-TAMRA 3′ (559–585)
Perforin	M28393	Sense	5′ GGACCAGTACAGCTTCAGCACTG 3′ (492–514)
		Antisense	5′ AGTCAGGGTGCAGCGGG 3′ (577–561)
		Probe	5′ FAM-TGCCGCTTCTACAGTTTCCATGTGGTACAC-TAMRA 3′ (526–555)
18S rRNA	K03432	Sense	5′ GCCCGAAGCGTTTACTTTGA 3′ (929–948)
		Antisense	5′ TCCATTATTCCTAGCTGCGGTATC 3′ (1009–985)
		Probe	5′ FAM-AAAGCAGGCCCGAGCCGCC-TAMRA 3′ (965–983)

Gene-specific oligonucleotide primers and probes were designed using Primer Express software (PE Applied Biosystems, Foster City, CA). The probes were labeled with 6-carboxy-fluorescein (FAM) at the 5′ end and with 6-carboxy-tetramethylrodamine (TAMRA) at the 3′ end. FAM functioned as the reporter dye, and TAMRA functioned as the quencher dye.

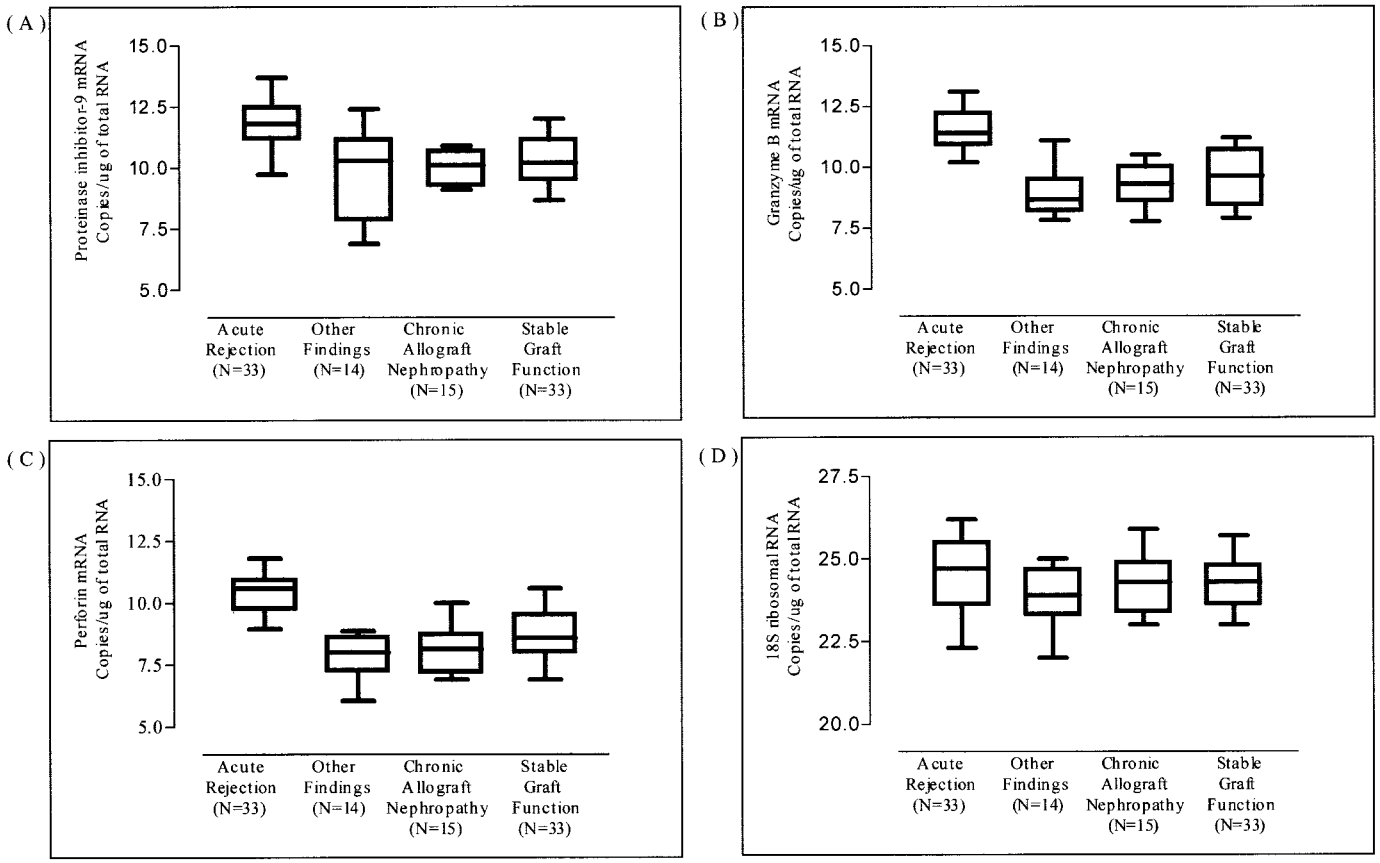

FIGURE 1. Levels of mRNA and rRNA in urinary cells. Box and whisker plots show the 10th, 25th, 50th (median), 75th, and 90th percentile copy numbers (log) for proteinase inhibitor-9 mRNA (A), granzyme B mRNA (B), perforin mRNA (C), and 18S rRNA (D) in urine samples. Copy numbers of proteinase inhibitor-9 mRNA (P=0.001), granzyme B mRNA (P<0.0001), and perforin mRNA (P<0.0001), but not those of 18S rRNA (P=0.54), were significantly higher in patients with an episode of acute rejection than in the patients in other groups (P value by one-way analysis of variance).

TABLE 3. Levels of mRNA in urinary cells[a]

Type of mRNA	Acute rejection (n=33)[b]	Other findings (n=14)	Chronic allograft nephropathy (n=15)	Stable graft function (n=33)	P value[c]
	Copies/μg of total RNA				
Proteinase inhibitor-9	11.7±0.3	9.7±0.7	10.4±0.4	10.4±0.3	0.001
Granzyme B	11.6±0.3	8.8±0.5	9.3±0.4	9.4±0.2	<0.0001
Perforin	10.4±0.2	7.7±0.4	8.0±0.5	8.7±0.3	<0.0001
18S rRNA	24.6±0.3	24.0±0.4	24.3±0.4	24.3±0.2	0.54

From patients with an episode of acute rejection and patients with other findings on graft biopsy, patients with biopsy evidence of chronic allograft nephropathy, and patients with stable graft function after transplantation.

