ORIGINAL ARTICLES

Matrix Metalloproteinases 2 & 9: novel biomarkers for sepsis severity?

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Abstract

Sepsis is a common clinical syndrome and a significant cause of death worldwide. The development of thrombocytopenia is a determinant prognostic factor in these patients. Matrix metalloproteinase (MMP) -2 and -9 are released from platelets during aggregation, and leukocytes during inflammation respectively. Upregulation of MMP-9 in plasma of septic patients has been demonstrated but little is known regarding MMP-2 levels. We aimed to identify if MMP-2 activity in the plasma and platelet membranes of septic patients correlated with increasing disease severity.

A platelet poor plasma fraction and a platelet pellet fraction were separated by differential centrifugation from blood samples of healthy controls (n=9), septic non-thrombocytopaenic (n=6) and septic thrombocytopaenic (n=3) patients. Following protein concentration standardisation, MMP -2 and -9 activity in each of the two donor fractions was determined using gelatin zymography and quantified by densitometry. MMP-2 and MMP-9 activity was upregulated in the plasma of septic patients compared to healthy controls as was MMP-9 activity in the platelet pellet of septic patient samples compared to healthy controls. There was a trend whereby MMP-2 activity was further upregulated in the thrombocytopaenic patients.We conclude that plasma MMP-2 activity may provide a novel non-specific marker for increasing severity of systemic inflammation.

Introduction

Sepsis describes a clinical syndrome whereby there is a non-specific systemic inflammatory response to an infection of suspected microbial origin. It may progress to severe sepsis, in which case the illness is accompanied by tissue hypoperfusion or dysfunction of at least one organ system. When further complicated by hyperlactataemia or hypotension requiring vasopressor therapy despite fluid resuscitation, it is termed "septic shock"[1].

Sepsis is the most common cause of death in non-coronary Intensive Care Units (ICU) worldwide and among the leading causes of death in North America[2].

Thrombocytopaenia is frequently seen among septic patients in ICU and is associated with poorer prognosis[3], prolonged treatment in the ICU, and increased mortality[4,5]. It is a marker of severity of the underlying pathological process and occurs due to increased consumption, (e.g. disseminated intravascular coagulation) reduced production (e.g. bone-marrow depression), or sequestration of platelets.

Activation of platelets is often seen in sepsis. Once activated, platelets release granules containing a number of pro-inflammatory substances that modulate the function of adjacent cells. They also react with neutrophils to form platelet-neutrophil complexes, which are elevated in sepsis[6].

Matrix Metalloproteinases (MMP)

are a group of calcium and zinc-dependent endopeptidases that are synthesised as pro-enzymes, requiring cleavage of their pro-peptide domain for activation[7]. They regulate extracellular matrix turnover through degradation of its components[8]. They are also heavily involved in the inflammatory process, being responsible for the release of CD40 ligand and various chemokines[9].

MMP-2 is constitutively expressed and found in the cytosol of platelets. Upon platelet activation, it is recruited to the membrane in endosome-like structures and released (schematic 1)[10,11]. MMP-2 mediates platelet aggregation independent of the classical thromboxane A2 and ADP-dependent pathways[10].

The concentration of the gelatinase MMP-9 is elevated in septic patients[12]. MMP-9 has been found within resting human platelets. MMP-9 inhibits platelet aggregation in a concentration-dependent manner, opposing the effects of MMP-2[13]. However, some controversy exists as to whether platelets are in fact a source of MMP-9, or if its detection may be due to contamination of the sample with leukocytes, which are known to release the enzyme (schematic 2)[14].

Objectives

It has been demonstrated that patients suffering from sepsis have elevated MMP-9 levelsand this is associated with increased mortality among critically ill patients[15,16]. Equally, numerous studies have shown that there are higher mortality rates in cohorts of septic thrombocytopaenic patients[4, 5]. Our aim was to measure MMP-2 and MMP-9 activity in plasma fractions from healthy, septic non-thrombocytopaenic and septic thrombocytopaenic patients. Furthermore, we sought to identify if there was differential activity of MMP-2 in the plasma of non-thrombocytopaenic and thrombocytopaenic septic patient cohorts.

