MMP-8 Genotypes Influence the Inflammatory Response in Human Endotoxemia

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Abstract—Clinical studies have reported associations between MMP-8 genotypes and clinical outcomes without exploring underlying mechanisms. This study aims to understand the influence of the rs1940475 SNP on downstream chemokine and cytokine response in human endotoxemia. Rs1940475 was genotyped in 44 healthy Caucasian males, who were challenged with an intravenous bolus of 2 ng/kg lipopolysaccharide (LPS). Plasma levels of tumor necrosis factor (TNF), interleukin (IL)-6, IL-8, and macrophage inflammatory protein (MIP)-1α were measured at baseline and 2, 4, 6, and 24 h after LPS infusion with high-sensitivity enzyme immunoassays. Peak TNF levels at 2 h after LPS infusion were significantly higher in subjects with AA genotype compared to subjects with AG or GG genotypes (185 pg/mL [IQR, 154–234] vs. 94 pg/mL [IQR, 65–125] vs. 107 pg/mL [IQR, 80–241], respectively; $p=0.03$ between groups). Peak IL-6 levels were trend-wise higher in subjects with AA genotype compared to those with AG or GG genotypes (566 pg/mL [IQR, 294–644] vs. 278 pg/mL [IQR, 184–539] and 329 pg/mL [IQR, 240–492], respectively; $p=0.15$ between groups). In contrast, peak MIP-1α at 2 h was highest in GG genotype carriers compared to those with AG or AA genotypes (602 pg/mL [IQR, 449–727] vs. 389 pg/mL [IQR, 375–490] and 510 pg/mL [425–813], respectively; $p<0.03$ between groups). AA genotype carriers had highest peak TNF and IL-6 levels after LPS challenge, whereas peak MIP-1α levels were highest in GG carriers. This indicates that the rs1940475 SNP modifies the host response to inflammatory stimuli, which may in part explain previously shown associations with clinical outcomes.

KEY WORDS: endotoxemia; inflammation; matrix-metalloproteinase 8; cytokines; SNP.

INTRODUCTION

Neutrophils (PMN) are crucial for clearing microbial pathogens [1]. Among many other proteins, PMNs produce matrix metalloproteinase (MMP)-8, a neutrophil collagenase, which is pivotal for initiation and consequent resolution of inflammation, presumably by altering chemotactic gradients [1]. In general, a lack of MMP-8 has been associated with increased neutrophil counts at inflammatory sites [2, 3].

Macrophage inflammatory protein (MIP)-1 α, also known as chemokine ligand 3 (CCL3), is a potent pro-inflammatory chemokine that is produced by macrophages in response to various stimuli including endotoxin. CCL3 propagates inflammation by further recruitment and activation of PMNs. Data from different experimental models, including a murine acute lung injury model, suggest that soluble as well as membrane bound MMP-8 degrades MIP-1α and therefore mitigates inflammation, resulting in improved survival [4]. In turn, genetically deleting MIP 1-α in MMP-8 knockout mice also reduced lung inflammation and mortality, thus suggesting an important role for an "MMP-8/MIP-1α axis" in acute inflammation [4].

Human studies to date have largely reported associations between MMP-8 plasma levels or MMP-8 genotypes and clinical outcomes, without exploring underlying

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mechanisms [5–7]. For example, preliminary analysis of the GenIMS study showed that the MMP-8 non-synonymous single-nucleotide polymorphism (SNP) rs1940475 was associated with 90-day mortality in patients with community-acquired pneumonia (CAP), and the presence of the A allele was associated with better survival [8]. The rs1940475 SNP causes a point mutation in the pro-domain of MMP-8. This results in an amino acid substitution of glutamic acid for lysine at position 87, thereby rendering it less amenable to activation, which results in functional suppression [9]. As such, its ability to function as a negative feedback regulator in inflammation is impaired, leading to an over-recruitment of PMNs which may lead to tissue and organ damage.

