Pancreatic stone protein is highly increased during posttraumatic sepsis and activates neutrophil granulocytes*

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Objectives: **The level of pancreatic stone protein/regenerating protein (PSP/reg), a secretory protein produced in the pancreas, increases dramatically during pancreatic disease. However, after stress (e.g., anesthesia), PSP/reg levels are increased transiently in animals without pancreatic injury. Therefore, we aimed to determine whether PSP/reg is an acute-phase protein after non-pancreatic trauma.**

Patients: **Eighty-three polytraumatic patients without pancreatic damage.**

Measurements and Main Results: **We compared serum PSP/reg levels from polytraumatic patients without pancreatic damage with those in healthy controls (n = 38). C-reactive protein, interleukin-6, procalcitonin, and leukocyte numbers were also compared. The expression of CD62L and CD11b on neutrophils after exposure to PSP/reg was analyzed by flow cytometry. Thirty-three patients (39%) developed sepsis, 32 (38%) had local infections, and 18 (21%) had no infections. At admission, PSP/reg serum levels (10.2 [6.2–14.5] ng/mL; median [interquartile range]) were comparable with those in healthy controls (10.4 [7.5–12.3] ng/mL). During hospital stay, PSP/reg levels were elevated significantly in patients with sepsis (146.4 ng/mL) and in patients with infections (111.4 ng/mL) compared with patients without infections (22.8 ng/mL). Furthermore, binding of fluorescein isothiocyanate–labeled recombinant PSP/reg to human neutrophils was demonstrated. Recombinant PSP/reg elicited a dose-dependent shedding of L-selectin (CD62L) and upregulation of β2-integrin (CD11b) in neutrophils, which indicates that PSP/reg activates neutrophils.**

Conclusions: **We conclude that PSP/reg is up-regulated in blood after trauma, and the PSP/reg level is related to the severity of inflammation. Furthermore, PSP/reg binds to and activates neutrophils. Therefore, PSP/reg might be an acute-phase protein that could serve as a marker for posttraumatic complications. (Crit Care Med 2009; 37:1642–1648)**

KEY WORDS: acute-phase protein; sepsis; severe trauma; infection

Systemic responses after severe trauma include a number of factors involved in innate immunity, inflammatory reactions, and cellular activities (1). Among the most commonly used markers of systemic infection and sepsis are leukocyte counts, C-reactive protein (CRP), and procalcitonin (PCT). The latter two serum proteins are highly induced after trauma, yet the functions of these proteins in this context are unknown (2). In addition, cytokines such as interleukin (IL)-6, IL-8 (3), and IL-18 have been measured to monitor patients. The posttraumatic course after severe injury can be complicated by sepsis and/or multiple organ failure, which are the conditions with high mortalities (4). Thus far, reliable predictors and indicators of posttraumatic sepsis are unavailable; hence, treatment can lag behind the onset of sepsis.

Pancreatic stone protein/regenerating protein (PSP/reg) belongs to a family of lectin-binding proteins that were identified initially in patients with pancreatitis (5). PSP/reg has been studied predominantly in the pancreas. In acute or chronic pancreatitis, PSP/reg is upregulated and may appear in the blood (6). Although this protein is a secretory product, its expression is not induced by diet alone. In animal experiments, we have shown that PSP/reg is induced upon stress, in the absence of pancreatic tissue damage (7). In an animal model of chronic pancreatitis, damaged acinar cells appeared to change their cellular architecture with a diverted localization of PSP/reg toward the basal pole of the cell (8).

This same protein has been associated with islet regeneration (9). PSP/reg is also synthesized in the Paneth cells of the small intestine (10, 11) and in the fundic cells of the stomach (12). PSP/reg is generally assumed to have a protective function. As an acute-phase protein, PSP/reg might be involved in promoting cell proliferation during regenerative processes (13). Members of the PSP/reg and pancreatitis-associated protein family are regulated by IL-6 and other cytokines that are released after tissue injury (14, 15). Therefore, we asked whether severe trauma that does not involve the pancreas leads to increased blood levels of PSP/reg. To this end, a set of patients with severe trauma but an apparent absence of pancreatic damage was chosen.

The presence of PSP/reg in peripheral blood during inflammation or after

***See also p. 1806.**

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trauma might point toward a specific regulatory response, as seen in acute-phase proteins, that could lead to activation of immune cells. Therefore, we investigated whether PSP/reg has an activating effect on leukocytes. Activation of leukocytes during inflammation is a highly regulated process that involves a sequence of events. The activation of polymorphonuclear neutrophil granulocytes (PMN or neutrophils) involves adherence to the vessel walls, i.e., endothelial cells, by selectins and integrins on the neutrophil surface. One of the major selectins is L-selectin (CD 62L), a protein expressed on the cellular surface that is shed after activation of the tethering process. On the other hand, integrins (e.g., CD 11b) are involved in the adhesion of granulocytes to vessel walls, and their appearance on the cell surface is dependent on neutrophil activation (16).

