Increased Plasma PCSK9 Levels Are Associated with Reduced Endotoxin Clearance and the Development of Acute Organ Failures during Sepsis

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Abstract
Purpose: We have recently shown that PCSK9 reduces the clearance of endotoxin and is therefore a critical regulator of the innate immune response during infection. However, plasma PCSK9 levels during human sepsis and their relationship to outcomes are not known. Our objective was to determine the relationship between plasma PCSK9 levels and the rate of endotoxin clearance, and then correlate PCSK9 levels with the development of acute organ failures in a cohort of patients with sepsis. Methods: Using human hepatocyte cells, we determined the threshold at which PCSK9 is able to reduce Escherichia coli endotoxin uptake by cultured human hepatocytes. In a single-centre observational cohort at St. Paul’s Hospital in Vancouver, Canada, we recruited 200 patients who activated our Emergency Department’s sepsis protocol and measured plasma PCSK9 and lipid levels at triage and throughout the admission. Outcomes were the development of sepsis-induced cardiovascular or respiratory failure. Results: We reviewed the literature and determined that the normal human range of PCSK9 found in plasma is 170–220 ng/ml, while levels of 250 ng/ml and above reduced E. coli endotoxin clearance in cultured human hepatocytes. In septic patients, the median levels associated with new-onset respiratory and cardiovascular failure were 370 (250–500) and 380 (270–530) ng/ml, respectively, versus 270 (220–380) ng/ml in patients who did not go on to develop any organ failure (p = 0.003 and 0.005, respectively). Conclusions: Plasma PCSK9 levels are greatly increased in sepsis. At normal levels, PCSK9 has no influence upon hepatocyte bacterial endotoxin clearance, but as levels rise, there is a progressive inhibition of clearance. During sepsis, PCSK9 levels are highly correlated with the development of subsequent multiple organ failure. Inhibition of PCSK9 activity is an attractive target for treating the spectrum of sepsis and septic shock.

Introduction

PCSK9 (proprotein convertase subtilisin/kexin type 9) plays a critical role in regulating circulating cholesterol levels through the reduction of the membrane-associated low-density lipoprotein (LDL) receptor. We have recent-
ly shown that PCSK9 increases systemic inflammation and organ failure during infection by reducing the hepatic clearance of bacterial endotoxins via the LDL receptor [1]. Produced and secreted from the liver, production of PCSK9 is normally tightly orchestrated via SREBP transactivation of the PCSK9 promoter region [2]. SREBP transcription factors are cleaved and translocate to the nucleus when there are low levels of cholesterol-derived sterols. In small cohorts of patients it has been reported that some septic patients may have low high-density lipoprotein (HDL), LDL and/or total cholesterol [3–7]. Suppression of cholesterol production has long been appreciated during acute illness, such as acute myocardial infarction; indeed, practice guidelines recommend against measuring lipid profiles following the first day of critical illness because values are lower than at pre-infarct [8].

We hypothesize that during sepsis there is a significant decline in plasma cholesterol with a compensatory increased hepatic PCSK9 production. Alternatively, there have been reports that systemic inflammation directly induces hepatic PCSK9 production [9]. As circulating PCSK9 exceeds a physiologic threshold, it could trap endotoxin in circulation and increase the likelihood of inflammatory organ failure. To test this hypothesis we reviewed the literature and determined the normal plasma levels of PCSK9. We then measured uptake of fluorescently labelled Escherichia coli endotoxin in hepatocytes as a bio-assay to determine the threshold level of PCSK9 at which endotoxin clearance becomes impaired, going on to elucidate the full dose-response curve. To correlate these in vitro findings with patient data we recruited a single-centre cohort of 200 patients who presented at the Emergency Department with sepsis and triggered our institutional sepsis protocol. We obtained plasma at triage and over the subsequent 11 days, measuring plasma PCSK9, LDL, HDL and non-HDL cholesterol and triglycerides. We then compared presenting PCSK9 levels in those who went on to develop sepsis-induced organ failure versus those who did not.

