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Elevated plasma matrix metalloproteinases and their tissue inhibitors in patients with severe sepsis

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Abstract

Purpose: Matrix metalloproteinases (MMPs) are essential for tissue remodeling. Our objectives were to determine (1) the concentrations of MMPs and their tissue inhibitors (TIMPs) in plasma obtained from patients with severe sepsis, (2) to correlate changes in MMP and TIMP levels with disease severity, and (3) to investigate recombinant activated protein C (rAPC) actions on plasma MMP2, 9 activities from severe sepsis patients.

Materials and methods: Matrix metalloproteinase and TIMP levels were quantified in plasma from patients with severe sepsis using antibody microarrays and gelatin zymography.

Results: Plasma MMPs (3, 7, 8, 9) and TIMPs (1, 2, 4) on microarray were increased in severe sepsis on intensive care unit (ICU) day 1, with more than 3-fold increases in MMP3, MMP7, MMP8, MMP9, and TIMP4. Latent forms of MMP2, 9 on zymography were increased in plasma from patients with severe sepsis, whereas only half of severe sepsis patients showed active MMP9. Elevated MMP7 and MMP9 on ICU days 1 and 3 negatively correlated with multiple organ dysfunctions. The temporal activity patterns of MMP2, 9 during 21 ICU days were not altered in patients treated with rAPC or by the addition of exogenous rAPC to plasma.

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Conclusion: Most plasma MMPs and TIMPS were elevated in patients with severe sepsis, but only a limited subset of MMPs (7, 9) negatively correlated with disease severity. Recombinant activated protein C does not appear to directly alter MMP2, 9 activities.

1. Introduction

Severe sepsis is a systemic inflammatory response to infection with acute organ dysfunction, hypoperfusion, and/or hypotension [1]. Despite advances in therapy, severe sepsis remains a leading cause of morbidity and mortality (28%-50%) in intensive care units (ICUs) [2,3]. An improved understanding of the molecular mechanisms underlying severe sepsis will lead to novel therapies that improve survival.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that may play an important role in severe sepsis. Matrix metalloproteinases are capable of degrading components of the extracellular matrix and play roles in both normal and pathophysiologic processes including wound healing, tissue repair, and remodeling [4]. Matrix metalloproteinases are secreted or associated with membranes and are initially latent zymogens that can be activated by protease-driven cleavage and/or posttranslational modification [5]. In humans, MMPs consist of a family of approximately 26 members including the collagenases (MMP1, 8, 13), stromelysins (MMP3, 10), matrilysins (MMP7, 26), and gelatinases (MMP2, 9) [6,7]. The gelatinases, MMP2 and MMP9, are critical for normal vascular development, functioning, and remodeling, as evidenced by their important roles in processes such as vasomotor tone, angiogenesis, and tumor invasion [8,9]. Furthermore, through the processing of cytokines and chemokines, these particular MMPs regulate inflammation, with MMP2 having anti-inflammatory actions and MMP9 thought to be proinflammatory [10-14].

Matrix metalloproteinases are regulated endogenously by binding to their tissue inhibitors of metalloproteinases (TIMPs, numbered 1-4) [15]. Different TIMPs inhibit MMPs to varying degrees based on structural affinity by forming 1:1 complexes [16]. The MMP/TIMP ratio may be important in specific tissues, as altered MMP/TIMP profiles are observed in various organs after experimental endotoxemia [17].

The role of MMPs and TIMPs in severe sepsis remains largely unknown; however, several studies have been reported. For example, MMP9 activity is increased in human plasma after experimental administration of lipopolysaccharide (LPS) [18,19] and peptidoglycan [20]. Plasma MMP9 [21-24], MMP10 [24], TIMP1 [22,24], and TIMP2 [22] levels are also elevated in patients with severe sepsis. Matrix metalloproteinase 9 and TIMP1 levels may correlate with survival [21,24]. To further study MMPs and TIMPs during severe sepsis, we investigated a large panel of plasma MMPs and TIMPs in a well-defined severe sepsis patient population using MMP and TIMP antibody microarrays and gelatin zymography. We hypothesized that plasma MMP and TIMP levels are altered by severe sepsis and that these changes would correlate with disease severity.

Activated protein C (APC) modulates severe sepsis by its anti-inflammatory and antithrombotic effects; however, endogenous APC is often diminished in severe sepsis [25-31]. Administration of recombinant APC (rAPC) is the only approved drug therapy for severe sepsis, resulting in a significant reduction in absolute mortality [32,33]. Activated protein C is also an endogenous regulator of MMP2 and MMP9 expression. Activated protein C increases MMP2 expression in endothelial cells during angiogenesis [34] and keratinocytes during wound healing [35], whereas, rAPC decreases expression of MMP9 in ischemic brain endothelium [36] and in fibroblasts during arthritis [37]. It is not known if rAPC directly regulates MMP2, 9 activities, particularly in severe sepsis, thereby modulating disease severity. Thus, we also hypothesized that rAPC, used as a treatment for severe sepsis, directly alters plasma MMP2, 9 activities.

2. Materials and methods

The institutional review boards of Hamilton Health Sciences (Hamilton, Ontario) and the University of Western Ontario (London, Ontario) approved this study. Consistency of approach in the participating centers was monitored by a senior study investigator (P.L.).