[a] Copies of mRNA and rRNA were measured with the use of gene-specific primers and probes by real time PCR assay. Plus-minus values are means (±SE) of the natural logarithm of mRNA and rRNA copy number. The diagnoses of acute rejection, other findings, and chronic allograft nephropathy were made by histologic evaluation of renal-allograft-biopsy specimens. The clinical diagnosis of stable graft function was based on the finding that serum creatinine levels either had decreased or had not increased by more than 0.2 mg/dL during the 7 days before and the 7 days after the urine samples were collected.

[b] Values in parentheses are the numbers of urine samples.

[c] P values were calculated with the use of log-transformed mRNA copy numbers as the dependent variable in one-way analysis of variance. Dunnett's test was then used to compare the mRNA copy numbers in samples showing acute rejection with the mRNA copy numbers in each of the three other group of samples. It showed that copy number of PI-9 mRNA in urinary cells obtained during an episode of acute rejection was significantly higher than those in specimens with other findings (P<0.01), chronic allograft nephropathy (P<0.05), or stable function (P<0.01). Dunnett's procedure showed that the copy number of granzyme B mRNA in urinary cells obtained during an episode of acute rejection was significantly higher than those in specimens with other findings (P<0.01), chronic allograft nephropathy (P<0.01), or stable function (P<0.01). Dunnett's procedure showed that the copy number of perforin mRNA in urinary cells obtained during an episode of acute rejection was significantly higher than those in specimens with other findings (P<0.01), chronic allograft nephropathy (P<0.01), or stable function (P<0.01). None of the pair wise comparisons copy numbers of 18S rRNA in urinary cells were significant.