Methods

**Meta-Analysis of Plasma PCSK9 Levels**

We searched MEDLINE between January 1, 1998, and May 2015. The reference lists of retrieved articles and review articles were read manually to augment our search. The search criteria included the terms ‘PCSK9’ and ‘Proprotein convertase subtilisin/kexin type 9’. One author (J.A.R.) performed the electronic search and listed the studies that were eligible for inclusion in the study. Candidate abstracts were then reviewed and selected for data retrieval. Two authors (J.A.R. and J.H.B.) independently reviewed each study for quality assessment and extracted data on studies and patient characteristics, as well as outcomes, using standardized extraction forms. The studies were assessed for the presence of 2 features: description of the patient sample characteristics and description of the inclusion and exclusion criteria, including statin or fibrate use. Disagreements were resolved through discussion. For each study, the following individual data were extracted: general data (study design), patients (number of included patients, mean age, gender) and PCSK9 plasma levels prior to any intervention (baseline in interventional studies). In our initial search, 26 studies were found and we limited the analysis to those with subjects aged at least 18 years and acute medical process or intervention without a ‘baseline’ plasma draw. This eliminated 11 studies, leaving 15 for analysis [10–22]. Pooled medians were reported with 95% confidence intervals.

**Hepatocyte Endotoxin Clearance**

The methods have recently been published [1]. Briefly, immortalized human hepatocytes (HepG2 cell line, ATCC) were seeded into a 24-well plate and grown to 80% confluence. The culture medium for the duration of the experiment was 80% DMEM (Invitrogen 11965-065) and 20% human plasma from pooled healthy donors. For the addition of PCSK9, 3 h prior to LPS treatment, 4 h and 19 h post-treatment cells were treated with 0, 250, 500, 1,000, 3,000 and 10,000 ng/ml of recombinant wild-type human PCSK9 (AcroBiosystems PC9-H5223) or vehicle control. Cells were treated with Alexa Fluor 488-conjugated LPS (Invitrogen L-23351) or with standard non-fluorescent LPS (Sigma L2880) as a control. After 24 h of LPS treatment, each well was rinsed twice with PBS and detached with Accutase (BD 561527). The cells were collected, washed with PBS, then resuspended in 500 μl of PBS and analysed via flow cytometry (Beckman Coulter Gallios™ Flow Cytometer). Cells were gated via forward and side scatter for viability using previously determined parameters; 20,000 gated cells were counted per sample. The output of interest was the median fluorescence intensity from the instrument’s FL1 laser (ex: 488 nm, em: 525/20 nm). Background autofluorescence of cells treated with non-fluorescent LPS was subtracted to determine the fluorescence level resulting from the uptake of the LPS conjugate. Data analysis was performed using Kaluza Analysis 1.3 software (Beckman Coulter).

**Early-Sepsis Patient Cohort**

This study was approved by the University of British Columbia, ethics approval H11-00505, and all patients gave written informed consent to the use of both their clinical and analytical data. In a blinded, observational, cohort study, patients with suspected sepsis were identified when the attending Emergency Department physician activated the Institutional Severe Sepsis Order Set (online suppl. table 1; see www.karger.com/doi/10.1159/000442976). Patients were enrolled from January 2011 to July 2013 at St. Paul’s Hospital, Vancouver, Canada, at the time of the first microbiological culture drawn for suspected sepsis. Blood was collected in EDTA tubes at the time of initial blood culture and immediately placed on ice. Plasma was separated and two 1-ml aliquots were transferred into barcoded cryovials at −20°C until they were transferred to a secure, alarmed, −80°C freezer. Study identification numbers were assigned to the secured enrolment forms and clinical data was stored in an ORACLE-based database on a firewall ed, RSS-encrypted server at St. Paul’s Hospital. New cardiovascular dysfunction was defined as treatment with a vasopressor (norepi-
nephrine, epinephrine, phenylephrine), new respiratory dysfunction as the need for mechanical ventilation and PaO_2/FiO_2 < 300 mm Hg, coagulopathy as platelet count < 80/μl, and hepatic dysfunction as bilirubin > 34 μmol/l. For acute kidney injury (AKI) we considered organ failure as stage 2 or 3 AKI according to the current KDIGO guidelines based on serum creatinine (SCR, www.kdigo.org) which is a modification of the AKIN classification. An AKI (stage 1) was defined as an SCR rise of ≥ 26.5 μmol/l within 48 h or an SCR ≥ 1.5-fold increase from the baseline reference value. Stage 2 AKI was defined as a ≥ 2.0- to 2.9-fold increase from the baseline reference SCR. Stage 3 AKI was defined as a ≥ 3-fold increase from baseline reference SCR, or an increase of 354 μmol/l or commencement of renal replacement therapy irrespective of the stage of AKI. The reference SCR is defined as the lowest creatinine value recorded within 3 months of the event, or from repeat SCR within 24 h, or estimated from the nadir SCR value if a patient recovered from AKI, as previously applied. Patients with chronic kidney disease, defined as being on chronic dialysis at admission, were excluded. The urine output criterion for the diagnosis of AKI was not used in this study.

