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The Granzyme B Inhibitor SERPINB9 (Protease Inhibitor 9) Circulates in Blood and Increases on Primary Cytomegalovirus Infection after Renal Transplantation

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SERPINB9 is the only known human intracellular inhibitor of granzyme B (GrB), the effector molecule in immunity against cytomegalovirus (CMV) and in renal allograft rejection. Therefore, using specific enzyme-linked immunosorbent assays, we addressed the presence of circulating SERPINB9 during primary CMV infection, subclinical rejection, acute rejection, and uncomplicated posttransplantation course. Soluble (s) SERPINB9 circulates in blood and increases on primary CMV infection. This increase was significantly higher in symptomatic than in asymptomatic patients. In contrast, sSERPINB9 levels did not change in response to subclinical or acute rejection. We demonstrated the presence of circulating sSERPINB9/sGrB complexes, which suggests that SERPINB9 has extracellular functions as well.

After renal transplantation, recipients are threatened by cytomegalovirus (CMV) infection and clinical or subclinical rejection and, thus, are exposed to a higher risk of chronic rejection and long-term graft failure [1]. Granzyme B (GrB)–mediated cytotoxicity plays a key role in immunity against CMV and also in the effector phase of rejection [2, 3]. Previously, we have shown that expansion of a specific subset of CD8+GrB+ T cells coincides with a temporary increase in systemic soluble (s) GrB levels during primary CMV infection. In contrast, no such increase was observed during acute allograft rejection [4]. Rapid trapping of allospecific GrB+CD8+ T cells into the graft may account for the lack of increase in circulating sGrB levels during rejection.

The intracellular serpin SERPINB9 (proteinase inhibitor 9 [PI-9]) is the only known human protein that specifically inhibits GrB [5, 6]. The in vivo distribution of SERPINB9 suggests that this enzyme protects against unwanted GrB-mediated cytotoxicity. SERPINB9 is expressed at immune-privileged sites and by endothelial, dendritic, and mesothelial cells and malignant lymphomas [7, 8]. Ex vivo experiments using NK cell lines and peripheral blood cells from healthy persons have provided us with evidence for subcellular localization and up-regulation of SERPINB9 during maturation of accessory cells and degranulation of effector cells [6]. In such systems, SERPINB9 could associate with GrB-containing granules and form intracellular complexes, leading to inactivation of cytoplasmic GrB by SERPINB9. However, whether SERPINB9 associates with sGrB released in the circulation is not yet known.

Recently, we have shown that SERPINB9 expression by tubular cells is significantly higher during subclinical rejection than during acute rejection. In contrast to acute rejection, subclinical allograft rejection is defined as the presence of a GrB+ lymphoid cell infiltrate in a renal allograft, which causes clear-cut tubulitis at the histological level but does not, however, lead to an instant deterioration of allograft function. High SERPINB9 expression in these tubules, involved in subclinical rejection, correlates with the presence of GrB+ T cells in the graft. These data point to a protective role of SERPINB9 against cytotoxic T cells [9]. We wondered whether serum levels of SERPINB9 can be used to discriminate subclinical from acute rejection and can possibly be used as a noninvasive diagnostic tool for intragraft events.

In the present study, we addressed the presence of this serpin in peripheral blood from healthy persons and renal transplant recipients during primary CMV infection, subclinical rejection, acute rejection, or no rejection.

Subjects, materials, and methods. This longitudinal study was performed retrospectively on serum samples from 46 transplant recipients (30 men and 16 women; median age, 46 years [range, 21–73 years]). Samples were obtained before transplantation, 3 times/week during hospitalization, and 1 time/week after discharge for 3 months and regularly thereafter for up to 6 months. Patients were treated with prednisolone (10 mg/day),
cyclosporine (trough level monitoring), and mycophenolate mofetil (2 g/day). Nine patients with subclinical rejection (107 samples), 7 patients with biopsy-proven acute rejection (123 samples), 7 patients with an uncomplicated course (47 samples), 16 patients with symptomatic CMV infection (312 samples), and 7 patients with asymptomatic CMV infection (138 samples) were included. Symptomatic infection was defined as fever of >38°C lasting for ≥3 days and visceral CMV disease, treated with intravenous ganciclovir at 5 mg/kg for 10 days. This study was approved by the institutional medical ethic committee. Each patient had given written informed consent. Twenty-five healthy persons (14 men and 11 women; median age, 30 years [range, 21–49 years]) served as controls.