2.1. Study population and data collection

This pilot study used a convenient sample size. For all initial biochemical comparisons between severe sepsis patients and healthy controls (age, 34 ± 3 years; range, 23-42 years; 40% female), we used n = 15 per group. Plasma samples from an additional 5 severe sepsis patients were added to the initial 15 severe sepsis patients for zymography analyses within this cohort (n = 20). Patients admitted to 1 of 3 academic ICUs from March 2007 to March 2008 were identified with a diagnosis of severe sepsis using identical inclusion criteria to the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study group [33]. Thus, inclusion in this study required sepsis associated with signs of at least one acute organ dysfunction, hypoperfusion, or hypotension. In accordance with the research ethics boards, informed consent was obtained either directly from the patient or from a

substitute decision maker. The exclusion criteria used in this study were pregnancy or breast-feeding, age younger than 18 years, use of unfractionated heparin to treat an active thrombotic event within 8 hours of blood sampling, and use of low–molecular weight heparin at a dose higher than recommended for prophylactic use within 12 hours of blood sampling. Baseline characteristics of the patients were collected, including demographic information, Acute Physiology and Chronic Health Evaluation II (APACHE II) admissions scores [38], daily Multiple Organ Dysfunction (MOD) scores [39], comorbidities, organ function, site and type of infection, and hematologic tests. Recombinant APC was administered according to previous criteria [33].

2.2. Blood collection

Blood was collected from patients with severe sepsis on the first day of ICU admission, within 24 hours of meeting the definition of severe sepsis. Blood samples were obtained daily for the first week and once a week thereafter for the duration of the patients' stay in the ICU. Venous blood (4.5 mL) was drawn via indwelling catheters (severe sepsis patients) or fresh venipuncture (healthy controls) and immediately transferred into 15 mL polypropylene tubes containing 0.5 mL of 0.105 mol/L buffered trisodium citrate (pH 5.4) with 100 μL of 1 mol/L benzamidine HCl (approximately 20 mmol/L benzamidine), a serine protease inhibitor required to stabilize rAPC in plasma. The blood was immediately centrifuged at 1500*g* for 10 minutes at 20°C, and the plasma was stored in aliquots at −80°C. Care was taken to ensure that freeze/thaw cycles were avoided. Subsequent sample analysis was performed by an investigator blinded to the experimental cohort (severe sepsis vs healthy control) in one laboratory as batched samples (D.D.F.).

2.3. Matrix metalloproteinase/TIMP antibody microarrays

Plasma samples were diluted by 1:20 in Array Sample Diluent and were run on an ExcelArray Human MMP/TIMP Array (Thermo Fisher Scientific, Rockford, IL). Methodologies for microarray production and antibody cross reactivity assays have been described previously [40]. Briefly, commercially available MMP and TIMP antibodies were used to produce the ExcelArray Human MMP/TIMP array. Most of these antibodies recognize both the latent and active form of the MMP proteins, with the following exceptions. The MMP7 detector antibody only recognizes the proform of MMP7. The MMP12 capture antibody binds to the catalytic domain of MMP12. The MMP12 detector antibody recognizes forms of MMP12 containing the C-terminal hemopexin-like domain. The specificity of the MMP9 detector antibody is not determined. Cross-reactivity was determined by running each recombinant standard individually on the MMP/TIMP array and measuring the signal intensity for all 12 antibody pairs. Less than 5% cross-reactivity was observed in these experiments. The extent to which the antibody pairs recognize MMP/TIMP complexes is unknown. Standard curves were obtained for all 12 targets using a mixture of all 12 recombinant proteins. Matrix metalloproteinase/TIMP microarrays were preblocked with a BSA–containing blocking buffer to minimize nonspecific binding. The negative control spots printed on the array (containing mouse IgG) serve as indicators of any nonspecific binding to the array. The lack of fluorescent signal detected on the negative control spots indicated no nonspecific binding in the MMP/TIMP array assays with the control and severe sepsis samples. Microarrays were imaged using an Alphascan Microarray Imager (Alpha Innotech, San Leandro, CA), and spot densitometry was performed by Thermo Fisher (Rockford, IL) using ArrayVision Software (GE Healthcare, Piscataway, NJ).

2.4. Gelatin zymography

This sensitive technique is based on the incorporation of the enzymatic substrate gelatin into the electrophoretic gel and allows for accurate visualization of both latent and active forms of the gelatin-degrading enzymes MMP2, 9 [41]. Plasma samples were quantified for total protein concentration using the colorimetric DC Protein Assay (Bio-Rad Laboratories, Inc, Hercules, CA) and read on a Multiskan Ascent microplate reader (Thermo Labsystems, Franklin, MA). Plasma with a total undiluted protein concentration of 100 μg or diluted by 1:10 in sample buffer were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) gels containing 1% gelatin under nonreducing conditions at 140 V. All gels contained a known MMP standard consisting of conditioned media from MMP2 secreting baby hamster kidney (BHK) cells transfected with MMP9 complementary DNA. Enzymes were renatured in 50 mL 2.5% Triton X-100 (vol/vol) in deionized water for 1 hour at room temperature on a RotoMix (Type 48200, Thermolyne, Dubuque, IA). After washing the gels for 5 minutes in 50-mL deionized water on mixer, they were incubated (Model 12-140E, Quincy Lab, Inc, Chicago, IL) for 20 hours at 37°C in 45 mL developing buffer (50 mmol/L *tris*-HCl, pH 7.4, 200 mmol/L NaCl, 5 mmol/L $CaCl_2$) containing one complete EDTA-free Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN). Gels were stained using 0.5% Coomassie Brilliant Blue R-250 for 1 hour on a mixer followed by destaining in 30% methanol and 10% acetic acid until the wash solution was clear. The gels were imaged with a FluorChem SP imaging system and FluorChem 8800 software (Alpha Innotech Corp, San Leandro, CA). Optical densitometry (OD) of clear white bands on a blue background indicated gelatinolytic activity and was determined using Multi-Analyst Version 1.1 software (Bio-Rad Laboratories, Inc, Hercules, CA). Optical densitometry is expressed as a ratio of the MMP band of interest to the known standard on the same gel.