To better understand the role of MMP-8 in human inflammation, we investigated the influence of the rs1940475 SNP (K87E) on downstream chemokine and cytokine processing in human endotoxemia. The human endotoxemia model is a well-established model, in which a defined inflammatory stimulus allows to study the pathogenesis and potential treatments for systemic inflammation [10]. We hypothesized that subjects homozygous for the allele (A) would have lower peak MIP-1α levels compared to subjects with mutated alleles (G).

MATERIALS AND METHODS

Study Participants

The Medical University of Vienna Ethics Committee approved the study protocol, and all participants gave written informed consent prior to enrolment. We obtained complete data and mRNA samples from 44 healthy Caucasian males. Determination of health status included a medical history, physical examination, laboratory testing, and virus and standard drug screening. Exclusion criteria were regular or recent intake of medications, including over-the-counter medications as well as clinically relevant abnormal findings detected in the screening examinations.

The experimental procedures of our LPS infusion studies have been described in detail elsewhere [11]. Briefly, volunteers reported to the study ward at 8:00 a.m. after an overnight fast and received an intravenous bolus of 2 ng/kg LPS (National Reference Endotoxin, *Escherichia coli*, CC-RE-Lot 2; National Institutes of Health, Bethesda, MD, USA). Throughout the entire study period, participants were confined to bed rest, and vital parameters, i.e., electrocardiogram (ECG), heart rate, oxygen saturation, and blood pressure were continuously monitored.

MMP-8 Genotype Analysis

Based on our preliminary findings in patients with CAP, [8] we selected the non-synonymous SNP 259A>G (rs1940475 in the National Center for Biotechnology Single Nucleotide Polymorphism database, http://www.ncbi.nlm.nih.gov/SNP) for analysis. All blood samples were immediately processed to avoid storage-induced changes in mRNA levels. After isolating total RNA with an RNeasy minikit (Qiagen, Valencia, CA, USA), mRNA was directly transcribed into cDNA, using a TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA, USA), and stored at −80 °C until analysis. Consequently, cDNA samples were genotyped on an ABI 7700 Prism using a commercially available SNP assay (TaqMan MMP-8 assay, Applied Biosystems) and analyzed with Sequence Detection System software (Ver. 1.7, Applied Biosystems).

Blood Sampling and Analysis

Blood samples were collected into ethylenediaminetetraacetic acid (EDTA) anti-coagulated tubes before LPS infusion and 2, 4, 6, and 24 h after LPS infusion. All samples were immediately centrifuged at $2,000 \times g$ at 4 °C for 15 min and then stored at −80 °C until batch analysis.

Plasma levels of tumor necrosis factor (TNF), interleukin (IL)-6, IL-8, and MIP-1α were analyzed with high-sensitivity enzyme immunoassays (all R&D-Systems, Oxon, UK) [12]. Plasma concentrations of thrombin-anti-thrombin complexes (TAT) and fibrin split products (F1+2) were measured with immunoassays (Enzygnost TAT micro and Enzygnost F1+2 micro, both Dade Behring, Marburg, Germany) as described previously [13–15].

Differential blood counts were measured with an automatic cell counter (Sysmex, Milton Keynes, UK) [16].

Data Analysis

Data are expressed as medians and inter-quartile ranges (IQR). After repeated measures ANOVA, we applied nonparametric statistics for robustness. We used the Kruskal–Wallis ANOVA for all statistical comparisons between groups, and the Mann–Whitney U test for individual comparisons. Based on a study by Kumpf et al., [17] we

estimated a sample size of 40 sufficient to demonstrate 40 % different peak MIP-1a levels between subjects homozygous for the A and G alleles, respectively. A *p* value of <0.05 was considered statistically significant. All statistical calculations were performed with statistical software (Stata 12.1, College Station, TX, USA).