In the present study, we show that PSP/reg is highly increased in patients during sepsis. Furthermore, we show that recombinant PSP/reg induces shedding of L-selectins and recruitment of integrins to the cell membrane, which are both indicators of neutrophil granulocyte activation.

MATERIALS AND METHODS

Patients. A total of 83 severely injured trauma patients who were admitted to the Division of Trauma Surgery (level I trauma center), University Hospital Zurich, from January 2002 to September 2006 were included in this study. Inclusion criteria were an Injury Severity Score (17) $\geq$17 points, patient age $>$16 years, $<$4 hours between the accident and hospital admission, absence of pancreatic damage as assessed by an initial computed tomographic scan, and surveillance in the intensive care unit with a survival $>$5 days. All patients were treated according to the advanced trauma life support guidelines (18) and our standard trauma protocol (19). Antibiotics were administered if a septic focus was verified by a positive bacterial culture. In addition, standard antibiotics were administered for 5 days to patients with open fractures, and a single prophylactic injection of a cephalosporin was given before every osteosynthesis.

Analysis of serum samples for PSP/reg was part of a retrospective study. For analysis of the biological activity of PSP/reg, whole blood was obtained from volunteers and from patients with sepsis who were recruited under informed consent guidelines approved by the Human Ethical Committee of the Kanton of Zurich. The study was registered in the local (StV 10-2003) and international (ClinicalTrials.gov Identifier: NCT00564109) registries.

Definition of Systemic Inflammatory Response Syndrome, Local Infection, and Sepsis. Clinical data including laboratory and vital parameters were collected prospectively over a period of 21 days after the trauma. The occurrences of systemic inflammatory response syndrome (SIRS), local infection, and sepsis were determined by the following parameters. SIRS was defined according to the guidelines of the 1992 American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (20). The described criteria were modified inasmuch as they had to be fulfilled for at least three consecutive days to allow the diagnosis of SIRS or sepsis, respectively (2, 21). SIRS was graded as moderate (two positive criteria) and severe (three or four positive criteria) (2, 21). Patients were categorized *post hoc* into three groups according to their clinical data and to the described definitions: a) without infection, b) with local infection, and c) with sepsis. Sepsis was diagnosed if all four criteria of SIRS were met for $\geq$3 consecutive days in the presence of a septic focus with positive bacterial tissue culture or a positive blood culture (2, 21). If less than four SIRS criteria were observed over 3 days in the presence of a positive focus, a local infection was diagnosed.

Blood Monitoring of Patients' Immune Status. Leukocytes were counted by flow cytometry. CRP, pancreatic amylase, and lipase levels were determined with routine instruments of the Department of Clinical Chemistry, University Hospital Zurich.

The PCT level was determined with an immunoluminometric assay (Lumitest PCT, Brahms, Berlin, Germany) (2). IL-6 was determined with a commercially available enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK) according to the manufacturer's protocol.

Recombinant human PSP/reg 1α, as described in the supplemental data, was used to generate an isoform-specific enzyme-linked immunosorbent assay using the sandwich technique as described previously (7, 22) (see Text, Supplemental Digital Content 1, http://links.lww.com/A863). Endotoxin contamination of the recombinant PSP/reg was analyzed by limulus amoebocyte lysate assay (LAL Endosave; Charles River Lab, Wilmington, MA), which indicated a low endotoxin level (9.8 EU/mL) for 50 μg/mL PSP/reg, i.e., a 0.098 EU/mL working dilution. A Western blot of the recombinant PSP/reg protein and PSP/reg in pure pancreatic juice is shown in supplementary Figure 1 (see Figure, **Supplement Digital Content 2,** http://links.lww.com/A858).

Labeling of PSP/reg. Recombinant PSP/reg was labeled with a commercially available fluorescein isothiocyanate (FITC) labeling kit (Sigma) according to the manufacturer's protocol. Briefly, 1 mg recombinant PSP/reg protein (5 mg/mL in 0.1 M Na_2CO_3 buffer, pH 9.0) was incubated with 0.6 mg FITC (10 mg/mL in dimethyl sulfoxide) for 2 hours at room temperature in the dark. The labeled PSP/reg was separated from residual FITC by solid-phase chromatography (Sephadex G25 column, Sigma). Fractions containing the labeled PSP/reg were pooled, and the protein concentration and labeling intensity were measured photometrically (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA). The FITC-PSP/reg solution was supplemented with 0.1% BSA (Sigma) and stored at 4°C until use.

Collection of Blood. Whole blood from healthy controls or from patients within the first 4 hours after trauma (day 0) was collected into sterile tubes containing 143 United States Pharmacopeia units of lithium heparin (Becton Dickinson, Meylon Cedex, France) at days 0, 1, 3, 5, 7, 10, 14, and 21.

For serum collection, blood was collected in sterile glass tubes and allowed to clot at 4°C for 15 minutes before being centrifuged at 1500 $\times$ *g* for 20 minutes at 4°C. The serum was aliquoted and stored at $-$70°C until assayed.