CLINICAL POINTS

Sepsis is a common clinical syndrome caused by a systemic inflammatory response to microbial components in the body. Thrombocytopenia, a feature of a more severe sepsis, is associated with increased mortality

Matrix metalloproteinase (MMP) -2 and -9, which are stored in platelets and leukocytes, are upregulated in the plasma of septic patients

MMP-2 may be a novel biomarker for disease progression in sepsis as activity rises in the sicker, or thrombocytopenic septic patient cohort

MMP-2 activity can be measured in the blood sample drawn from the septic patients as part of the 'sepsis six' diagnostic protocol, meaning that no additional invasive test is required to quantify this

MMP-2 could potentially be incorporated into a sepsis screen that also considers other inflammatory markers such as C-Reactive Protein or Erythrocyte Sedimentation Rate for a more comprehensive overview of disease state

As CRP and ESR are elevated in a number of pathological processes, we believe that MMP-2 up regulation in septic patients requires further investigation to determine if it is a more sensitive and specific marker of inflammation

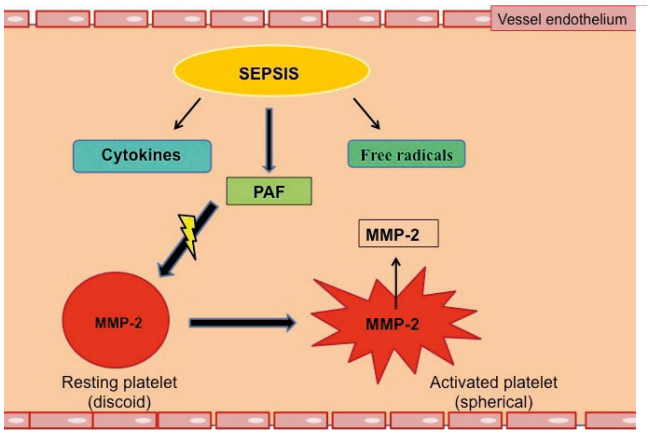
Materials and Methods

Recruitment of donors

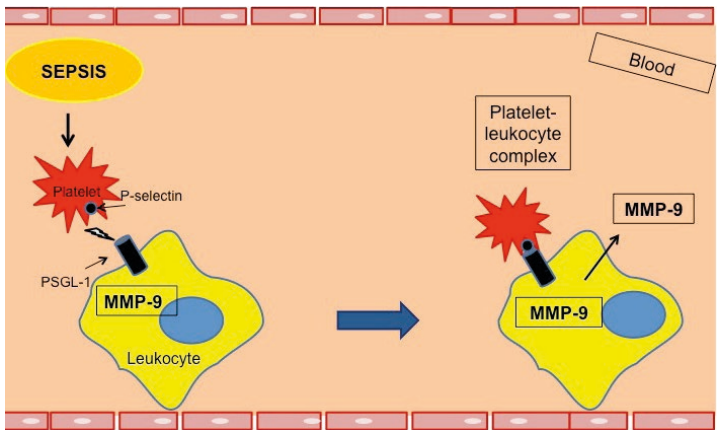
Three distinct groups were recruited for participation in this study – healthy controls (*n*=9), septic patients without thrombocytopaenia (*n*=6) and septic patients with thrombocytopaenia (*n*=3). The septic patients included were those admitted to the Intensive Care Unit (ICU) of St James' Hospital, Dublin from the 27th of February 2013 to the 10th of March 2014. The demographic and laboratory data relating to the patients is displayed in table 1.

Sepsis was defined as patients having at least one positive blood culture and/or an identified focus of infection. Severe sepsis, or septic shock, was defined as the presence of at least one organ failure attributable to sepsis necessitating the use of vasopressor therapy. Thrombocytopaenia was diagnosed when platelet count was below 100,000/ µL of blood.