2.5. Statistics and data analysis

Experiments were performed by researchers blinded to the experimental groups and patient characteristics. Data were analyzed using SigmaStat 3.5 Systat Software, Inc., San Jose, CA. Continuous variables are reported with mean ± SE. Groups were prescreened for normality and compared with either a Student *t* test or Mann-Whitney *U* test. Correlations between continuous variables were determined with a Pearson product-moment correlation coefficient and, in some cases, displayed graphically with both a linear regression and 95% confidence intervals. $P \leq .05$ was considered statistically significant.

3. Results

The baseline characteristics of the patients admitted with severe sepsis including APACHE II and MOD scores, absolute neutrophils counts (ANCs), comorbidities, and infection data are shown in Table 1. Steroids were administered to 10 of 20 severe sepsis patients (50%). Of 20 patients with severe sepsis, 18 had septic shock (90%) and 4 died (20%). There was a statistically significant difference in the highest daily MOD scores between patients who either survived (10.1 ± 0.8; n = 16) or died (14.5 ± 0.5; n = 4; $P = .01$). In contrast, baseline APACHE II scores did not significantly differ between survivors (25.2 ± 2.6; n = 16) and nonsurvivors (28.3 ± 1.7; n = 4; $P = .57$).

Table 1 Baseline characteristics of 20 patients with severe sepsis on ICU day 1

Age, y	59 ± 4 (20, 80)
Sex, female	6 (30%)
APACHE II score	25.8 ± 2.1 (6, 42)
MOD score	10.4 ± 0.86 (3, 15)
ANC [a]	11.5 ± 0.9 (3.7, 19.5)
Comorbidities, no. (% of total)	
Cardiovascular	9 (45%)
Pulmonary	5 (25%)
Hepatitis/pancreatitis	5 (25%)
Inflammatory	3 (15%)
Neurologic	3 (15%)
Renal	3 (15%)
Diabetes	2 (10%)
Primary site of infection, no. (% of total)	
Lung	11 (55%)
Abdomen	3 (15%)
Blood	1 (5%)
Urinary tract	0 (0%)
Other	4 (20%)
Unknown	1 (5%)
Total no. of positive cultures (% of total) [b]	
Gram-negative bacteria	10 (50%)
Gram-positive bacteria	9 (45%)
Fungus	2 (10%)

Data are presented as mean ± SE (minimum, maximum) or n (%).
[a] Normal ANC = 1.5 to 8.0.
[b] Includes blood and nonblood cultures. Four polymicrobial nonblood cultures were not included in table.

The levels of MMP and TIMP proteins in healthy control and severe sepsis patient plasma samples were compared using MMP/TIMP antibody microarrays (n = 15 per group). As shown in Fig. 1, severe sepsis plasma samples obtained on the first day of admission to the ICU displayed significantly elevated levels of multiple MMPs (MMP3, 7, 8, 9) and TIMPs (TIMP1, 2, 4) relative to plasma obtained from healthy controls. With the exception of moderate TIMP1 levels, healthy controls had universally low levels of MMPs and TIMPs with limited variability (Fig. 1). Notable increases in severe sepsis included MMP3 (3.2-fold), MMP7 (3.2-fold), MMP8 (35-fold), MMP9 (4.3-fold), and TIMP4 (3.2-fold) (Table 2). Matrix metalloproteinase 2 increased 1.5-fold in severe sepsis, but this change was nonsignificant ($P = .125$, 1-β: 21%).

Correlation analyses were performed between elevated MMP/TIMP levels in plasma from patients with severe sepsis on ICU day 1 and either baseline APACHE II (27.1 ± 2.4, n = 15) or MOD scores, with the highest MOD scores (10.6 ± 1.0, n = 15) occurring on ICU day 3.1 ± 0.9. Although no correlations between either MMPs or TIMPs and APACHE II were established, a negative correlation was found between MMP7 levels on ICU day 1 and both the ICU day 1 MOD scores (n = 15, $P = .06$) and the highest daily MOD scores (n = 15, $P = .008$, Table 3). Matrix metalloproteinase 9 showed trends toward negative correlations with the highest MOD scores, whereas MMP8 showed a trend toward a positive correlation with APACHE II score (Table 3).

Given that the gelatinases (MMP2, 9) had been specifically implicated in the pathogenesis of severe sepsis, we specifically examined these activity levels using gelatin zymography (n = 15 per group, Fig. 2). Plasma samples were diluted 1:10 to allow for resolution of changes in both MMP2 and MMP9, without MMP2 saturation on OD. Latent MMP9 was observed as an isolated 92-kd bright band on zymography in diluted plasma from all patients with severe sepsis ($P < .001$, Fig. 2). In contrast, the 92-kd band observed in plasma from healthy controls was uniformly weak (Fig. 2). These MMP9 zymography findings are consistent with increased MMP9 observed on the antibody microarray (Fig. 1). Latent MMP2 was also observed as a distinct 72-kd band on zymography and mildly elevated in diluted plasma from patients with severe sepsis as compared to healthy controls ($P = .026$, Fig. 2).