rejection were significantly higher than in those classified as stable posttransplant group ($P<0.01$), other ($P<0.01$), or chronic allograft nephropathy ($P<0.05$).

The levels of granzyme B mRNA were higher in urinary cells from patients with acute rejection compared with those without acute rejection (Fig. 1) (Table 3). Dunnett's test showed that granzyme B mRNA levels in urinary cells obtained during acute rejection were significantly higher than those classified as stable posttransplant ($P<0.01$), other ($P<0.01$), or chronic allograft nephropathy ($P<0.01$).

The levels of perforin B mRNA were higher in urinary cells from patients with acute rejection compared with those without acute rejection (Fig. 1) (Table 3). Dunnett's multiple comparison procedure revealed that perforin mRNA levels in urinary cells obtained during acute rejection were significantly higher than in those classified as stable posttransplant group ($P<0.01$), other ($P<0.01$), or chronic allograft nephropathy ($P<0.01$).

The levels of constitutively expressed 18S rRNA did not vary significantly among the four diagnostic categories ($P=0.54$) (Fig. 1) (Table 3).

Correlation Between mRNA Levels

We examined whether there is a correlation between the levels of expression of PI-9 mRNA and mRNA encoding cytotoxic attack proteins granzyme B/perforin in urinary cells. Granzyme B and perforin collaborate to induce target-cell demise. We also determined whether there is a correlation between the levels of expression of granzyme B mRNA and perforin mRNA in urinary cells. A positive correlation between the levels of expression of PI-9 mRNA and granzyme B mRNA in urinary cells (Fig. 2A) ($r=0.57$, $P<0.0001$) and a positive correlation between the levels PI-9 mRNA and perforin mRNA (Fig. 2B) ($r=0.54$, $P<0.0001$) were observed; a positive correlation between the levels of expression of granzyme B mRNA and perforin mRNA in urinary cells (Fig. 2C) ($r=0.83$, $P<0.0001$) was also observed

Receiver Operating Characteristic Curve Analysis of mRNA Levels

The receiver operating characteristic curves (Fig. 3) show the true-positive fractions (sensitivity) and false-positive fractions (1-specificity) for various cut points for mRNA levels of PI-9, granzyme B, perforin, and 18S rRNA. The natural log-transformed cut point (threshold) that maximized the combined sensitivity and specificity for PI-9 was 11.01 copies per microgram of RNA, and at this threshold, the estimated sensitivity and specificity for predicting acute rejection were 76% and 79%, respectively ($P<0.0001$)(Fig. 3A). The natural log-transformed cutpoint was 10.11 copies per microgram of RNA for granzyme B and at this threshold sensitivity was 88% and specificity was 79% ($P<0.0001$) (Fig. 3B). The natural log-transformed cutpoint was 8.87 copies per microgram of RNA for perforin, and at this threshold, sensitivity was 88% and specificity was 79% ($P<0.0001$) (Fig. 3C). Figure 3D shows the receiver operating characteristic curve for 18S rRNA with respect to presence or absence of acute rejection. 18S rRNA levels did not discriminate acute rejection from other renal diagnoses.

The calculated area under the curve was 0.76 (95% confidence intervals [CI] 0.64–0.88) for PI-9 mRNA levels, 0.88 (95% CI 0.79–0.97) for granzyme B mRNA levels, 0.90 (95% CI 0.80–0.96) for perforin mRNA levels, and 0.58 (95% CI 0.44–0.72) for 18S rRNA level.