Isolation of Neutrophil Granulocytes. Neutrophil granulocytes (PMN) were isolated from heparinized blood according to previous reports (23, 24). The final neutrophil preparation (1×10^6/mL) contained $>$95% neutrophils, as determined by flow cytometry analysis using phycoerythrin (PE)-labeled anti-CD15 monoclonal antibody (Dako, Glostrup, Denmark). Cell viability was $>$98%, as determined by trypan blue exclusion.

Stimulation Protocol and Flow Cytometry Analysis. Whole blood (1 mL) or isolated PMN (4×10^6/mL) was stimulated with or without PSP/reg for 1 hour at 37°C and 5% CO_2 atmosphere. After stimulation, whole blood or isolated PMN (50 μL each) was stained with respective antibody solutions, and fluorescence was measured by flow cytometry.

PE fluorescence of individual cells was measured with a FACSCalibur flow cytometer (Becton Dickinson AG, Basel, Switzerland), gating on physical parameters to exclude cell debris. In whole blood, the neutrophil population was detected by staining with PE-anti-CD15 antibodies, and the monocyte population was identified by staining with anti-CD14 antibodies (Dako, Glostrup, Denmark). Expression of CD11b (Dako, Glostrup, Denmark) and CD62L (eBioscience, Wembley, UK) by these cells was measured by gating on the respective population in the forward-scatter and side-scatter scan. A minimum of 10,000 events per gate were counted per sample. Results are reported as the mean fluorescence intensity (MFI) corrected by subtracting the fluorescence of cells stained with the respective PE-labeled isotype control antibody (25).

For measurement of PSP/reg binding, leukocytes were incubated with serial dilutions of FITC-labeled PSP/reg (1–10 μL, corresponding to 6.6–66.6 μg/mL) for 1 hour on ice. FITC-PSP/reg fluorescence on the surfaces of monocytes or PMN was measured in gates set in the forward-scatter and side-scatter scan that were identified previously by staining with anti-CD14 or anti-CD15, respectively.

Data Presentation and Statistics. Results are presented as means and SD (mean ± SD) or medians and interquartile ranges [IQR]. SigmaPlot Version 10.0 was used for figures. After a logarithmic transformation, the time courses of PSP/reg were compared between groups using an analysis of variance for repeated measures with *post hoc* Bonferroni test in which p values were multiplied accordingly. No Bonferroni correction was performed for comparisons between groups at single days. A forward stepwise (likelihood ratio, entry $p \geq 0.05$) logistic regression analysis was performed to test the predictive value for the occurrence of sepsis of PSP/reg concentrations in comparison to PCT, CRP, or IL-6 levels. Continuous data were compared by the Mann-Whitney test and the Kruskal-Wallis test. Comparisons of stimulated blood samples were done by paired Student's t test. Categorical variables were compared by the chi-square test. Differences were considered significant if p values were <0.05. Statistical analysis was performed using the SPSS software package (SPSS 13.0 for Macintosh, SPSS Inc., Chicago, IL).

RESULTS

Demographic and Clinical Data of Study Population. The mean age, Injury Severity Score (17), Glasgow Coma Scale, Acute Physiology and Chronic Health Evaluation II score (26), intensive care unit stay, and gender distribution of the three enrolled populations are listed in Table 1. The three patient groups were comparable with regard to age, Injury Severity Score, Glasgow Coma Scale, and Acute Physiology and Chronic Health Evaluation II score but differed in number and days in the intensive care unit. The group without infection had fewer patients (n = 18) than the other two groups (n = 32 and 33, respectively). The mean intensive care unit stay in the three groups was related to the severity of posttraumatic complications, with the lowest number of days in the "no infection" group (9.6 ± 5.7 days) and the highest in the sepsis group (30.3 ± 14.9 days) (Table 1).

The incidence of injury site and the mean Abbreviated Injury Scale (27) for each group are shown in Table 2. The Abbreviated Injury Scale for head, thorax, and abdomen increased slightly with the severity of complications, whereas differences were marginal for the other injury sites (extremities, pelvis, and spine). The incidence and grade of SIRS and the incidence of sepsis in each group (Table 2) reflected the severity of posttraumatic complications, whereas mortality did not. The mortality rate of the no infection group (11%) was higher than that of the

Table 1. Demographic data of enrolled patients

Parameter	No Infection	Local Infection	Sepsis
Number	18	32	33
Age (yrs)	38.8 ± 15.6	37.1 ± 14.4	38.5 ± 16.1
Males	15 (83%)	20 (62%)	28 (85%)
ISS (points)	35.1 ± 9.6	32.3 ± 13.8	38.9 ± 14.7
GCS (points)	9.3 ± 5.2	9.7 ± 5.0	8.7 ± 5.1
APACHE II (points)	14.4 ± 6.5	14.9 ± 7.2	17.8 ± 7.4
ICU (d)	9.6 ± 5.7[a]	18.7 ± 11.9[a]	30.3 ± 14.9[a]

ISS, Injury Severity Score (20); GCS, Glasgow Coma Scale; APACHE II, Acute Physiology and Chronic Health Evaluation II (26); ICU, intensive care unit.