Activated MMP9 was not observed on zymography in undiluted plasma from any of the healthy controls but was present in undiluted plasma in half of the patients with severe sepsis on ICU day 1 (n = 10/20, Fig. 3). There were no correlations between the presence of activated MMP9 and disease severity on ICU day 1. However, when total MMP9 (latent and active) in undiluted plasma of patients with

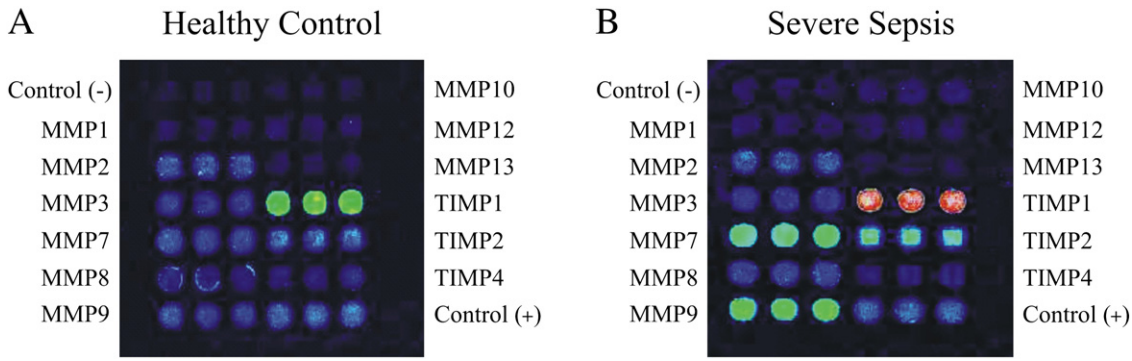

Fig. 1 Matrix metalloproteinase and TIMP antibody microarray analysis of human plasma. A, Representative microarray false-color image of an MMP/TIMP subarray of plasma from a healthy control. With the exception of moderate TIMP1 levels, MMPs and TIMPs were universally low in plasma obtained from healthy controls. Each antibody is printed in triplicate, and both negative and positive controls are illustrated. B, Representative microarray false-color image of a MMP and TIMP subarray of plasma from a patient with severe sepsis. Elevated levels of multiple MMPs and TIMPs can be seen as an increased fluorescence signal (blue→green→red) on the respective antibody spots. Plasma dilutions were 1:20 for all plasma samples as per the manufacturer recommendations, and all experiments were performed by a researcher blinded to experimental groups.

severe sepsis on ICU day 1 was correlated with measures of disease severity, a statistically significant negative correlation with the highest MOD scores was observed (n = 20, P = .028, Fig. 4). The statistical significance established for total MMP9 using zymography was likely due to a slightly larger patient cohort relative to the antibody microarray studies (n = 20 vs n = 15, respectively). Despite a likely neutrophilic source for at least some MMP9, no correlation was observed between total MMP9 and ANC (Table 1, P = .845).

Serial plasma samples from patients with severe sepsis were also examined with gelatin zymography (Fig. 5). Total MMP9 was typically greatest on ICU day 1 and then decreased significantly for 21 days. Matrix metalloproteinase 2 levels were constant for 21 days (Fig. 5). Patients with severe sepsis were subdivided into those who received rAPC treatment (n = 8/20, Table 4) and those who did not receive rAPC (n = 12/20, Fig. 5). Recombinant APC was administered as an infusion for 81.0 ± 7.8 (range, 48-96; n = 8/20) hours [33]. The 2 groups were evenly matched with regard to age and disease severity, although ANC was slightly greater in the rAPC group (Table 4). Gelatin zymography did not show an appreciable effect of rAPC on MMP9 activities for 21 days relative to patients who did not receive therapy (1-β: 5%-38%). However, it is possible that our sample size was too small to establish significant differences between the 2 treatment groups or that rAPC was dissociated from the MMP during the denaturing conditions of zymography. Thus, increasing concentrations of rAPC from 1 to 100 nM were added directly to the *Tris* incubation buffer after enzyme renaturing to test for direct alterations in MMP9 activity by rAPC. Under these in vitro conditions, up to 100 nM rAPC again failed to alter MMP9 activity (n = 6

Table 2 Plasma MMP and TIMP antibody microarray results

Analyte	Healthy control	Severe sepsis	Fold-increase	P
MMP2	70 ± 9	105 ± 29	1.5	.125
MMP3	11 ± 2	35 ± 6	3.2	.002
MMP7	38 ± 4	120 ± 18	3.2	<.001
MMP8	0.4 ± 0.3	14 ± 4	35.0	<.001
MMP9	32 ± 5	138 ± 17	4.3	<.001
TIMP1	162 ± 30	429 ± 33	2.6	<.001
TIMP2	51 ± 4	71 ± 6	1.4	.01
TIMP4	2.0 ± 0.6	6.4 ± 1.9	3.2	.024

All plasmas were obtained from patients with severe sepsis on ICU day 1, and values are presented as ng/mL (1:20 dilution, n = 15 per group). Matrix metalloproteinase 1, MMP10, MMP12, and MMP13 were undetectable in plasma from both healthy controls and patients with severe sepsis. Data were prescreened for normality and then compared with either a Student t test or a Mann-Whitney U test. Patient APACHE II and MOD scores were 27.1 ± 2.4 (range, 12-42) and 10.6 ± 1.0 (range, 4-16), respectively. Data are presented as mean ± SE.