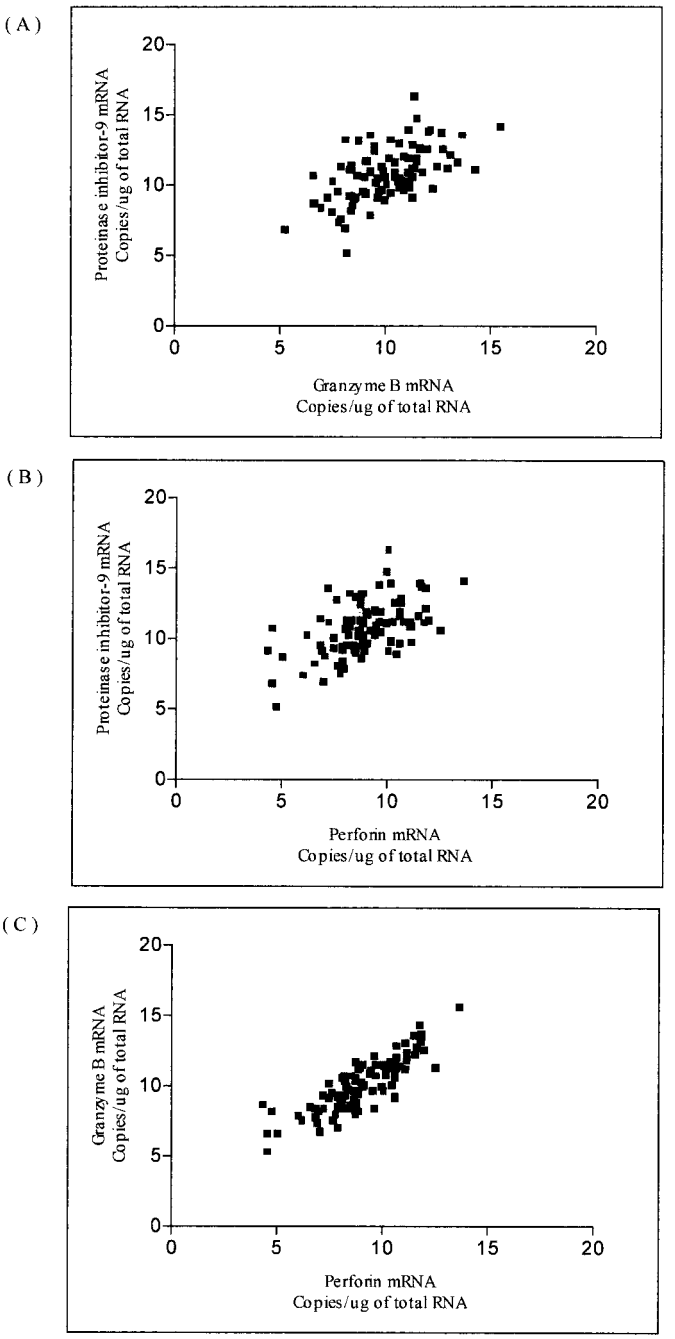

FIGURE 2. Scatterplot of mRNA levels. Correlation between proteinase inhibitor-9 and granzyme B mRNA copy numbers (A)($r=0.57$, $P<0.0001$), proteinase inhibitor-9 and perforin (B)($r=0.54$, $P<0.0001$), and granzyme B/perforin (C)($r=0.83$, $P<0.0001$).