[a] $p < 0.001$ (Kruskal-Wallis test). Mean ± SD. Values in parentheses are percentages.

Table 2. Injury pattern and posttraumatic course of enrolled patients

Parameter	No Infection (n = 18)	Local Infection (n = 32)	Sepsis (n = 33)
AIS head (mean points)	78% (3.4)	75% (3.4)	67% (3.7)
AIS thorax (mean points)	83% (3.0)	41% (3.4)	67% (3.5)
AIS abdomen (mean points)	50% (3.9)	41% (4.1)	49% (4.3)
AIS extremities (mean points)	67% (3.0)	72% (2.5)	67% (2.6)
AIS pelvis (mean points)	22% (3.0)	25% (2.8)	18% (2.8)
AIS spine (mean points)	56% (2.9)	38% (2.8)	30% (2.3)
No SIRS	2 (11%)	0	0
SIRS 2	6 (33%)	4 (12%)	0
SIRS 3/4	10 (56%)	28 (88%)	0
Sepsis	0	0	33 (100%)
Mortality	2 (11%)	2 (6%)	6 (18%)

AIS, abbreviated injury scale (27); SIRS, systemic inflammatory response syndrome (20). Percentage (%) of patients who scored positive.

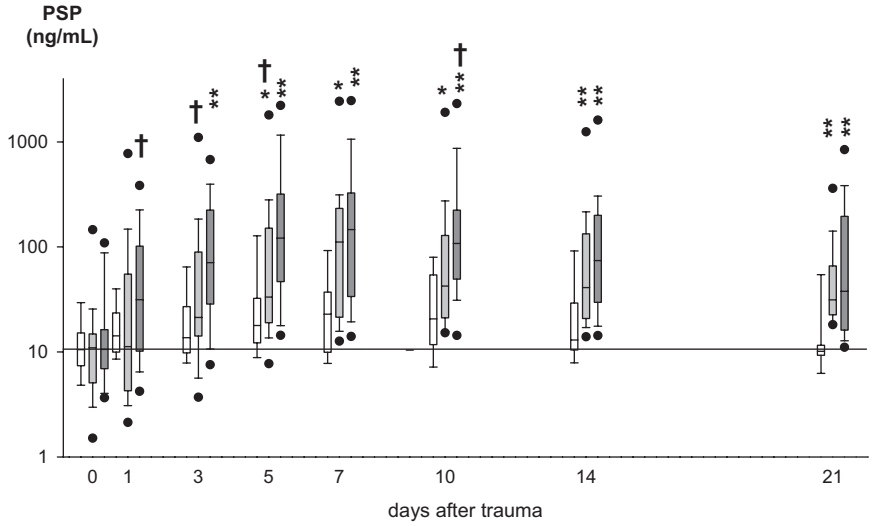

Figure 1. Kinetics of pancreatic stone protein (*PSP*)/reg serum levels in polytrauma patients. PSP/reg serum levels from polytrauma patients without infection (*white bars*, n = 18), with local infection (*gray bars*, n = 32), or with sepsis (*dark gray bars*, n = 33) were measured by enzyme-linked immunosorbent assay from the day of admission until day 21. Data are represented as *box plots*. *Whiskers* and *black circles* indicate 10th/90th percentiles and outlying points. The *horizontal line* indicates normal values of healthy volunteers. *p value <0.05 and **p value <0.01 for the comparison of patients with local infection or sepsis vs. noninfected patients. †p value <0.05 for comparison of patients with local infection vs. patients with sepsis.

"local infection" group (6%); however, this difference was not significant. The highest mortality rate was observed in patients with sepsis (18%) (Table 2).

Posttraumatic Upregulation of PSP/reg. Initial (day 0) serum levels of PSP/reg in all three patient groups (10.5 [7.4–15.2]; 10.9 [5.1–14.8]; 10.6 [6.9–16.3] ng/mL; median [interquartile range]) (Fig. 1) were comparable to those of healthy controls (n = 38; 10.4 [7.5–12.3] ng/mL). In the group without infectious complications, PSP/reg increased from 10.5 to 22.8 ng/mL during the hospital stay. In patients with a local infection, PSP/reg increased to 111.4 ng/mL, and in septic patients, PSP/reg levels reached 146.4 ng/mL, which represents more than a 15-fold increase over baseline (Fig. 1, days 5–10). PSP/reg levels in septic patients were elevated significantly compared with the group without infections from days 3 to 21 and were elevated significantly compared with the group with local infections on days 1, 3, 5, and 10. The time courses of PSP/reg elevation in the three groups were significantly different (all $p < 0.0005$ after Bonferroni correction). Therefore, we conclude that PSP/reg is an acute-phase protein that responds to injury, specifically to systemic cues, during the early, covert phase of infection.