Table 3 Correlation analyses between elevated plasma MMPs and TIMPs from patients with severe sepsis and APACHE II and MOD scores

Analyte	APACHE II score R, R^2 (P)	MOD score R, R^2 (P)
MMP3	0.077, 0.006 (.79)	0.003, 0.000 (.99)
MMP7	−0.142, 0.020 (.61)	−0.657, 0.432 (.008)
MMP8	0.456, 0.208 (.08)	0.419, 0.178 (.12)
MMP9	−0.109, 0.036 (.70)	−0.442, 0.195 (.09)
TIMP1	0.010, 0.000 (.97)	−0.047, 0.002 (.87)
TIMP2	−0.006, 0.000 (.98)	0.334, 0.112 (.22)
TIMP4	−0.326, 0.106 (.24)	−0.430, 0.185 (.11)

Plasma samples obtained from patients with severe sepsis on ICU day 1 and MMP and TIMP levels analyzed with antibody microarrays (R = correlation coefficient; 15 per group). Acute Physiology and Chronic Health Evaluation II and highest MOD scores were 27.1 ± 2.4 (mean ± SE; range, 12-42) and 10.6 ± 1.0 (mean ± SE; range, 4-16; ICU day 3.1 ± 0.9), respectively.

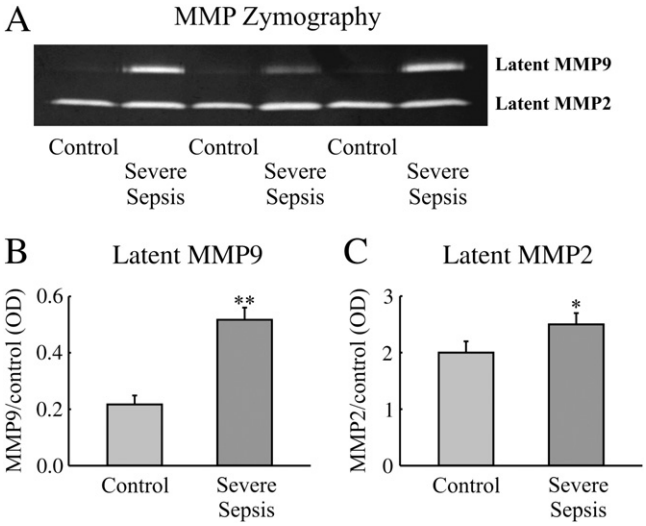

Fig. 2 Matrix metalloproteinase 2, 9 zymography of human diluted plasma. A, A standard zymography gel illustrating increased MMP9 (latent) in plasma obtained from 3 patients with severe sepsis (ICU day 1) as compared to 3 healthy controls. Matrix metalloproteinase 9 was universally low in plasma obtained from healthy controls, consistent with the antibody microarray analyses. B, A plot showing the average increase in MMP9 (latent) from patients with severe sepsis on ICU day 1 as compared to healthy controls (**$P < .001$, n = 15 per group). C, A second plot showing MMP2 (latent) levels from patients with severe sepsis and healthy controls ($P = .026$, n = 15 per group). Plasma was diluted 1:10 for all plasma samples to ensure that MMP2 was not saturated on OD, and all experiments were performed by a researcher blinded to experimental groups.

per rAPC concentration, $P = .172$, Fig. 5). As therapeutic levels of rAPC in blood are approximately 1 nM, these combined data suggest that rAPC does not directly alter MMP9 activity.

4. Discussion

Studies on MMPs and TIMPs in severe sepsis are limited, with only a small number of published reports available [21-24]. We show that most plasma MMPs and TIMPs increase significantly during severe sepsis and that increased MMP7 and MMP9 on ICU day 1 negatively correlated, albeit modestly, with the highest MOD scores. The elevated MMP9 levels detected in the plasma from patients with severe sepsis decreased over time, coincident with clinical recovery.

Increased MMP9 was observed in plasma obtained from patients with severe sepsis on ICU day 1 as compared to plasma from healthy controls. Matrix metalloproteinase 9 in patients with severe sepsis may have a neutrophilic source [42], as studies show early rises in MMP9 to be associated with neutrophil degranulation of stored metalloproteinase [19]. Other possible sources of