To determine the molecular weight of sSERPINB9 and sGrB, serum proteins were separated by gel filtration, using an AcA54 ultragel column (Biosepra). This column is able to separate proteins in the range of 5–70 kDa. A flow rate of 0.5 mL/min was used routinely. PBS with 0.1% Tween and 2 mol/L NaCl was used as running buffer. Before each run, the column was equilibrated with 2 column volumes of running buffer. In each experiment, serum samples (500 µL) diluted 1:1 with running buffer were applied to the column. Fractions of 0.5 mL were collected, and the concentrations of sSERPINB9 or sGrB were determined by ELISA. The column was calibrated with phenol red (5 kDa), albumin (66 kDa), IgG (160 kDa), and dextran blue (void volume).

sSERPINB9 and sGrB antigen levels were measured by specific ELISA, as described elsewhere [10, 11]. Cell lysate of YT-indy, an NK cell line expressing SERPINB9 and GrB, was used as standard. YT-indy cells were cultured and lysed as described elsewhere [8]. 1 U of GrB represents ∼1 pg.

sSERPINB9/sGrB complexes were measured essentially as described above for sSERPINB9 and sGrB antigen except that polyclonal anti-PI-9 antibodies (1 µg/mL) were used for coating the microtiter plates, and biotinylated anti-GrB monoclonal antibody GB11 (~1 µg/mL) was used for detection. Cell lysate of YT-indy, also containing sSERPINB9/sGrB complexes, served as the control.

Data are presented as median and range unless indicated otherwise. The correlation between parameters was assessed by Spearman rank correlation analysis, whereas differences between groups were analyzed by Mann-Whitney U test. Tests were 2-sided; P < .05 was considered significant.

**Results.** sSERPINB9 was detectable in serum from healthy persons (n = 25; median level, 530 pg/mL [range, 122–1263 pg/mL]). sSERPINB9 levels from patients before transplantation (n = 46; median, 928 pg/mL; range, 240–6565 pg/mL) were significantly higher than those in healthy subjects (P < .0001). No correlation was found between creatinine clearance (Cockroft-Gault formula) and sSERPINB9 levels (R = 0.08; data not shown), which is consistent with the high molecular weight of SERPINB9, 42 kDa. This can also be explained by the possibility that this molecule, in part, circulates as complexes with macromolecular proteins.

Using gel filtration, we were able to demonstrate the presence of a high-molecular-weight form of SERPINB9 in the circulation, corresponding to 79 kDa. The 79-kDa peak may represent a SERPINB9 dimer or a complex with another protein(s), such as GrB. An ELISA for sSERPINB9/sGrB complexes was developed using anti-SERPINB9 polyclonal antibodies as catching antibodies and anti-GrB monoclonal antibody GB-11 as detecting antibody. With this assay, sSERPINB9/sGrB complexes could be detected in some patients. Also, sSERPINB9/sGrB complexes were demonstrated in the above-mentioned high-molecular-weight peak of sSERPINB9. These data indicate that sSERPINB9 circulates in serum at least partially complexed to GrB.

sSERPINB9 levels increased significantly, from a median of 275 pg/mL (range, 83–688 pg/mL) at 1 week after transplantation to a median of 2324 pg/mL (range, 532–10,046 pg/mL) at the time of the peak CMV DNA load, as determined by polymerase chain reaction (PCR). This increase paralleled the increases in CMV DNA load and sGrB level. As shown in figure 1, the peak levels of sSERPINB9 in patients with symptomatic CMV infection (n = 16; median, 2780 pg/mL [range, 532–10,046 pg/mL]) were significantly higher than those in asymptomatic patients (n = 7; median, 1112 pg/mL [range, 620–2367 pg/mL]) (P = .01).

Figure 2 shows that sSERPINB9 levels rose in parallel with increases in sGrB level and CMV DNA load, as measured by PCR. In the majority of patients, the peak levels of sSERPINB9, sGrB, and CMV DNA coincided. However, the extent of the increase in sSERPINB9 did not correlate with that of sGrB (n = 16).

**Figure 1.** Soluble (s) SERPINB9 serum levels in primary posttransplantation cytomegalovirus infection. sSERPINB9 levels in patients with symptomatic infection (n = 16) were significantly higher than those in patients with an asymptomatic course (n = 7) (P = .01). Median and ranges are shown.
Figure 2. Kinetics of soluble (s) SERPINB9 and granzyme B (sGrB) levels in serum from patients with symptomatic and asymptomatic primary posttransplantation cytomegalovirus (CMV) infection. CMV DNA load, as determined by polymerase chain reaction (PCR), is expressed as copies per milliliter of whole blood. A parallel increase and nearly coinciding peak levels of sSERPINB9, sGrB, and CMV DNA were observed in symptomatic and asymptomatic patients.

sSERPINB9 levels did not differ significantly between patients with subclinical rejection, acute rejection, and no rejection. On the basis of the median of repeated measurements of SERPINB9 levels in a given patient, we determined a median sSERPINB9 level for each group of patients. In patients experiencing a subclinical rejection, sSERPINB9 and sGrB levels were, respectively, 936 pg/mL (range, 130–8565 pg/mL) and 40 pg/mL (range, 2–4453 pg/mL). In patients experiencing an acute rejection, sSERPINB9 and sGrB levels were 555 pg/mL (range, 35–10,046 pg/mL) and 38 pg/mL (range, 3–1256 pg/mL). In patients with no rejection, sSERPINB9 and sGrB levels were 852 pg/mL (range, 162–27,300 pg/mL) and 19 pg/mL (range, 5–1244 pg/mL). No significant differences were found.

sSERPINB9 and sGrB levels on or near days on which biopsy was performed were quite low in all patients with subclinical or acute rejection and showed no significant differences between these groups. No correlation was found between sSERPINB9 levels measured on the day before transplantation and posttransplantation clinical course (data not shown).