PI-9 mRNA Levels Vary with the Severity of Acute Rejection

Twenty-five of 33 acute rejection biopsies showed less than type II histologic changes. The mean (±SE) PI-9 mRNA level was 11.3±0.3 copies per microgram of RNA in samples from patients with less than type II changes, and 13.0±0.6 copies per microgram of RNA in samples from patients with type II

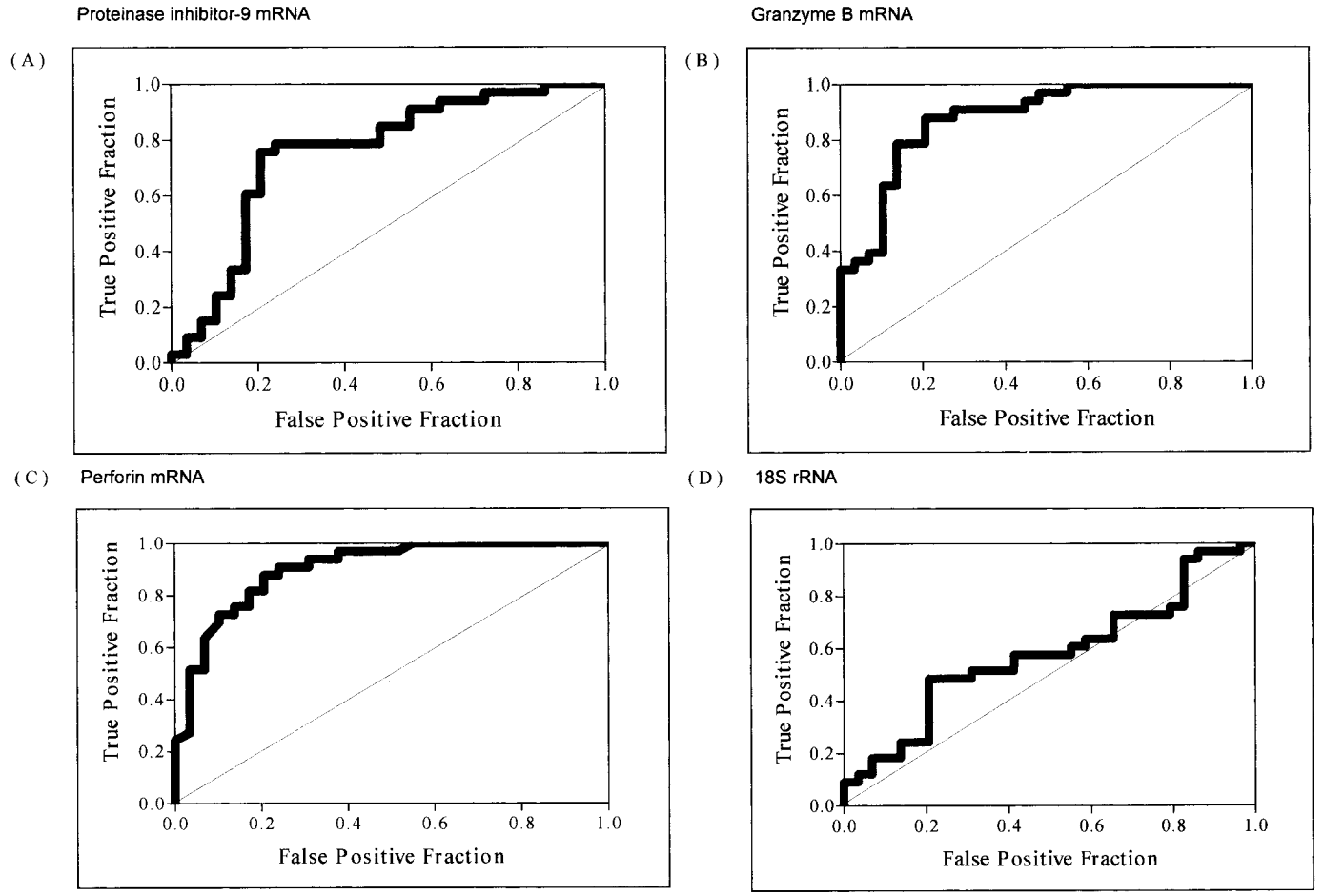

FIGURE 3. Receiver operating characteristic curve of mRNA and rRNA Levels. Fraction of true-positive results (sensitivity) and false-positive results (1-specificity) for proteinase inhibitor-9 mRNA copy numbers (A), granzyme B mRNA copy numbers (B), perforin mRNA copy numbers (C), and 18S rRNA copy numbers (D) as markers of acute rejection. The calculated area under the curve was 0.757 for proteinase inhibitor-9 mRNA levels, 0.879 for granzyme B mRNA levels, 0.903 for perforin mRNA levels, and 0.578 for 18S rRNA levels. A value of 0.5 is no better than expected by chance, and a value of 1.0 reflects a perfect indicator.