RESULTS

MMP-8 Genotype Distribution and Subject Characteristics

Of the 44 subjects genotyped, 9 (20.5 %) had the AA genotype, 9 (20.5 %) had the GG genotype, and the remaining 26 (59 %) had the AG genotype and thus were in Hardy–Weinberg equilibrium (*p*=0.23). All participating subjects were healthy male nonsmokers, with a median age of 25.5 years (IQR, 23 to 30 years) and a median BMI of 23.4 kg/m2 (IQR, 21.8 to 24.2 kg/m^2), without any differences between different genotype carriers (Table 1).

Vital Parameters

LPS infusion induced a transient increase in heart rate and a transient decrease in mean arterial blood pressure (MAP; all *p*<0.05 versus time), as previously described in this model [11]. Interestingly, subjects homozygous for the A allele showed the largest decrease in MAP at 4 h compared to the other groups (69, 79, and 81 mmHg, respectively, *p*=0.04). LPS infusion also induced a transient increase in body temperature that reached its maximum after 4 h. Subjects homozygous for the A allele had the largest increase in body temperature (37.8 vs. 37.3 °C and 37.3 °C, *p*=0.02)

MMP-8 Genotypes and Plasma Cytokine and Chemokine Levels

LPS infusion transiently increased plasma concentrations of TNF, IL-6, IL-8, and MIP-1α (all *p*<0.05 vs. time), which returned to baseline after 24 h.

Plasma TNF levels peaked 2 h after LPS infusion (106 pg/mL; IQR, 75–210 pg/mL). Subjects homozygous for the A allele had significantly higher peak values compared to those with GG or AG genotypes (185 pg/mL [IQR, 154–234] vs. 107 pg/mL [IQR, 80–241] and 94 pg/mL [IQR, 65–125, respectively; *p*=0.03). The remaining time course did not differ between genotypes (Fig. 1).
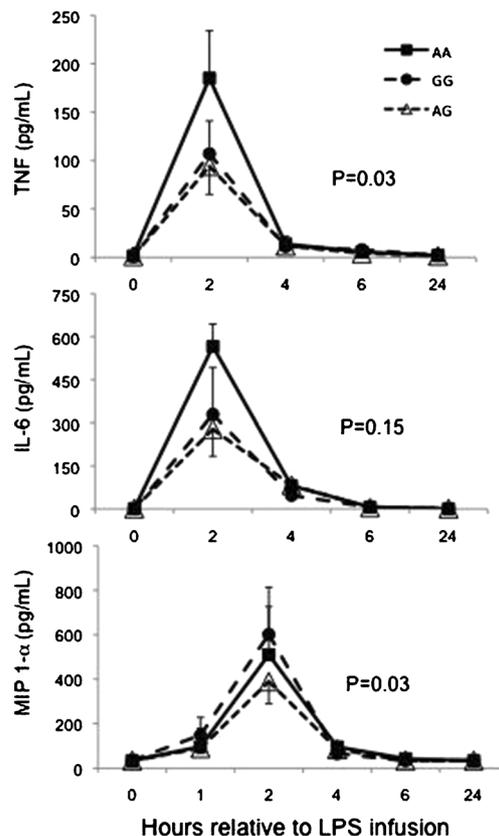

Fig. 1. Subjects homozygous for the A allele had a stronger pro-inflammatory response to LPS in terms of peak TNF and IL-6 levels. In contrast, peak MIP-1 α levels were highest in subjects with the GG genotype. Data presented as medians and inter-quartile ranges.

Peak IL-6 values were observed 2 h after LPS infusion (329 pg/mL; IQR, 216 to 541 pg/mL]. Subjects homozygous for the A allele had trend-wise higher peak values compared to those with GG or AG genotypes (566 pg/mL [IQR, 294–644] vs. 329 pg/mL [IQR, 240–492] and 278 pg/mL [IQR, 184–593], *p*=0.15; Fig. 1).