**Plasma Lipids and PCSK9 Levels**

Plasma PCSK9 was measured via ELISA (R&D Systems DPC900) using the manufacturer’s recommended protocol. As per the manufacturer’s product information and direct communication, the upper limit of healthy volunteer plasma PCSK9 using this assay is less than 250 ng/ml. Lipid profiles (total cholesterol, HDL cholesterol and triglycerides, TG) were measured by the clinical laboratory at St. Paul’s Hospital using the ADIVA 1800 Chemistry System. LDL cholesterol was calculated using the Friedewald equation, whereby LDL cholesterol = total cholesterol – HDL-cholesterol – TG/2.2, with all concentrations in mmol/l. We used the last observation carried forward method to account for missing measurements.

**Results**

Pooling 3,556 subjects from 15 studies published between 2010 and 2014, we found that the normal range of plasma PCSK9 in patients not on statins or fibrates to be 170–220 ng/ml (fig. 1). The highest reported value in these outpatient cohorts was 2,500 ng/ml. In our previous work we found that at supra-physiological doses of PCSK9 (3,000 ng/ml) there is nearly complete inhibition of hepatocyte LDLR expression and a consequent 60–65% reduction in bacterial endotoxin uptake [1]. Here we extend our work to analyse how concentrations between 0 and 3,000 ng/ml (reflecting human plasma PCSK9) altered *E. coli* endotoxin uptake (fig. 2). In preliminary dose-response experiments, we determined that 250 ng/ml was the lowest concentration of PCSK9 capable of reducing hepatocyte endotoxin uptake. While statistically significant (p < 0.001) due to the 20,000 cells per measurement, the biological effect at 250 ng/ml was a modest 4% reduc-
We saw a steep reduction in endotoxin uptake as PCSK9 levels exceeded 250 ng/ml—in the plasma PCSK9 range of 250–1,000 ng/ml there was a linear reduction in endotoxin uptake achieved at 3,000 ng/ml PCSK9 (fig. 2). Given this mechanistic knowledge, we went on to examine whether increased PCSK9 plasma levels in a cohort of patients with early sepsis were correlated with the development of acute cardiovascular and/or respiratory failure.

We recruited 200 patients. Patient baseline demographics upon presentation to the Emergency Department and their subsequent organ failures during the admission are shown in table 1. In keeping with our institution’s aggressive approach to activating the sepsis protocol for patients at risk of developing organ failure, this cohort of patients with sepsis had a low (5%) 28-day mortality.

In our cohort of patients with sepsis, 30% progressed to respiratory failure, 18% to cardiovascular failure, 30% to AKI stage 2 or stage 3, 14% to liver failure and 27% to hematologic failure. Like most sepsis cohorts [1, 26–28], this group of patients was 65% male and middle-aged, with a median age of 57 years.

In these patients with early sepsis blood was obtained within 1 h after Emergency Department admission as part of the blood culture mandated by our Institutional Sepsis Protocol. This time-point represents the earliest possible assessment of patient plasma following triage and is a unique aspect of this cohort. The majority of patients had very low cholesterol at presentation. Only 31 of 200 patients had a normal lipid profile. Figure 3 demonstrates graphically the individual components of the lipid profile along with our laboratory’s normal ranges. The most pronounced shift is a reduction in HDL cholesterol. Total cholesterol and LDL cholesterol are also suppressed, although slightly less so than HDL. Triglycerides remained largely unaffected early in sepsis. In a striking negative correlation with cholesterol, PCSK9 is greatly increased in this cohort (fig. 1). This pattern is consistent with the knowledge that low cholesterol could cause the rise in PCSK9; in contrast, a primary rise in PCSK9 production would increase plasma cholesterol. Chronic statin use is also known to increase PCSK9 levels. We found an exaggerated effect of statins; those on statins had PCSK9 levels at presentation of 381 (260–585) ng/ml versus 281 (228–376) ng/ml in patients not on statins (p < 0.001).