The exclusion criteria used in this



schematic 1 Sepsis manifests as a number of inflammatory responses in the body including cytokine and PAF release and free radical generation. PAF stimulates the resting platelet, which is discoid in shape, to become activated and hence, spherical in shape. Platelet activation is the stimulus for the translocation of MMP-2 from the platelet cytosol to the membrane and its subsequent release into the plasma



schematic 2 During sepsis, platelets and leukocytes interact to form platelet-leukocyte complexes via P-selectin on the platelet and PSGL-1 on the leukocyte membrane. It is hypothesised that MMP-9 translocates from the cytosol of the leukocyte to the plasma, accounting for the increased activity in the septic state

table 1 Septic-patient data

Diagnosis	Culture	Sex	APACHE II score (% mortality)	SOFA score	Age	Platelets (number/µl blood)	WCC
LRTI following lobectomy	Klebsiella in sputum	M	22 - 42.4	10	63	174	7.9
Necrotizing Fasciitis in right hand	Coagulose-negative staphylococcus	M	32 - 76	12	64	355	14.5
Intra-abdominal sepsis following appendicectomy	Enterococcus avium + Escherichia coli	M	22 - 42.4	9	65	395	12.2
Colectomy	Clostridium difficile colitis	F	36 - 85.1	11	80	151	15.7
Abdominal sepsis - perforated colon	Staphylococcus hominis	M	27 - 60.5	10	75	143	17.1
Influenza A H1N1		M	19 - 32.2	9	48	261	9.8
Mediastinits	Saccharmyces cerevisiae in chest drain fluid	M	19 - 32.2	9	50	84	8
Cellulitis + parastomal abscess	Escherichia coli	F	28 - 76.4	11	67	84	10.5
Abdominal sepsis	Candida albicans	M	23 - 48.9	15	46	59	15.5

Abbreviations: LRTI - lower respiratory tract infection, WCC - white cell count, APACHE II - acute physiology and chronic health evaluation, SOFA - sequential organ failure assessment

study were age below 18 years, presence of a haematological malignancy, patients post-cardiopulmonary bypass, history of thrombocytopaenia thrombotic purpura, history of idiopathic thrombocytopaenia purpura, massive haemorrhage, and current antiplatelet agent use (e.g. clopidogrel/aspirin, GPIIb/IIIa inhibitors).

Reagents

Human HT 1080 fibrosarcoma cells were purchased from American Type Culture Collection (ATCC). They release MMP-2 and MMP-9 after stimulation with phorbol 12 myristate 13-acetate (PMA) and their conditioned media was used as a positive control for MMP activity. All other reagents were purchased from Sigma-Aldrich.

Preparation of samples

Blood was collected from subjects in the three study groups and platelet suspensions washed in prostacyclin were prepared as previously described[17] to yield a platelet poor plasma (PPP) fraction and a platelet pellet. Blood from septic patients was collected on admission to the ICU. The PPP and the platelet pellet samples were stored at -20 °C. The platelet pellets were lysed using 0.2% NaCl and centrifuged at 13,000 RPM to yield a pellet containing the platelet membrane only. Homogenising buffer containing 1% Triton X-100 was also added to each sample to solubilise the proteins.

Protein assay

Protein concentrations in each of the samples was determined using the Bradford Protein Assay as previously described[18].

Gelatin zymography

The standardised PPP and platelet membrane pellet samples were subject to gel electrophoresis at 150V for 2 hours and 20 minutes in an 8% sodium dodecyl sulphate (SDS) polyacrylamide gel co-polymerised with 2 mg ml-1 gelatin. The gels were then washed with 2.5% Triton X-100 to remove SDS. They were subsequently incubated with zymography buffer (50 mL of 2 M Tris HCl, 18 g NaCl, 1.47 g CaCl2, 1 g NaN3 made up to 2000 mL) at 37°C for 17 hours. Following incubation, the gels were stained with a solution of 0.05% Comassie Brilliant Blue G-250, 25% methanol and 10% acetic acid for one hour and destained with a solution of 4% methanol, 8% acetic acid. Gels were visualised using the gel documentation system from Biorad. The software "Quantity One" used densitometric analysis to measure the intensity of the bands which reflected MMP-2 and -9 activity.