Baseline MIP-1α levels did not differ between the three genotypes (Table 1). Peak MIP-1α plasma levels were measured 2 h after LPS infusion (439 pg/mL; IQR, 376–614). In contrast to TNF and IL-6 values, subjects homozygous for the mutant G allele had the highest peak levels compared to subjects with AA or AG genotypes (602 pg/mL [IQR, 449–727] vs. 510 pg/mL [IQR, 425–813] and 389 pg/mL [IQR, 375–490], *p*=0.03). The remaining time course was similar between genotypes (Fig. 1). Peak IL-8 values were detectable 2 h after LPS infusion (222 pg/mL; IQR, 167–486) but did not differ between genotypes.

Table 1. Subject Characteristics (Data Are Presented as Medians and Inter-quartile Ranges (IQR)) and Baseline Cytokine Values

	GG genotype (n=9)	AG genotype (n=26)	AA genotype (n=9)	P
Age, years	28 (23–29)	26 (23–31)	25 (23–27)	0.84
BMI (kg/m^2)	23.3 (21.3–24.3)	23.5 (21.9–24.1)	23.2 (22.0–24.2)	0.91
MAP (mm Hg)	91 (84–95)	84 (80–91)	89 (85–99)	0.32
HR (min^{-1})	76 (72–83)	67 (59–74)	75 (62–76)	0.11
Temperature (°C)	35.9 (35.7–36.3)	36.0 (35.6–36.3)	36.0 (35.9–36.3)	0.85
PMN (G/L)	2.9 (2.5–3.0)	2.4 (1.8–3.3)	3.1 (2.8–3.6)	0.06
TNF (pg/mL)	2.7 (2.0–10.0)	3.7 (1.6–7.6)	3.8 (2.7–5.1)	0.91
IL-6 (pg/mL)	0.7 (0.9–1.1)	0.7 (1.0–1.6)	1.4 (0.9–1.7)	0.24
IL-8 (pg/mL)	2.9 (2.7–4.5)	3.2 (2.3–4.0)	3.5 (2.9–4.0)	0.62
MIP-1α pg/mL	34 (34–34)	34 (34–34)	34 (34–34)	0.54
F$_{1+2}$ (pmol/L)	128 (121–152)	102 (79–150)	129 (100–157)	0.46
TAT (μg/L)	2.1 (1.9–2.8)	2.4 (1.9–2.6)	2.2 (1.9–2.4)	0.81

MMP-8 Genotypes and Coagulation Activation

As expected, [13] LPS infusion was associated with a potent activation of the coagulation system as reflected by increases in the plasma concentrations of thrombin–anti-thrombin complexes and prothrombin fragments F$_{1+2}$, both peaking after 4 h (54.5 pmol/L [IQR, 28.2–111.8] and 1,130 μg/L [IQR, 668–1,918], respectively, both $p<0.05$ vs. time). This LPS-induced coagulant response did not differ between different genotypes.

MMP-8 Genotypes and Neutrophil Counts

As expected, neutrophil counts increased about threefold compared to baseline levels ($p<0.05$ vs. time) after LPS infusion. Nadir levels were reached 1.5 h after LPS infusion (2.0 G/L [IQR, 1.7–3.4]), whereas peak levels were observed 6 h after LPS infusion (9.2 G/L [IQR, 8.2–11.8]). Subjects homozygous for the A allele displayed a lesser drop in neutrophils at 1.5 h compared to subjects with AG or GG genotypes (3.7 G/L [IQR, 2.3–4.6] vs. 1.9 G/L [IQR, 1.6–2.7] and 1.8 G/L [IQR, 1.4–3.3], $p=0.07$ between groups). Similarly, subjects with the AA genotype had trend-wise higher peak neutrophil levels at 6 h compared to AG and GG individuals, respectively (12.2 G/L [IQR, 8.6–12.7] vs. 9.1 G/L [IQR, 7.2–11.1] and 9.2 G/L [IQR, 8.3–11.6]); $p=0.09$ between groups).