or higher changes ($P=0.01$). In contrast, the levels of mRNA for granzyme B or perforin did not vary with the type of acute rejection changes. The mean (±SE) granzyme B mRNA level was 11.6±0.3 copies per microgram of RNA in those with less than type II changes, and it was 11.5±0.7 copies/μg RNA in those with type II or higher changes ($P=0.86$). The mean (±SE) perforin mRNA level was 10.4±0.3 copies per microgram of RNA in those with less than type II changes, and it was 10.5±0.5 copies per microgram of RNA in those with type II or higher changes ($P=0.82$).

PI-9 mRNA Levels Predict Renal Functional Outcome

We investigated whether the mRNA levels of PI-9 were a correlate of renal functional outcome as assessed by serum creatinine levels 6 months after the acute rejection episode. For this analysis, we assigned a value of 10.0 mg of creatinine for those whose grafts had failed. Our evaluation of the relationship between mRNA levels and functional outcome showed that PI-9 mRNA levels are a correlate of serum creatinine at 6 months following the acute rejection episode ($r=0.43$, $P=0.02$). In contrast, mRNA levels of granzyme B ($r=0.19$, $P=0.30$) or perforin ($r=0.26$, $P=0.15$) were not a correlate of renal functional outcome.

DISCUSSION

A major biological puzzle is how CTLs protect themselves from their own fatal arsenal of cytotoxic attack molecules. An equally important unresolved issue is how allografts may protect themselves from CTL effector mechanisms. PI-9, found in our investigation to be overexpressed during an episode of acute rejection, might provide a mechanistic explanation.

PI-9 is a 42 Kda protein originally identified using a human placental lambda gt11 cDNA library (*4*). It is an intracellular protein and belongs to the serpin family of serine proteinase inhibitors and to the viral serpin cytokine response modifier A. PI-9 has been detected in several cell types including lymphocytes, dendritic cells, and endothelial cells (*5*, *10*, *11*). It is distinguished by the reactive center P1 (Glu)-P1', and this unusual reactive center is responsible for its exquisite specificity for granzyme B (*5*, *12*).

Compelling evidence exists that granzyme B is a vital component of cytotoxic effector machinery of CTLs (*1*). Genetic support for the role of granzyme B is provided by the observation that CTLs from mice with homozygous null mutation in the granzyme B gene have a profound deficiency in inducing apoptosis of target cells (*13*). Current data suggest that overexpression of PI-9 in tumor cells is a robust mechanism for blocking CTL attack by way of the granzyme B/perforin pathway (*6*) The PI-9 afforded protection is distinct from that provided by c-FLIP protein that antagonizes tumor cell death by way of the Fas receptor pathway (*2*, *3*).

To the best of our knowledge, our study represents the first time evaluation of expression of a natural and endogenous antagonist of granzyme B/perforin pathway in organ transplantation. We realize that the heightened expression of PI-9 during an acute rejection episode, identified in the current study, might have a dual or even an antagonistic role. Over abundance of PI-9 in the alloreactive CTL might serve to enhance CTL function by limiting self-damage from the CTLs granzyme B; on the other hand, over expression of PI-9 in target cells such as endothelial cells may serve a protective function or may reflect endothelial-cell activation during the acute rejection process. Moreover, the endogenous defense afforded by PI-9 may not be restricted to a single cell type (e.g., CTLs) but offered to dueling partners (e.g., CTLs as well as their targets endothelial cells).