We reasoned that if PCSK9 levels causally contributed to the pathophysiology of human sepsis, then there should be a relationship between PCSK9 levels and sepsis...
severity. Accordingly, we analysed the odds ratio of developing more than one acute organ failures over the range of plasma PCSK9 levels (fig. 4). Due to the very significant effect of statins on PCSK9, we only analysed patients not on statins (n = 143). As PCSK9 levels exceed the normal range of 170–220 ng/ml, there is a rapid and large increase in the odds of developing multi-organ failure. We went on to analyse PCSK9 levels over the admission according to individual organ failures. In large epidemiological studies of sepsis, cardiovascular and respi-
Cardiovascular failure is responsible for the vast majority of early organ failure [29]. Therefore, to increase the clinical relevance of our analysis we selected patients who developed cardiorespiratory failure as a result of their sepsis and compared their PCSK9 levels to the patients who did not go on to develop these organ failures. PCSK9 levels are significantly higher upon presentation in those who progress to cardiovascular and respiratory failure compared to those who do not develop these organ dysfunctions (fig. 5a, b). Interestingly, following admission, PCSK9 levels continued to increase in those with cardiovascular or respiratory failure.

We also sought to confirm the findings of other authors [3–7] that very low levels of total, HDL and LDL cholesterol are associated with the development of severe sepsis. At presentation to the Emergency Department, we found much lower levels of total cholesterol, HDL and LDL in patients who went on to develop cardiovascular or respiratory failure as a result of their sepsis compared to patients with sepsis but without subsequent cardiovascular or respiratory failure (table 2). Given our recent insight into the critical role played by PCSK9 [1], this suggests that the early decline in cholesterol as well as hepatic inflammatory transcriptional activation may trigger increases in PCSK9, thus reducing the LDLR-mediated clearance of bacterial toxins.

**Discussion**

To our knowledge, this is the first report describing markedly increased plasma PCSK9 levels in human sepsis and the correlation of plasma PSCK9 levels with later cardiovascular and respiratory failure. PCSK9 levels associated with new onset respiratory and cardiovascular failure were 370 (250–500) and 380 (270–530) ng/ml, respectively, versus 270 (220–380) ng/ml in patients who did not go on to develop organ failure (p = 0.003 and 0.005, respectively). We demonstrated that this effect has biological plausibility in that PCSK9 levels above 250 ng/ml progressively inhibited human hepatocyte endotoxin uptake.
PCSK9 was discovered from genetic association studies. Loss of function genetic variants in PCSK9 resulted in a reduced sequestration of the LDL receptor and, as a result, decreased plasma LDL cholesterol concentrations [36, 37] and improved patient outcomes in atherosclerotic disease [38]; gain-of-function mutations have the opposite effect [30]. These observations led to intense interest in PCSK9 and to rapid development of PCSK9 inhibitors which, indeed, markedly reduce LDL cholesterol levels [39]. We recently discovered a marked benefit of reduced PCSK9 activity across a variety of murine models and in patients who have septic shock, implicating the LDLR (the primary target of PCSK9) as the most relevant pathway affected by PCSK9 [1]. PCSK9 inhibition using an anti-PCSK9 antibody increased the clearance of circulating pathogen toxins, reduced the septic in-
flammatory response, and improved cardiovascular and survival outcomes in mice [1].