DISCUSSION

Data from MMP-8 knockout animals suggest that MMP-8 assists in early PMN recruitment and elimination of chemotactic gradients. For example, acute lung inflammation (ALI) is less pronounced in MMP-8 wild-type mice compared to knockout mice due to inactivation MIP 1-α [4]. Compared to knockout animals, MMP-8 wild-type (WT) mice had higher levels of PMN membrane bound MMP, which is likely to be the key bioactive form of the enzyme. In turn, MMP-8 inactivated MIP 1-α, and genetically deleting MIP 1-α in MMP-8 knockout mice decreased the increased lung inflammation and mortality, thus emphasizing a possible role for a "MMP-8/MIP-1α axis" in acute inflammation.

Our understanding of MMP-8 in human inflammation and infection is less clear, in part due to the fact that we cannot selectively silence the MMP-8 gene. Studies to date have largely reported associations between MMP-8 plasma levels and/or MMP-8 genotypes and clinical outcomes without elaborating the underlying mechanisms. Most of these studies report a similar trend, i.e., higher MMP-8 levels are associated with worse clinical disease. For instance, MMP-8 is not only strongly associated with acute inflammatory states in humans such as ARDS [18] but also states with chronic inflammation and neutrophilia such as cystic fibrosis, rheumatoid arthritis, periodontal, and chronic skin diseases [19–22]. For example, MMP-8 levels correlated well with C-reactive protein (CRP) levels in a study of patients with ARDS [23]. Alternatively, several functional MMP-8 single-nucleotide polymorphisms (SNPs) have been linked to clinical outcomes as well. For instance, our preliminary analysis from the GenIMS study showed that the rs1940475 genotype was associated with 90-day mortality in patients with CAP, and patients homozygous for the A allele had the best outcomes [8].

In this study, we show that subjects with the A allele had higher peak TNF and IL-6 levels after LPS challenge

than those homozygous for the G allele, whereas MIP-1 α levels were higher in subjects homozygous for the G allele. In addition, subjects with the A allele also showed a stronger clinical response to LPS infusion in terms of drop in MAP and increase in body temperature. As clinical studies have shown a higher pro-inflammatory response to be associated with poorer survival [24], our findings of higher peak TNF and IL-6 levels in AA homozygous individuals are not consistent with higher survival rates in our preliminary analysis of AA homozygous individuals with CAP [8]. Conversely, patients homozygous for the G allele had the highest peak MIP-1 alpha levels. MIP-1α usually parallels TNF and IL-6 response in a TNF independent way [25]. Bronchoalveolar fluid levels of MIP-1 α were inversely correlated with lung compliance in a small human cohort of ARDS patients but not associated with outcomes. These preliminary findings warrant further study in larger cohorts.

Our study has several limitations. First, although we calculated a sample size of 40 to be sufficient to detect a 40 % difference in peak MIP-1α levels between AA and GG genotypes, our study was not adequately powered to assess any differences beyond that; hence, all additional analyses must be viewed as exploratory. Second, while our results suggest an association between peak plasma cytokine levels and MMP-8 genotypes and thus a role for MMP-8 in cytokine regulation, we do not have insight how this affects downstream cell trafficking to actual sites of infection. Hence, additional studies using alternative compartmental models such as inhaled LPS will be helpful to better understand the role of MMP-8 in ALI and pneumonia. Third, we did not measure MMP-8 levels in our healthy volunteers. Fourth, our study was conducted in healthy male volunteers, and thus, our results may not apply to females. Currently, the exact link between MMP-8 genotypes and plasma levels/activity levels is not well understood. While some studies report a clear dose–response relationship between MMP-8 genotypes and activity levels, we failed to show a significant association in *ex vivo* incubation experiments (unpublished data). However, our preliminary data suggest that the rs1940475 SNP results in a conformational change of the active site, which makes the enzyme less amenable to activation [9]. This needs to be verified in further studies.

In conclusion, subjects homozygous for the ancestral A allele had a stronger pro-inflammatory response to LPS compared to other genotypes, whereas peak MIP-1α levels were highest in subjects homozygous for the G allele. These findings indicate that the rs1940475 SNP modifies the host response to inflammatory stimuli, which

may in part explain previously shown associations with clinical outcomes.

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