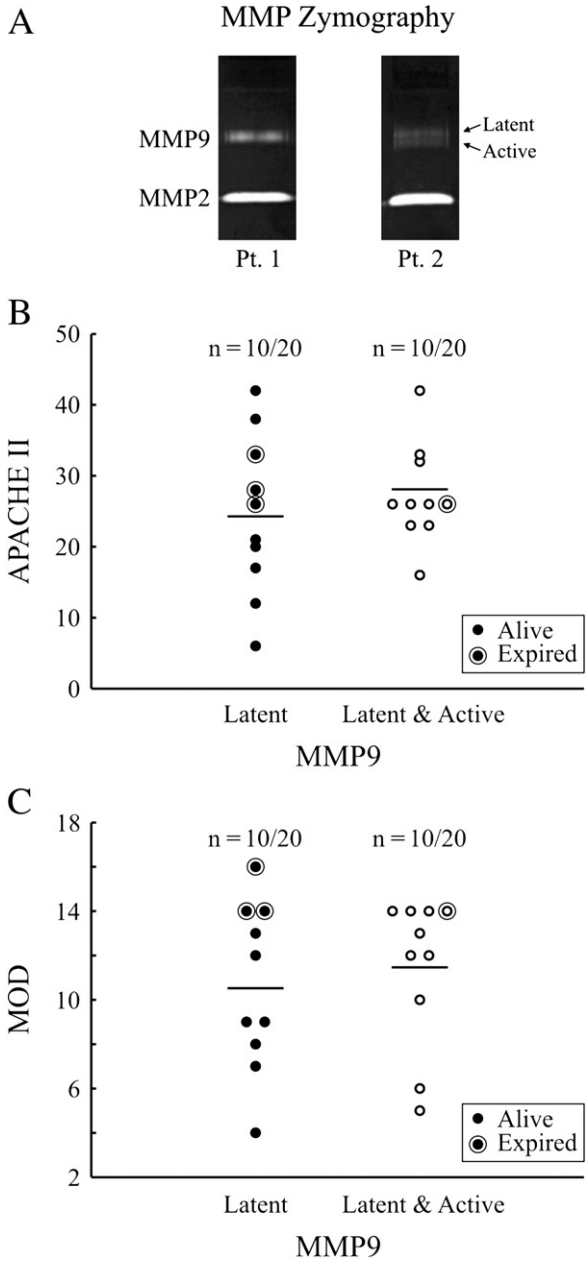

Fig. 3 Latent and active MMP9 zymography of undiluted plasma obtained from patients with severe sepsis. A, Zymography gels of undiluted plasma from 2 patients with severe sepsis. Plasma obtained from Pt.1 displayed only latent MMP9, whereas plasma obtained from Pt. 2 showed both latent and active MMP9. B, A plot illustrating that 50% of patients with severe sepsis had only latent MMP9, whereas 50% of patients with severe sepsis had both latent and active MMP9 in their plasma. All plasma samples were obtained from patients with severe sepsis on ICU day 1. The mean APACHE II and MOD scores were similar between groups, as illustrated with the horizontal bar. C, A second plot also illustrating that 50% of patients with severe sepsis had only latent MMP9, whereas 50% of patients with severe sepsis had both latent and active MMP9 in their plasma. All plasma samples were obtained from patients with severe sepsis on ICU day 1. The mean APACHE II and MOD scores were similar between groups, as illustrated with the horizontal bars. Pt. 1 indicates patient 1; Pt. 2, patient 2.

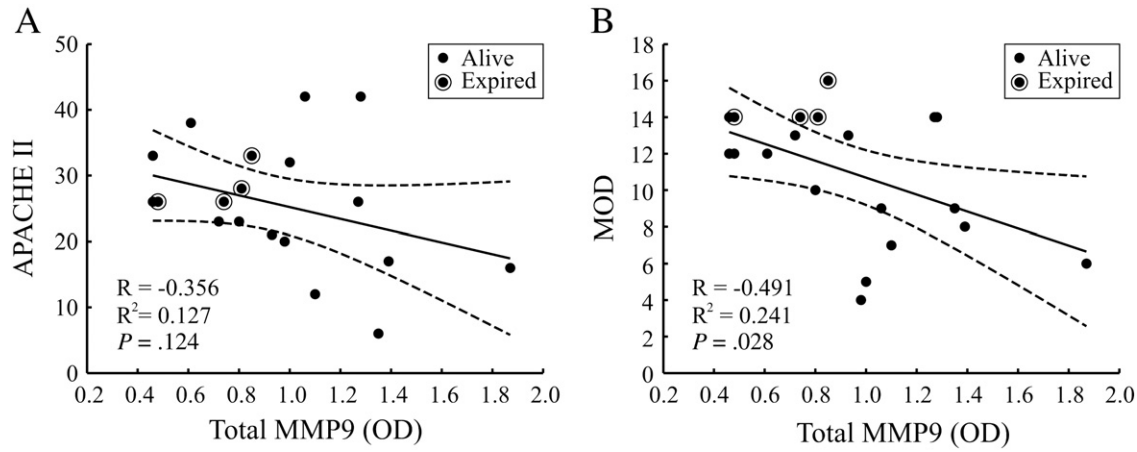

Fig. 4 Total MMP9 in plasma from patients with severe sepsis negatively correlates with the highest daily MOD scores. Total MMP9 in undiluted plasma from patients with severe sepsis on ICU day 1 was determined with zymography gels and quantified with OD. A, A plot illustrating total MMP9 does not correlate with APACHE II scores (linear regression with 95% confidence intervals is shown). B, A plot illustrating that total MMP9 negatively correlates with the highest daily MOD scores (linear regression with 95% confidence intervals is shown). Notice that patients who died had lower MMP9 levels in plasma that were associated with high APACHE II and MOD scores.

increased levels of MMP9 include de novo synthesis via monocytes, lymphocytes, dendritic cells, and endothelial cells, but these sources are likely to contribute later in the disease process [43]. Matrix metalloproteinase is secreted as an inactive, or latent, higher molecular weight proform with pericellular activation via proteolysis [16]. Although latent MMP9 lacks significant biologic activity, its elevation in plasma provides a pool of circulating enzyme available for immediate activation.