Statistical analysis

Unpaired two-tailed t-tests and ANOVA and Tukey's post tests were performed to analyse the data using the programme GraphPad Prism 6 (GraphPad Software, San Diego, CA). The data are shown as mean ± standard deviation (SD). A *p*-value of less than 0.05 was considered statistically significant.

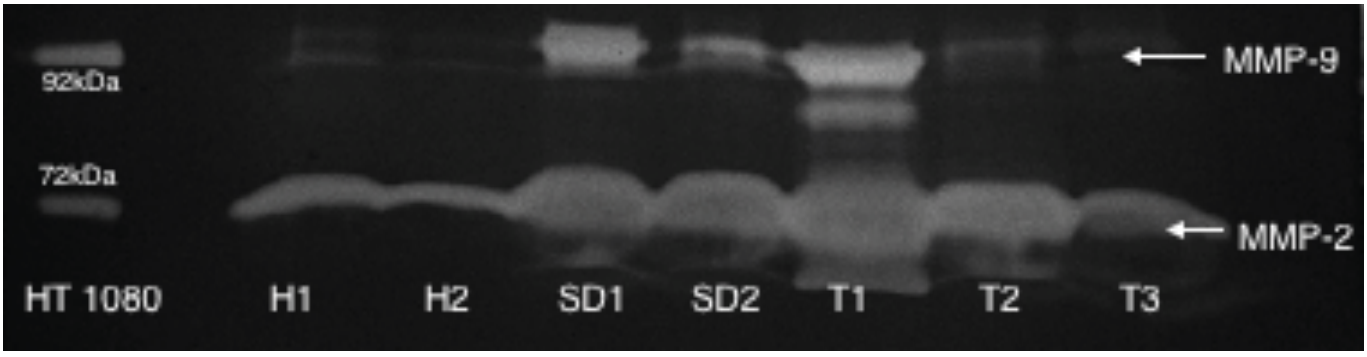
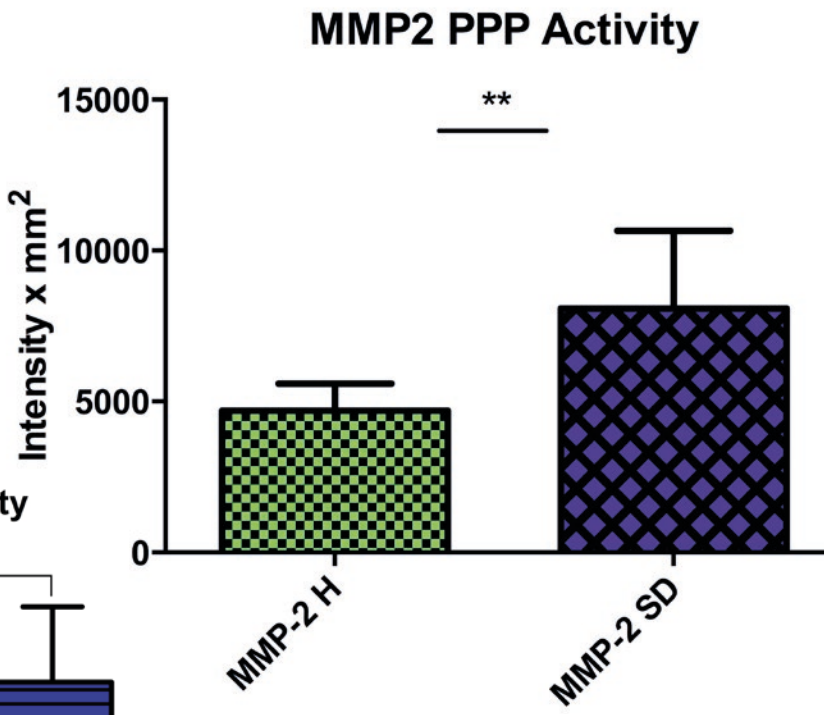
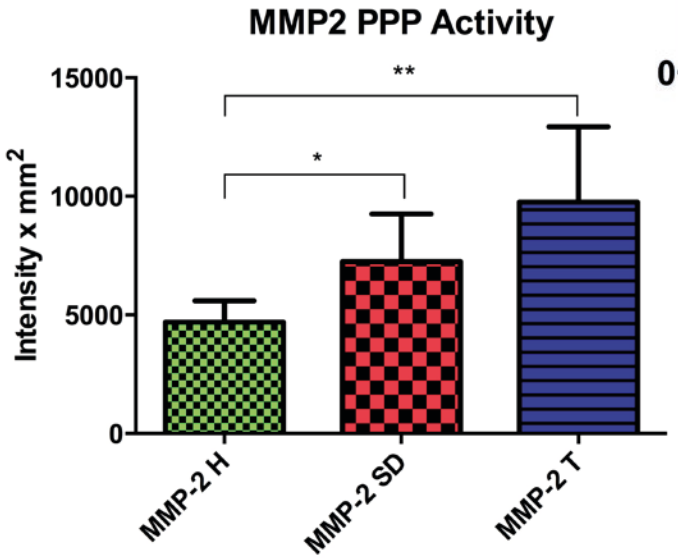