Courses of Other Pancreatic Proteins After Trauma. At admission, pancreatic amylase was within normal levels (13–53 U/L) in all patients (19.5 [15–32.5]; 21 [15–39.8]; 20 [14.8–28.5] U/L). In the septic patient group, the amylase level increased from 20 U/L to a maximum of 132.5 U/L, a level that was maintained throughout hospitalization. The strongest amylase increase occurred in patients with local infections on day 14 (157.5 U/L). In the noninfected group, amylase levels rose to a maximum of 90 U/L on day 7 but were well below this level during most of the hospitalization period (see Figure, Supplemental Digital Content 3, http://links.lww.com/A859). For pancreatic lipase, similar courses were observed, with levels appearing to increase over the period of hospital stay (see Figure, Supplemental Digital Content 4, http://links.lww.com/A860).

Posttraumatic Course of CRP and Leukocyte Numbers. CRP showed a gradual increase from low levels at admission (1 [1–6]; 2 [1.5–3]; 2 [2–3] μg/mL) to ~168 μg/mL on day 3 in all groups (Fig. 2*A*). Significant differences in the course of CRP expression were found only after

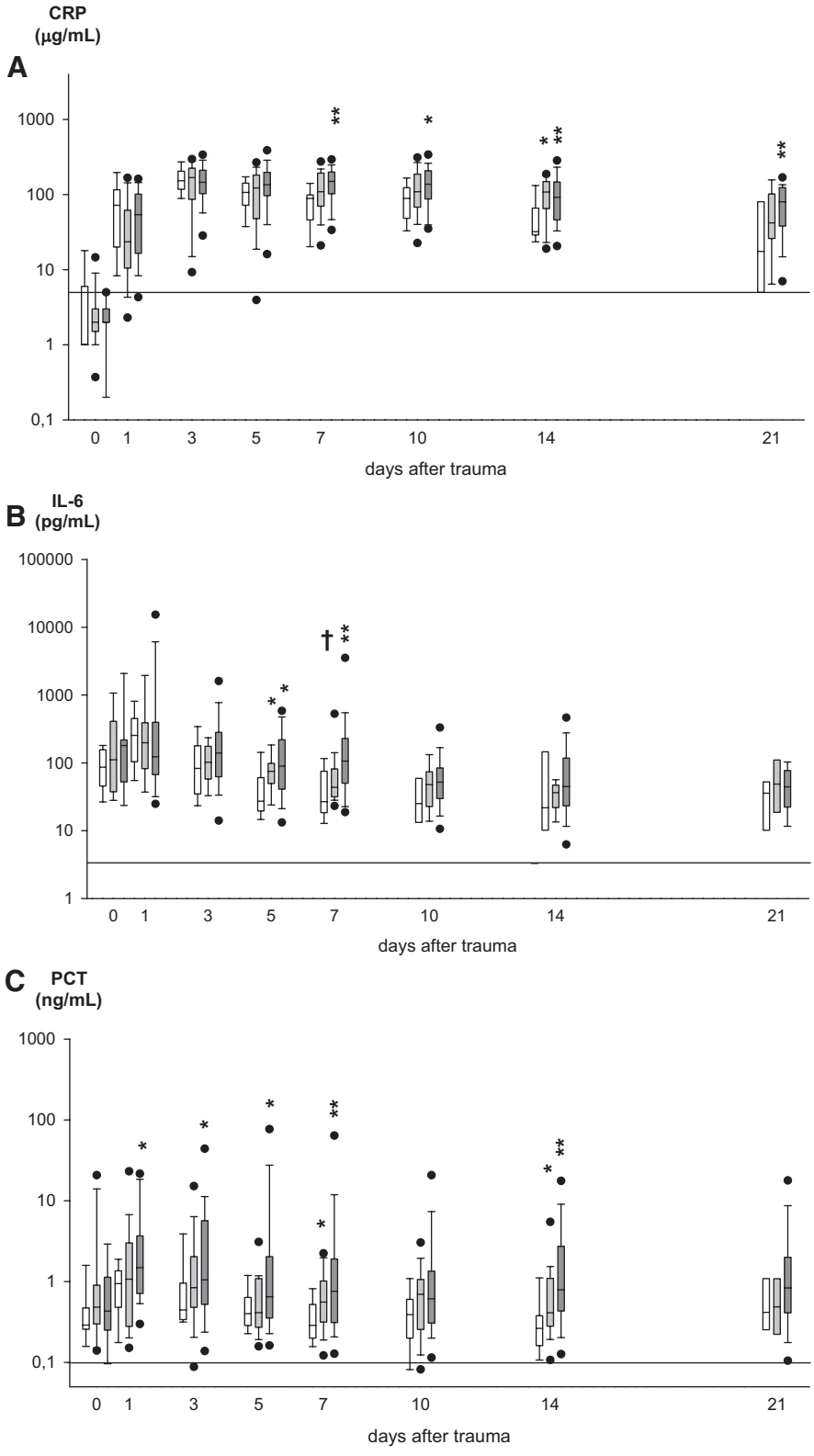

Figure 2. Kinetics of C-reactive protein (*CRP*), interleukin (*IL*)-6, and procalcitonin (*PCT*) serum levels in polytrauma patients. *A*, Serum CRP levels from polytrauma patients without infection (*white bars*, n = 18), with local infection (*gray bars*, n = 32), or with sepsis (*dark gray bars*, n = 33) were measured by an immunoturbidimetric assay from the day of admission until day 21. *B*, Serum IL-6 levels from the same polytrauma patients were measured by enzyme-linked immunosorbent assay from the day of admission until day 21. *C*, Serum PCT levels were measured by immunoluminometric assay. Data presentation is described in Figure 1. The *horizontal line* represents normal values.