A negative correlation, albeit modest, was found between plasma MMP9 on ICU day 1 and the highest daily MOD score in patients with severe sepsis. Hence, relatively higher levels of MMP9 on ICU day 1 appeared to be associated with reduced disease severity. Our data are consistent with studies that suggest a protective role of MMP9 in sepsis [24], peritonitis [44], lung injury [45], and asthma [46]. However, it is suggested that increased MMP9 may also aggravate severe sepsis [21-23]. Patients with septic shock, for example, have elevated MMP9 that correlates with increased mortality [21]. In animal sepsis studies, MMP9 reduction or inhibition correlated with increased survival rates and improved outcomes [47,48]. These contradictory results reported with MMP9 in severe sepsis may relate to differences in patient populations, timing of plasma sampling, MMP9 measuring techniques, and/or clinical end points. In addition, MMP9 might primarily be a bystander in severe sepsis without significant biologic actions.

A negative correlation between MMP7 on ICU day 1 and highest daily MOD scores was also demonstrated, again, modest, which corroborates with previous studies that allude to a protective role for MMP7. Matrix metalloproteinase 7 colocalizes and proteolytically activates α-defensins, which are neutrophil and macrophage proteins that insert into bacterial, fungal, and viral membranes, causing their destruction by increased permeability [49]. In animal studies, MMP7 deficiency resulted in greater numbers of surviving and virulent bacteria in the small intestine after bacterial challenge [49], suggesting that MMP7 may function to modulate antimicrobial activity at the level of innate immunity in the abdomen. Matrix metalloproteinase 7 has also been implicated as a mediator in the lung injury–response where inhibition or depletion of MMP7 significantly reduces epithelial migration and tracheal re-epithelialization [50].

Produced almost exclusively by granulocytes [51], MMP8 is a neutrophil collagenase that degrades the extracellular matrix and is critical for wound healing [52]. Our antibody microarray showed a 35-fold increase in MMP8 in plasma from patients with severe sepsis compared to plasma from healthy adult controls. Analysis of correlation between MMP8 and APACHE II score trended toward statistical significance. Although the actions of MMP8 are largely unknown, MMP8 coordinates leukocyte trafficking during inflammation and orchestrates the initial inflammatory response to LPS [53,54].

Higher plasma levels of TIMP1, 2, and 3 were measured in patients with severe sepsis. A previous study showed that elevated plasma TIMP1 was associated with higher mortality in patients with severe sepsis [22]. Because TIMPs endogenously inhibit MMPs, this may be further suggestive of a protective role of MMPs. An appropriate balance between MMPs and TIMPs is likely an important determinant of disease severity. Matrix metalloproteinase/TIMP ratios were not calculated in this study, however, because each MMP can be inhibited by virtually all TIMPs with varying degrees of affinity, making the predictive value of these calculations in plasma questionable.

Steroids were administered to 50% of our severe sepsis patients. Expression of MMPs are altered by steroids; however, opposing actions have been documented depending on the cell lines examined and steroid concentrations

applied [55,56]. A single intravenous injection of hydrocortisone 100 mg in healthy subjects resulted in reductions of MMP2, 9 in mononuclear cells secondary to suppression of the proinflammatory transcription factor AP1; however, MMP2, 9 levels rapidly recovered [57]. Such transient reductions in MMP2, 9 expressions by steroids would be unlikely to alter our results.

Previous evidence favors rAPC as a treatment for severe sepsis [31,32], with a reduction in relative risk of death of 19.4% [33]. The mechanisms by which rAPC exerts its beneficial effects, however, have been mostly attributed to its anti-inflammatory, anticoagulatory, and antiapoptotic effects [58]. Activated protein C directly activates latent MMP2 in human endothelial cells [34], whereas it decreases

Table 4 Baseline characteristics of 20 patients with severe sepsis considered for rAPC therapy

	No rAPC (n = 12)	Received rAPC (n = 8)	*P*
Age, y	62 ± 5 (20, 80)	54 ± 5 (36, 73)	.31
Sex, female	2 (17%)	4 (50%)	.51
APACHE II score	24.2 ± 2.2 (12, 38)	28.2 ± 4.1 (6, 42)	.40
MOD score	9.2 ± 1.2 (3, 14)	12.2 ± 0.98 (8, 15)	.07
ANC	9.8 ± 1.1 (3.7, 17.9)	14.1 ± 1.4 (8.1, 19.5)	.03
Primary site of infection			
Lung	5 (42%)	6 (75%)	
Abdomen	3 (25%)	0 (0%)	
Blood	1 (8%)	0 (0%)	
Urinary tract	0 (0%)	0 (0%)	
Other	2 (17%)	2 (25%)	
Unknown	1 (8%)	0 (0%)	
Blood cultures			
Gram-negative bacteria	2 (17%)	2 (25%)	
Gram-positive bacteria	1 (8%)	0 (0%)	
Fungus	0 (0%)	0 (0%)	
Negative	9 (75%)	6 (75%)	
Nonblood cultures			
Gram-negative bacteria	4 (33%)	2 (25%)	
Gram-positive bacteria	2 (17%)	6 (75%)	
Fungus	2 (17%)	0 (0%)	
Polymicrobial	3 (25%)	1 (13%)	

Data are presented as mean ± SE (minimum, maximum) or n (%). Normal ANC = 1.5 to 8.0.