image 1 Gel under UV light showing MMP-2 and MMP-9 activity in the PPP. Gelatin is dark. Areas where gelatin has been digested appear light. These are areas of MMP activity. Quantification of MMP activity is achieved through absorbance measurements, indicating degree of gel digestion and therefore activity of enzyme

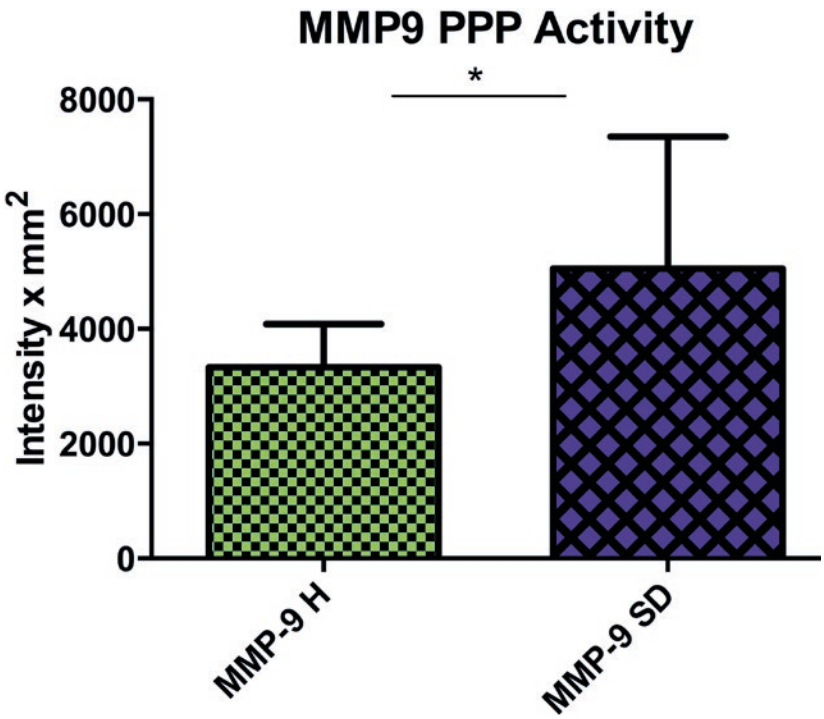


▶ figure 1 Comparison of MMP-2 activity in the platelet poor plasma of septic patients versus healthy subjects. MMP-2 activity, detected by densitometric analysis of the gelatin zymography, was found to be significantly increased in septic subjects (**p=0.0018, healthy n=9 and septic n=9) when the results were analysed using an unpaired t-test. Bars represent mean + standard deviation



▲ figure 2 Comparison of MMP-2 activity in the platelet poor plasma of healthy (n=9), septic (n=9) and septic thrombocytopaenic (n=3) subjects. MMP-2 activity, detected by densitometric analysis of the gelatin zymography, was found to be significantly increased in septic non-thrombocytopaenic compared with healthy subjects (* - $p \leq 0.05$). MMP-2 activity was also significantly increased in septic thrombocytopaenic patients compared with healthy subjects (** - $p \leq 0.01$). Non-significantly elevated activity levels of MMP-2 were observed in septic thrombocytopaenic patients compared with septic subjects. The results were analysed using one way ANOVA. A Tukey post-test was applied to generate the p-value. Bars represent mean + standard deviation