day 7 in the group of septic patients compared with patients without infection and at day 14 between infected and noninfected patients. CRP levels could not discriminate between the patients with infection and those with sepsis (Fig. 2*A*).

Leukocyte numbers at admission were comparable in all three groups (10.5 [6.6–16.5]; 12.4 [9.2–13.7]; 12.2 [9.1–17.5] $\times$ $10^3/\mu L$). Significant differences were seen between septic patients and patients without infection from day 7 to day 21 and between infected and noninfected patients at day 21 only. At days 7 and 14, differences in leukocyte numbers between infected and septic patients were significant (see Figure, Supplemental Digital Content 5, http://links.lww.com/A861).

Release of IL-6 and PCT. IL-6 increased posttraumatically on the day of admission (79.8 [37.9–155.4]; 68.9 [31.9–384.5]; 177.2 [26.1210.6] pg/mL), reaching the highest levels on day 0 in the group with sepsis and on day 1 in the groups with and without infection (174.3 [49.2–345.3] and 253.8 [77.7–445.9] pg/mL, respectively) (Fig. 2*B*). During the first 3 days, the IL-6 values in the three severity groups were different from those of healthy subjects, but IL-6 levels could not discriminate among no infection, local infection, and sepsis. IL-6 levels were significantly higher after day 5 in septic patients and after day 14 in patients with infection compared with the group without infection (Fig. 2*B*).

PCT levels clearly increased in the septic patient group (1.5 [0.7–3.7] ng/mL), whereas in the other groups PCT remained at ~0.4–1.1 ng/mL (Fig. 2*C*). However, in septic patients the range was very high [0.3–5.7 ng/mL], and the values for PCT in septic patients differed significantly from the patient group without infection at days 1, 3, 5, 7, and 14. The PCT values for patients with infection differed significantly from those for patients without infection on days 7 and 14 only. No significant differences in PCT levels were observed between patients with sepsis and patients with infection. Thus, commonly used markers of inflammation, such as CRP, IL-6, and PCT, increased during the early phase after severe trauma, but these values did not discriminate between infected and septic patients in this study.

Comparison of Infected and Noninfected Patients. For comparison of infected and noninfected patients, data for groups 2 and 3 were pooled. Statistical analysis revealed significant differences in PSP/reg levels on days 3–21; in CRP on days 7, 10, and 14; in IL-6 on days 5 and 7; and in PCT levels on days 7 and 14 (data not shown). Logistic regression analysis for PSP/reg, IL-6, CRP, and PCT data between noninfected and infected patients identified PSP/reg as an independent predictor of infection on days 3, 7, and 10. IL-6 and PCT were found to be independent predictors on day 5 and day 14, respectively. No predictor was found for days 0, 1, and 21.

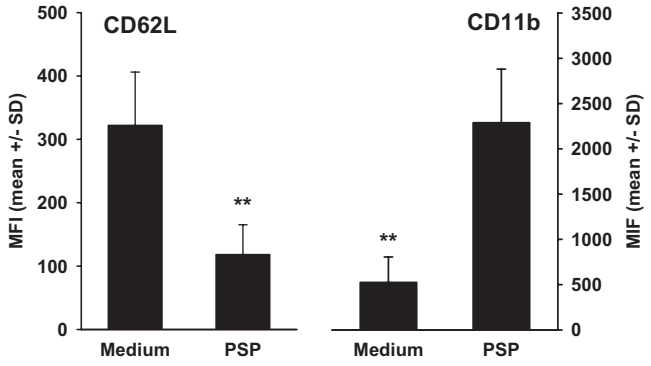

Figure 3. Expression of CD62L and CD11b on the polymorphonuclear neutrophil granulocytes surface. Whole blood from healthy volunteers was incubated with medium or with pancreatic stone protein (*PSP*)/regenerating protein (500 ng/mL) for 1 hour, and expression of CD62L (*left*) and CD11b (*right*) on polymorphonuclear neutrophil granulocytes was analyzed by flow cytometry after staining with phycoerythrin-labeled antibodies (mean $\pm$ SD *black bars*, n = 8). **p value <0.01 for comparison of medium-treated vs. PSP-treated cells. *MFI*, mean fluorescence intensity.