Our observation that that there is a positive correlation between the levels of expression of PI-9 and cytotoxic attack molecules suggests that activation of lymphocytes results in the up-regulation of PI-9. Indeed, we have found that stimulation of human peripheral blood mononuclear cells with the T-cell mitogen phytohemagglutinin results in the up-regulation of granzyme B as well as PI-9.

Our findings that levels of mRNA for granzyme B/perforin in urinary cells are predictive of acute rejection confirm and extend our earlier demonstration that measurement of mRNA for cytotoxic attack molecules in urine with the use of competitive quantitative PCR assay is a noninvasive means of diagnosing acute rejection of renal allografts (*9*). Our measurement of levels mRNA for PI-9 suggest that PI-9 levels may provide diagnostic and prognostic parameters hitherto unavailable from measurement of mRNA for cytotoxic attack molecules granzyme B/perforin. In the current investigation, PI-9 mRNA levels, but not the levels of expression of granzyme B or perforin, reflected histologic severity of a given acute rejection episode. Moreover, PI-9 levels, measured in urine specimens obtained at the time of acute rejection, predicted subsequent renal graft functional outcome. Should these observations be replicated in a larger cohort of patients, reliable biomarkers for predicting renal acute rejection grade as well as for prognosticating the outcome of a given episode of acute rejection may emerge.

Our study was not designed to resolve the cellular basis for the heightened expression of PI-9 during an episode of acute rejection. It is unresolved at this stage whether the high levels measured in urinary cells are caused by overexpression of PI-9 in CTL or endothelial or both. Also, whether PI-9 and granzyme B exhibit differential sensitivity to antirejection therapy remains unresolved.

In summary, we demonstrate for the first time that PI-9, a natural and endogenous antagonist of granzyme B/perforin cytolytic pathway, is hyperexpressed in urinary cells of patients with acute rejection. Moreover, PI-9 levels appear to predict renal-allograft acute rejection histology grade as well as predict functional outcome following an episode of acute rejection.

Acknowledgments. The authors thank Linda Stackhouse for meticulous preparation of the manuscript.

REFERENCES

1. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol 2002; 2(6): 401.
2. Djerbi M, Screpanti V, Catrina AI, et al. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. J Exp Med 1999; 190(7): 1025.
3. Medema JP, de Jong J, van Hall T, et al. Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. J Exp Med 1999; 190(7) 891.
4. Sprecher CA, Morgenstern KA, Mathewes S, et al. Molecular cloning, expression, and partial characterization of two novel members of the ovalbumin family of serine proteinase inhibitors. J Biol Chem 1995; 270(50): 29854.
5. Sun J, Bird CH, Sutton V, et al. A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. J Biol Chem 1996; 271(44): 27802.
6. Medema JP, deJong J, Peltenburg LT, et al. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci U S A 2001; 98(20): 11515.
7. Strom TB, Tilney NL, Carpenter CB, et al. Identity and cytotoxic capacity of cells infiltrating renal allografts. N Engl J Med 1975; 292(24): 1257.
8. Racusen LC, Solez K, Colvin RB, et al. The Banff 97 working classification of renal allograft pathology. Kidney Int 1999; 55(2): 713.
9. Li B, Hartono C, Ding R, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. N Engl J Med 2001; 344(13): 947.
10. Buzza MS, Hirst CE, Bird CH, et al. The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses. Cell Immunol 2001; 210(1): 21.
11. Bladergroen BA, Strik MC, Bovenschen N, et al. The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites. J Immunol 2001; 166(5): 3218.
12. Bird CH, Sutton VR, Sun J, et al. Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. Mol Cell Biol 1998; 18(11): 6387.
13. Heusel JW, Wesselschmidt RL, Shresta S, et al. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell 1994; 76(6): 977.