▼ figure 3 Comparison of MMP-9 activity in the platelet poor plasma of healthy (n=9) versus septic subjects (n=9). MMP-9 activity, detected by densitometric analysis of the gelatin zymography, was found to be significantly increased (* - p=0.0495) in septic versus healthy subjects. The results were analysed using an unpaired t-test. Bars represent mean + standard deviation



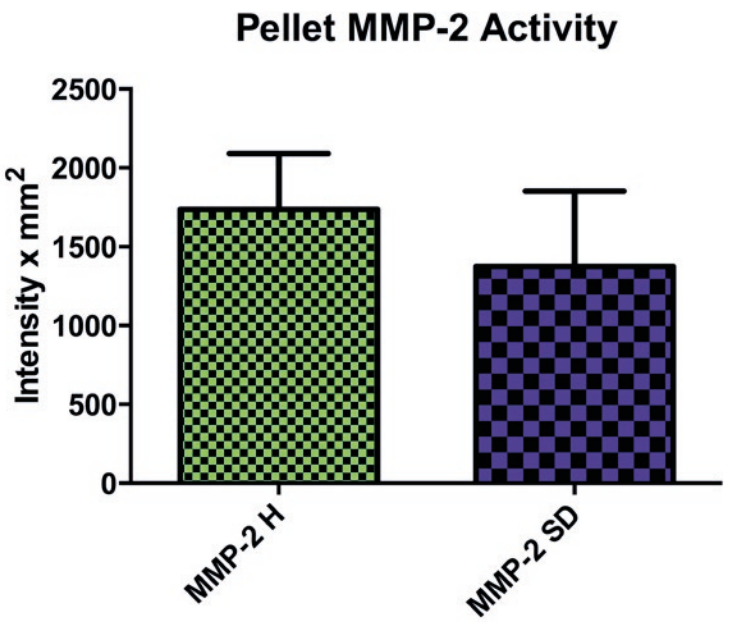


figure 4 Comparison of MMP-2 activity in the platelet pellet of septic patients versus healthy subjects. No statistically significant difference in MMP-2 activity, detected by densitometric analysis of the gelatin zymography, was found between septic and healthy subjects (p=0.1836, healthy n=6 and septic n=5) when the results were analysed using an unpaired t-test. Bars represent mean + standard deviation