Biological Function of PSP/reg. To test whether PSP/reg has a biological effect on target cells, whole blood from healthy volunteers was incubated for 1 hour in the presence of PSP/reg concentrations of up to 1000 ng/mL. Leukocytes were analyzed by flow cytometry for extracellular markers of rolling (CD62L) and adhesion (CD11b). The leukocyte subpopulations were well separated and identified by size in the forward scatter/side scatter gate (see Figure, Supplemental Digital Content 6, http://links.lww.com/A862) and after specific staining with PE-labeled anti-CD15 and anti-CD14 antibodies.

Expression of CD62L and CD11b on PMN was affected by PSP/reg in a dose-dependent manner (Supplementary Digital Content 7, http://links.lww.com/A864). PSP/reg caused a clear reduction in CD62L expression, which implies shedding of this protein. In contrast, CD11b was upregulated in PMN. To exclude that endotoxin contamination of the recombinant PSP/reg preparation was responsible for activation of PMN, PSP/reg was boiled for 15 minutes. The stimulating effect was completely lost, indicating that PSP/reg, and not the heat-resistant lipopolysaccharide, is responsible for this effect (see Figure, Supplemental Digital Content 7, http://links.lww.com/A864).

For future tests, 500 ng/mL was used. In an independent series, 500 ng/mL PSP/reg reduced CD62L expression, whereas CD11b expression significantly increased (Fig. 3). These findings support the hypothesis that PSP/reg is involved in the activation and recruitment of PMN.

Binding of PSP/reg to Neutrophil Granulocytes. To evaluate whether PSP/reg interacts directly or indirectly with PMN, purified PMN were incubated with FITC-labeled, recombinant PSP/reg, and FITC fluorescence was analyzed by flow cytometry. A dose-dependent (6.6–66.6 μg/mL) increase in FITC fluorescence (from 7.7 $\pm$ 0.8 to 28.2 $\pm$ 0.8 MFI) was detected on PMN, whereas autofluorescence was low (4.6 $\pm$ 0.2 MFI) (Fig. 4). After coincubation with a 100-fold (6.66 mg/mL) excess of unlabeled PSP/reg (n = 3), the FITC-PSP/reg fluorescence in PMN (MFI 28.2 $\pm$ 0.8) was reduced significantly (8.9 $\pm$ 2.4 MFI) (Fig. 4, inset), which demonstrates specific binding of PSP/reg to PMN.

Constitutive Exposure of Granulocytes to PSP/reg. The observation that PSP/reg was highly increased in serum during sepsis led us to question whether PMN from septic patients who were exposed to a high level of PSP/reg for several days would respond similarly to PSP/reg stimulation as naïve cells from healthy controls. Whole blood from patients with sepsis (n = 8) was stimulated with 500 ng/mL PSP/reg for 1 hour. Under these conditions, PSP/reg could not induce shedding of CD62L (Fig. 5*A*) or CD11b upregulation (Fig. 5*B*) compared

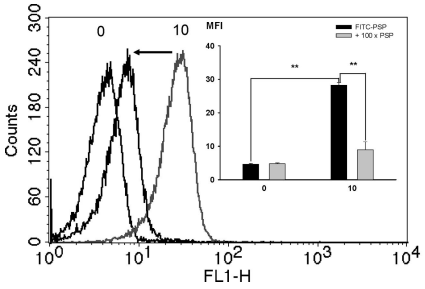

Figure 4. Flow cytometric analysis of fluorescein isothiocyanate (*FITC*)-labeled pancreatic stone protein/regenerating protein (*PSP*/reg) binding to isolated polymorphonuclear neutrophil granulocytes (PMN). Isolated PMN from healthy controls were stained with FITC-labeled PSP/reg, with or without a 20-fold molar excess of unlabeled PSP/reg, and fluorescence was analyzed by FACS. The histogram analysis for the PMN shows the fluorescence for nonspecific binding (*black line*), specific binding (*gray line*), and competition with a 20-fold molar excess of unlabeled PSP/reg (*dark gray line*). *Inset* depicts the mean fluorescence intensity ± SD for three separate experiments. $^{**}p$ value <0.01 for comparison of medium vs. FITC-labeled PSP or of FITC-labeled PSP with vs. without a 100-fold excess of unlabeled PSP/reg.