Discussion. Here, we show that the intracellular serpin SERPINB9 can be detected in the circulation. Serum levels of SERPINB9 increase during primary CMV infection after transplantation, showing a higher increase in symptomatic than in asymptomatic patients.

SERPINB9 belongs to the subfamily of intracellular serpins, which are unique in that they lack a cleavable N-terminal signal sequence and, therefore, reside and function mainly intracellularly [12]. Although intracellular serpins exert their functions primarily intracellularly, at least 2 other intracellular serpins, SERPINB2 (PAI-2) and SERPINB3 (SCCA1), are also found extracellularly. SERPINB2 is partially secreted through a yet-unidentified secretory pathway [13], whereas SERPINB3 is thought to be passively released into the circulation [14]. In this study, we demonstrate the existence of sSERPINB9 in the circulation. Whether sSERPINB9 is actively secreted or passively released remains to be elucidated.

In addition, we show that sSERPINB9 in serum partially occurs as complexes with GrB. Because inhibition of target proteinases by serpins yields covalently linked complexes between these compounds, these sSERPINB9/sGrB complexes provide us with extra evidence that sSERPINB9 indeed inhibits GrB in vivo. Until now, such complex formation between serpins and their cognate proteinases, such as thrombin-ATIII, plasmin-antiplasmin, and complement factor 1 esterase–complement factor 1 esterase inhibitor, has been thought to be required as the first step in the performance of serpin’s inhibitory function.

Previously, we showed a marked and temporal increase in sGrA and sGrB levels during CMV infection after renal transplantation [4]. sSERPINB9 levels increased in parallel with the increase in CMV DNA load, as determined by PCR, and fol-
lowed or coincided in time with that of sGrB. The peak levels of CMV DNA, sGrB, and sSERPINB9 occurred on approximately the same day. In patients with symptomatic infection, sSERPINB9 levels rose to a significantly higher degree than in asymptomatic patients. A more protracted exposure to the virus could offer an explanation for this difference. Higher numbers of virally infected endothelial cells in symptomatic patients may also underlie this difference, because these cells are principal CMV reservoirs and contain high amounts of SERPINB9 [2, 7]. To escape unwanted cytotoxicity, these cells probably express SERPINB9 to a high degree. Other cell types, such as cytotoxic T lymphocytes (CTLs) and dendritic cells, may also be the source of sSERPINB9 [5, 6]. CTLs produce high levels of SERPINB9 during inflammatory responses [5]. Because we could not demonstrate a correlation between the number of CMV-specific CTLs and sSERPINB9 levels in our recipients (data not shown), these cells seem not to be the source. Whether the increase in systemic sSERPINB9 levels is specific for CMV infection is unknown but seems unlikely in view of its antagonistic function toward GrB that is released on degranulation of cytotoxic cells irrespective of their target.

sSERPINB9 levels did not relate to subclinical rejection, acute rejection, or uncomplicated posttransplant course. Also, levels measured on the day before transplantation did not discriminate between these clinical conditions. Apparently, hyperexpression of SERPINB9 by tubular cells during subclinical rejection, as has been shown recently by immunohistochemical studies of graft biopsy samples [9], is not reflected by increased systemic sSERPINB9 levels. Similar data were shown previously for systemic sGrB levels during acute rejection. During acute rejection, increased numbers of GrB+ CTLs in allograft biopsy samples have been found [3]; however, sGrB levels in the circulation have not been found to be elevated [4]. Accordingly, in the present study, low GrB levels were measured during acute rejection (data not shown). Because the levels of mRNA encoding either SERPINB9 or GrB have been shown to be increased in urinary cells in recipients with acute rejection versus no rejection [15], we speculate that measurement of SERPINB9 mRNA and protein in urinary cells from patients with subclinical, acute, or no rejection may constitute a noninvasive diagnostic tool. To address this question, further studies are in progress.

In conclusion, the GrB inhibitor SERPINB9 can be detected in blood, and levels of SERPINB9 increase temporarily on primary posttransplantation CMV infection. However, because levels of sSERPINB9 are not increased during subclinical rejection, levels in the circulation do not reflect intragraft events.

References