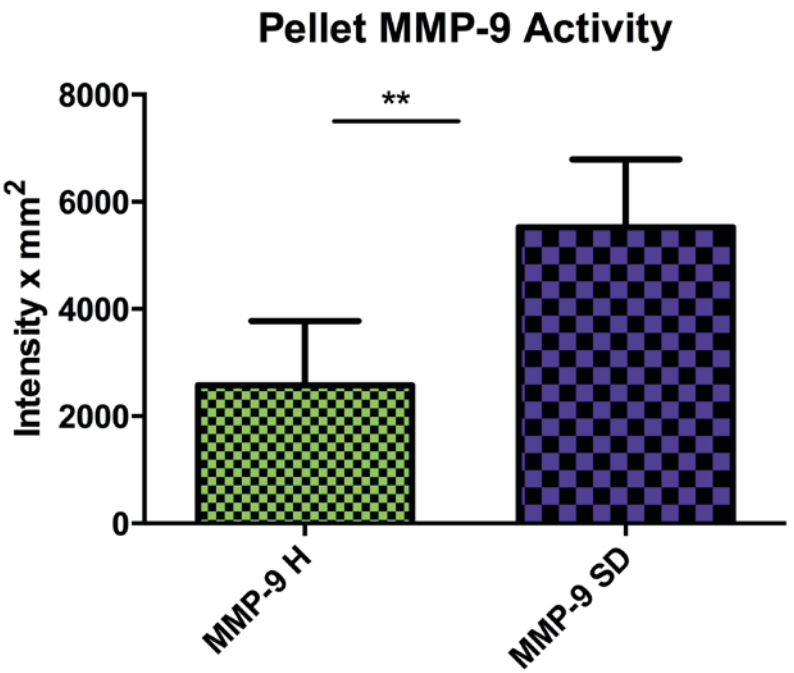


figure 5 Comparison of MMP-9 activity in the platelet pellet of healthy (n=6) versus septic subjects (n=5). MMP-9 activity, detected by densitometric analysis of the gelatin zymography, was found to be significantly increased in septic compared with healthy patients (**p=0.0034). The results were analysed using an unpaired t-test. Bars represent mean + standard deviation

Results

Platelet-poor plasma

Nine healthy, six septic non-thrombocytopaenic and three septic thrombocytopaenic samples were analysed. Following standardisation, zymography was performed on the samples to determine the activity of MMP-2 and MMP-9 (image 1) in the plasma of septic and healthy donors.

MMP-2 activity was found to be significantly upregulated in septic donors (n=9) compared with healthy ones (n=9) (p=0.018 (figure 1). ANOVA was used to compare MMP-2 activity across all three groups. MMP-2 activity was significantly increased (p≤0.01) in septic thrombocytopaenic patients (n=3) compared to healthy donors (n=9) as was MMP-2 activity in septic non-thrombocytopaenic patients (n=6) compared to healthy subjects (p≤0.05) (figure 1). No statistically significant difference in MMP-2 activity between thrombocytopaenic and non-thrombocytopaenic septic patients was found (figure 2).

MMP-9 activity was also significantly upregulated in septic donors (n=9) compared with healthy donors (n=9) (p=0.0495) (figure

3). No difference in MMP-9 activity between thrombocytopaenic (*n*=3) and non-thrombocytopaenic septic donors (*n*=9) was found.

Platelet pellet

There were six healthy, four septic non-thrombocytopaenic and two septic thrombocytopaenic samples of sufficient size to use in the investigation. As before, the samples were standardised and zymography performed. Due to the very small concentration of protein in some samples, certain septic and septic thrombocytopaenic samples were not of sufficient quantity for zymography. Only one thrombocytopaenic sample was suitable for use in zymography, therefore statistical analysis could not be performed on septic non-thrombocytopaenic versus septic thrombocytopaenic activity levels.

There were no significant differences in MMP-2 levels between septic (*n*=5) and healthy samples (*n*=6) (*p*=0.1836) (figure 4). MMP-9 levels were significantly increased in septic donors (*n*=6) compared to healthy donors (*n*=5) (*p*=0.0034) (figure 5).

Discussion

In this study, the activity of the enzymes MMP-2 and MMP-9 were compared in the plasma and the platelet membranes of septic and healthy donors. Furthermore, the MMP-2 and MMP-9 activities in the plasma were compared between two subsets of septic donors – thrombocytopaenic and non-thrombocytopaenic.

Under normal physiological circumstances, MMP-2 is released into the blood by activated platelets, as one of the mechanisms by which other platelets are recruited to the site of injury[13]. In sepsis, due to the systemic inflammatory response to infection, leukocytes release many cytokines, which in turn may stimulate platelet activation. It would appear that due to increased platelet activation, more MMP-2 is released by platelets. Ongoing functional studies using a quartz crystal microbalance have further confirmed that platelets are more labile in sepsis. We have demonstrated that MMP-2 is upregulated in sepsis and are the first, to our knowledge, to discuss this finding. Upregulation of MMP-2 may also be a contributing factor in the common septic condition of disseminated intravascular coagulation, as it is already known that MMP-2 is a pro-aggregatory agent[13].