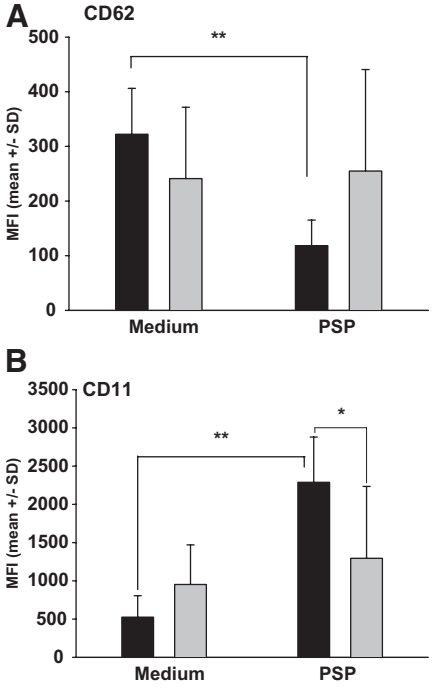

Figure 5. Expression of CD62L and CD11b on polymorphonuclear neutrophil granulocytes in whole blood from healthy controls and septic patients. Whole blood was incubated with medium or pancreatic stone protein (*PSP*)/reg (500 ng/mL) for 1 hour, and expression of CD62L (*A*) and CD11b (*B*) on polymorphonuclear neutrophil granulocytes was analyzed by flow cytometry after staining with phycoerythrin-labeled antibodies. Shown is the mean ± SD for healthy controls (*black bars*, n = 8) or for septic patients (*gray bars*, n = 8). $^{**}p$ value <0.01 for comparison of medium vs. PSP/reg, $^{*}p$ value <0.05 for comparison of controls vs. patients with sepsis. *MFI*, mean fluorescence intensity.

with the values from healthy controls (n = 8), which suggests that repeated exposure reduces responsiveness to PSP/reg.

DISCUSSION

In this study, we have shown that PSP/reg is highly increased in the blood of severely injured trauma patients. The appearance of PSP/reg in the blood might be explained by several independent processes. On the one hand, PSP/reg could be synthesized by healthy acinar cells in response to nonpancreatic trauma and released into the bloodstream. On the other hand, damaged acinar cells might release their secretory proteins into the bloodstream. This process would lead to a parallel increase in serum amylase after a trauma and would reflect pancreatic cellular damage. In comparison to pancreas-specific inflammation, where amylase levels can surpass 1000 U/L, the maximal amylase level in our septic patients (132.5 U/L) was just a little above the upper limit of normal (13–53 U/L), which indicates that pancreatic damage was not present or very limited. Furthermore, relevant pancreatic trauma was excluded by radiologic examination (computed tomographic scan, data not shown).

Another potential source of PSP/reg is the intestine (28), in which members of the pancreatitis-associated protein and PSP/reg family of proteins are expressed constitutively (29). Paneth cells at the base of the crypts produce these proteins, and these cells are thought to be related to pancreatic acinar cells on the basis of their ability to secrete a number of proteins known to be produced in the pancreas, e.g., trypsin and pancreatic secretory trypsin inhibitor (30, 31). After an inflammatory insult, the intestine activates secretion of these proteins, and expression of PSP/reg can also be observed in enterocytes. Thus, it is likely that during inflammation and even more so in sepsis, the production of PSP/reg in the intestine is activated.

In patients with severe trauma, the PSP/reg levels began to increase at day 3. In a consistent bell-shaped curve, these increased levels mirrored the onset and peak of sepsis, which usually occurred between days 7 and 10. In our study, the onset of sepsis (n = 33) was observed 8.7 ± 4.3 days after trauma. PCT levels demonstrated a similar curve; however, because of the limited patient number, the data were not as consistent as the PSP/reg data. In a previous study that analyzed a larger number of patients (n = 405), PCT increased significantly by day 3 after trauma (2). Because of the lower total number of patients in our study (n = 83), the observed differences in PCT levels in the patient subgroups were not significant. However, with a higher patient number we expect that PSP/reg levels would distinguish the subgroups more clearly than PCT. This situation would make PSP/reg a suitable predictive marker for sepsis in posttraumatic patients.

In contrast to the most commonly used inflammation markers CRP and PCT, whose functions still have to be determined (32), we found clear evidence for a function of PSP/reg. PSP/reg not only binds to neutrophils (Fig. 4), but also elicits an activating response in PMN (Fig. 5). In these cells, we showed shedding of L-selectin (CD62L) and increased exposure of the β_2-integrin (CD11b) in response to incubation with PSP/reg levels found in serum from patients with sepsis. The functions of CD62L and CD11b have been investigated thoroughly, and both proteins are involved in the early phase of PMN activation, i.e., rolling along and sticking to the endothelium (16). The endotoxin contamination found in our recombinant PSP/reg preparations was much too low (0.098 EU/mL) to be responsible for the biological effects of PSP/reg, and PSP/reg boiled for 15 minutes did not induce neutrophil activation. Therefore, our findings could place PSP/reg in the group of danger signals (33) similar to the heat shock proteins (34). The reduced responsiveness of PMN toward restimulation with PSP (Fig. 5*A*, *B*) indicates a desensitization of PMN toward PSP during inflammation. A similar desensitization has been shown for other proinflammatory stimuli, e.g., lipopolysaccharide and IL-12 in critically ill patients (35).

Although we did not elucidate completely the role of PSP/reg in sepsis, we showed that PSP/reg is detectable in the serum of polytraumatized patients and is related to the severity of posttraumatic inflammation, which qualifies PSP/reg as a prognostic marker for posttraumatic complications, such as sepsis. High levels of PSP/reg in the blood of septic patients seem to induce and/or maintain the activation of neutrophils;

thus, PSP/reg might serve as an acute-phase protein.

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