To understand the early effects of sepsis on PCSK9 and lipids, we studied early sepsis in the Emergency Department and obtained blood within 1 h of ED admission and subsequently over 11 days during the hospitalization. We found that plasma PCSK9 is rapidly increased in sepsis. These patients represent the broad base of the ‘pyramid of sepsis’, which includes sepsis, severe sepsis and septic shock. In this sepsis pyramid the base refers to all patients with suspected infection who have low blood pressure, and elevated heart and respiratory rates upon presentation. We understand that a new consensus definition will collapse to sepsis and septic shock. Prior reports of plasma PCSK9 in acute conditions are limited to trauma [40] and acute myocardial infarction [10]. Le Bras et al. [40] found that plasma PCSK9 doubled from an admission level of 231 to 480 ng/ml in severe trauma, and the peak levels were correlated with poor outcome, including length of stay in the intensive care unit and duration of mechanical ventilation. PCSK9 levels were more modestly increased by 20–30% during angiography for acute myocardial infarction in a two-cohort, case-control retrospective review [10]. Similar to values reported in the literature [41] for later sepsis, lipids in our cohort were dramatically decreased in very early sepsis. We speculate that the decrease in lipids led to the increase in plasma PCSK9 because an increase of PCSK9 would be expected to increase lipid levels. However, this remains an association rather than proven causation.

To understand the effects of increasing PCSK9 levels on endotoxin uptake, we evaluated E. coli endotoxin uptake in cultured human hepatocytes. We found that PCSK9 greater than 250 ng/ml led to progressive inhibition of E. coli endotoxin uptake and a plateau at 60% inhibition at levels of about 3,000 ng/ml. What constitutes a significant change in endotoxin levels with respect to the development or resolution of inflammatory organ failures? In data presented regarding the efficacy of endotoxin removal by the polymyxin blood perfusion device used in the EUPHAS and EUPHAS2 trial [42] presented by Dr. Massimo Antonelli (Canadian Critical Care Forum, Toronto, Canada, 2013), the therapy, when most efficacious, results in a 25% decline in endotoxin activity. This degree of removal was associated with rapid improvements in blood pressure and the need for vasopressors [42]. At the median levels of PCSK9 seen in our patient cohort the expected suppressive effect upon endotoxin clearance was nearly 20%. Thus, it seems very plausible that high levels of PCSK9 are biologically active in early sepsis.

This study extends our knowledge of the role played by PCSK9 in sepsis in that we show a dramatic increase in circulating PCSK9 at the very earliest clinical encounter. We suggest that as PCSK9 levels increase in response to low cholesterol and direct inflammatory effects upon the liver, the probability of organ failure increases. Taken together, we believe that acute inhibition of PCSK9 activity is an attractive target for treating the spectrum of sepsis, severe sepsis and septic shock.

The strengths of our study include mechanistic evidence of PCSK9 being active at levels seen during sepsis, the extensive literature review involving a large sample size, and the recruitment of patients with early sepsis who are at increased risk of progressing to cardiorespiratory failure. Traditionally these patients are very difficult to recruit into clinical trials and our institutional deferred consent process was crucial in allowing us access to very early biological samples. The limitations to our study include its single-centre observational nature, which only allowed us to speculate regarding the mechanism through which low cholesterol and elevated PCSK9 are linked to the development of organ failures.

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Disclosure Statement

J.A.R., K.R.W. and J.H.B. are founders of Cyon Therapeutics Inc., a biotechnology company focused on the inhibition of PCSK9 for the treatment of sepsis. Sepsis Therapeutics had no role in the funding or interpretation of these data.

Dr. Russell reports patents owned by the University of British Columbia (UBC) that are related to PCSK9 inhibitor(s) and sepsis and related to the use of vasopressin in septic shock. Dr. Russell is an inventor on these patents. Dr. Russell is a founder, Director and shareholder in Cyon Therapeutics Inc. (developing a sepsis therapy). Dr. Russell has share options in Leading Biosciences Inc. (developing a sepsis therapeutic). Dr. Russell reports receiving consulting fees from Cubist Pharmaceuticals (now owned by Merck, formerly Trius Pharmaceuticals, developing antibiotics), Ferring
Pharmaceuticals (which manufactures vasopressin and is developing selepressin), Grifols (which sells albumin), MedImmune (regarding sepsis), Leading Biosciences (developing a sepsis therapeutic), La Jolla Pharmaceuticals (developing a sepsis therapeutic), CytoVale Inc. (developing a sepsis diagnostic), Asahi Kesai (developing a sepsis therapeutic), and Sirius Genomics Inc. (now closed, formerly involved in pharmacogenomics research in sepsis). Dr. Russell reports having received grant support from Sirius Genomics and Ferring Pharmaceuticals that was provided to and administered by UBC.

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