Although this study could not confirm that MMP-2 activity is further upregulated in thrombocytopaenic patients compared to the non-thrombocytopaenic septic cohort due to the low number of donors available, we hypothesise that with a higher powered study, this may be demonstrated. Thrombocytopaenic donors have fewer platelets, but whether they are sequestered within organs, or have been activated and have aggregated, is unclear. Although thrombocytopaenic patients have fewer circulating platelets, we believe those remaining are more labile and prone to aggregation as they are very active in terms of MMP-2 release. It is known that thrombocytopaenia is indicative of poorer prognosis among the critically ill[5]. Therefore the fact that MMP-2 is likely to be upregulated among thrombocytopaenic patients indicates that upregulation may be indicative of illness severity and independent of platelet count.

Although it has previously been shown that MMP-9 is upregulated in septic cohorts[19], our findings further confirmed those results.

Our investigation yielded a number of interesting results with regard to MMP activity in the platelet membrane. We noticed a trend in which MMP-2 activity was upregulated in the platelet membrane of healthy donors compared to the septic sample. As previously mentioned, MMP-2 is a constitutively expressed enzyme, and platelet activation results in the translocation of its inactive pro- form to the membrane, where it is cleaved and activated prior to its release into the plasma[11]. Platelets are anucleate

cells and do not contain mRNA for MMP-2[20]. Therefore they cannot synthesise new MMP-2, so when MMP-2 is recruited to the platelet membrane, the stores in the cytosol are irreversibly depleted, reflecting this translocation. Our observation may be explained by such a depletion of the MMP-2 stores within the platelet cytosol, meaning that little remains to be translocated to the membrane and subsequently released into the plasma. This would be consistent with our findings of MMP-2 activity upregulation in the plasma of septic patients. There may be greater MMP-2 activity in the platelet membrane of septic patients than we detected should the investigation be carried out prior to this huge release of MMP-2 into the blood.

As we found less MMP-2 activity in the plasma of healthy donors we hypothesise that the MMP-2 is still located in the cytosol awaiting platelet activation and subsequent release. The platelet lysate could be analysed to confirm this.

Our results also revealed that MMP-9 activity in the platelet membrane is significantly upregulated in septic patients compared to healthy controls. However, there is continuing debate regarding the expression of MMP-9 in platelets and their capacity to release this enzyme. Certain studies have found evidence of MMP-9 in human platelets and its translocation to the membrane upon stimulation, similar to the mechanism of MMP-2 release[13,21]. A number of others dispute this and believe that platelets play a central role in leukocyte recruitment to the site of vascular damage, linking the processes of thrombosis and inflammation. Platelet-leukocyte complexes are formed when P-selectin glycoprotein ligand-1 on the surface of the leukocyte interacts with P-selectin expressed on the surface of activated platelets[14]. The presence of these complexes is significantly increased in the septic process[6] due to the inflammatory mechanisms at play in the pathogenesis of the disorder.

Interaction of platelets is thought to regulate the synthesis of the inducible MMP-9 in leukocytes in a paracrine manner. Platelet binding stimulates the leukocytes to release MMP-9 among other inflammatory mediators[22]. No method exists to isolate the separate components of the complexes and so, the debate continues as to whether it is the platelet or the leukocyte that releases the MMP-9. We believe that as platelets are more labile in septic patients, there may be increased likelihood of such platelet-leukocyte interactions occurring. This could possibly account for the upregulation in MMP-9 activity seen in the platelet membrane of septic patients.

As previously mentioned, we believe a higher powered study is required to confirm if the trend in MMP-2 activity that we have identified is of significance. An unfortunate limitation in our investigation was inadequate protein concentrations for analysis in four of the septic thrombocytopaenic samples.

Conclusion

In conclusion, we have shown that MMP-2 and MMP-9 activity is upregulated in the plasma of septic patients. Our finding of MMP-9 upregulation is in accordance with previous studies. However, the finding of upregulation in the activity of MMP-2 in septic patients has not previously been discussed. Furthermore, we postulate that with a larger study, increasing upregulation of MMP-2 activity in the thrombocytopaenic cohort of septic patients would be found. Thrombocytopaenia is an indicator of sepsis severity. In this study, the transition from non-thrombocytopaenic to thrombocytopaenic was paralleled by an increase in MMP-2 activity. We hypothesise that the progressive upregulation of MMP-2 with increasing severity of sepsis could be a novel marker for disease progression.

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