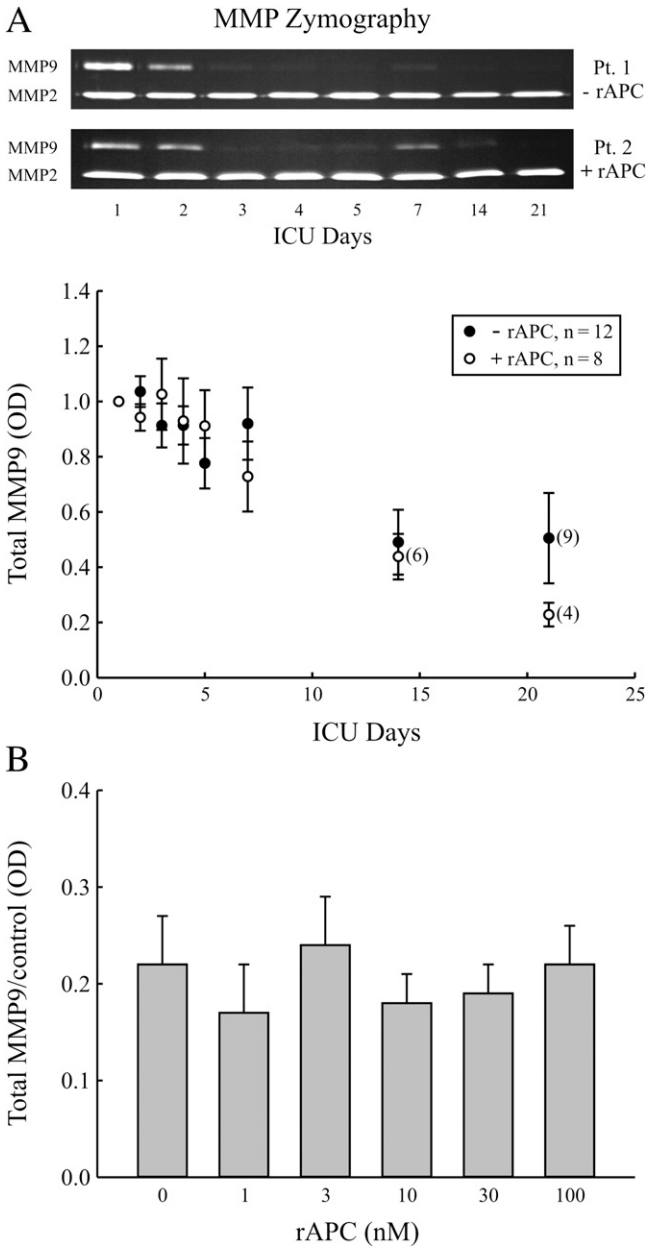

Fig. 5 Recombinant APC does not alter MMP9 activities in plasma obtained from severe sepsis patients investigated on zymography gels. Plasma samples were obtained from patients with severe sepsis at 8 time points and subsequently analyzed with zymography gels (days 14 and 21 are ±1 day). A, Two zymography gels showing similar patterns of plasma MMP2, 9 activities at 8 time points. Plasma on the top gel (Pt. 1) was obtained from a patient not treated with rAPC, whereas plasma on the lower gel (Pt. 2) was obtained from a patient treated with rAPC. A plot illustrates the averaged data for total plasma MMP9 activity at 8 time points and shows the subset of patients who received rAPC therapy (n = 8/20). The numbers in brackets on days 14 and 21 illustrate the number of plasma samples tested and reflect reduced patient numbers in the indicated treatment groups secondary to ICU discharge or death. Plasma MMP9 samples were normalized to day 1 levels for illustrative purposes. Zymography gels were quantified with OD. B, A plot illustrating the total MMP9 activity on zymography gels with increasing concentrations of rAPC added to the *Tris* incubation buffer after enzyme renaturing (n = 6 per group). Recombinant APC up to concentrations of 100 nM failed to alter MMP9 activity in this in vitro assay. Pt. 1 indicates patient 1; Pt. 2, patient 2.

MMP9 expression in human fibroblasts and monocytes [37]. Recombinant APC modulation of MMP2, 9 activities in plasma from patients with severe sepsis was not observed in our study either for 21 ICU days or by addition of exogenous rAPC to plasma. Although these combined data suggest that rAPC does not directly alter MMP2, 9 activities, our experiments do not address rAPC effects on MMP2, 9 expression.

This study has several limitations. First, we did not control for either comorbidities or genetic and lifestyle factors. Furthermore, patients were likely admitted to the ICU at different stages of the disease process, thereby exacerbating this variability. Second, the use of scoring systems such as APACHE II and MOD do offer standardized predictions, but, as with any system of this nature, they are imperfect. Third, our data on elevated plasma MMPs and TIMPs do not discriminate between increased production and decreased clearance (ie, decreased endocytic activity and/or renal failure). Fourth, we cannot exclude additional factors that may have influenced our measurements of plasma MMPs and TIMPs, including coexisting medical therapies (ie, heparin-based anticoagulants) and/or post-sampling autodegradation. Fifth, we cannot exclude that the measured MMP and TIMP levels might have been dampened by epitope masking in the antibody microarray assay by MMP-TIMP interactions. Finally, we used a convenience sample of patients for whom tissues were available. We concede that patient heterogeneity and potential confounding factors listed above mandate further investigation on the nonsignificant MMP experiments to determine their relation to disease severity and outcome.

5. Conclusions

Our data suggest that multiple MMPs and TIMPs are elevated in severe sepsis on ICU day 1 and that MMP7 and MMP9 negatively correlate, albeit modestly, with MOD scores. Further studies on MMPs in severe sepsis may eventually lead to their use both as prognostic biomarkers (as members of a larger biomarker panel) and as possible therapeutics. Recombinant APC in our study did not significantly alter MMP2, 9 activities in plasma from patients with severe sepsis, suggesting other mechanisms underlie the beneficial effects of rAPC in this condition.

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