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Full Length Article

Extracellular histone H3 levels are inversely correlated with antithrombin levels and platelet counts and are associated with mortality in sepsis patients

Karin C.A.A. Wildhagen [a], Maryse A. Wiewel [b], Marcus J. Schultz [c], Janneke Horn [c], Roy Schrijver [a], Chris P.M. Reutelingsperger [a], Tom van der Poll [b], Gerry A.F. Nicolaes [a,*]

[a] Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands
[b] Center for Experimental and Molecular Medicine (CEMM), Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
[c] Department of Intensive Care, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

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ABSTRACT

Objective: Sepsis is a leading cause of death worldwide. Extracellular histones are cytotoxic compounds mediating death in murine sepsis and circulating nucleosome levels predict mortality in human inflammation and sepsis. Whether or not circulating extracellular histone H3 correlates with other plasma parameters and/or ICU scoring systems has not been completely established, nor if levels of circulating extracellular histones can be used as predictive markers for clinical outcome in sepsis.
Methods: We measured plasma histone H3 (H3) levels in the plasma of 43 sepsis patients who were admitted to the Intensive Care Unit and determined their correlation with disease severity, organ failure, mortality and coagulation- and tissue homeostasis parameters including LDH levels, thrombin potential (ETP), prothrombin levels, antithrombin levels and platelet counts.
Results: Median H3 levels of sepsis patients at the ICU were significantly increased in non-survivors as compared to survivors with levels found being 3.15 μg/ml versus 0.57 μg/ml respectively, $P = 0.04$. H3 levels are positively correlated with lactate dehydrogenase (LDH) activity (Spearman's rho = 0.49, $P < 0.001$), and negatively correlated with antithrombin levels (rho = −0.34, $P = 0.027$) and platelet counts (rho = −0.33, $P = 0.031$).
Conclusions: We conclude that circulating H3 levels correlate with mortality in sepsis patients and inversely correlate with antithrombin levels and platelet counts.

1. Introduction

Sepsis is a syndrome caused by a deregulated host response to infection. Extrapolated from incidence rates in the United States, worldwide there are currently at least 19 million cases of sepsis per year [1]. Despite advances in supportive therapy for organ failure and the use of potent antibiotics, mortality rates are currently 20-30% for sepsis patients and even higher for severe sepsis and septic shock patients [2].

Recent studies that used animal sepsis models revealed that extracellular histones are major mediators of death in sepsis and that mainly the nuclear proteins histones H3 and H4 are cytotoxic [3]. Histones are highly alkaline proteins that form nucleosomes and thus organize the structure of chromatin. A nucleosome comprises two copies of histones H2A, H2B, H3 and H4 and is surrounded by a superhelix of 146 bp DNA [4]. Histones can be released into the extracellular compartment during apoptosis and necrosis [5], as well as during NETosis [6,7]. Upon release, these extracellular histones cause endothelial dysfunction and may eventually cause organ failure [3,8]. Predominantly H3 and H4 exhibit cytotoxic activity due to a molecular mechanism that is still not fully understood. A potential factor contributing to the cytotoxic properties of histones is the fact that histones can interact directly with phospholipid bilayers of cellular membranes, promote channel formation and cause loss of membrane barrier function [9–11]. Alternatively, it has been described that H3 and H4 can activate Toll-like receptors (TLR) 2 and 4 resulting in MyD88 signaling, NF-κB activation and downstream cytokine production, leukocyte recruitment, microvascular leakage, inflammation, tissue injury and finally death in murine models [12–14].

Elevated levels of circulating nucleosomes have been reported in patients with systemic inflammation and sepsis [5,15,16] and correlate with mortality in traumatic injury patients [17]. In addition, extracellular histones have been implicated in the pathogenesis of sepsis, thrombosis, peritonitis, lung injury, autoimmune diseases and brain, liver and kidney disease [18].

In this study we determined circulating H3 concentrations in the plasma of a group of 43 intensive care unit (ICU) sepsis patients. We

* Corresponding author at: Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University. P.O. Box 616, 6200 MD Maastricht, the Netherlands.
E-mail address: g.nicolaes@maastrichtuniversity.nl (G.A.F. Nicolaes).

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determined correlations between the H3 levels found and ICU-mortality, disease severity and organ failure and between H3 levels and a number of variables reflecting the coagulation status and tissue homeostasis in the patients included in this study.

2. Materials & methods

2.1. Study Design

43 Sepsis patients admitted to the Intensive Care Unit (ICU) of the Academic Medical Center (AMC) in Amsterdam, The Netherlands, were included between May 2012 and January 2013. The inclusion criteria were: age > 18 years and a diagnosis of sepsis according to the Bone criteria [19]: clinical suspicion of infection and at least 2 Systemic Inflammatory Response Syndrome criteria: 1) a body temperature greater than 38 °C or less than 36 °C; 2) a heart rate greater than 90 beats/minute; 3) tachypnea, manifested by a respiratory rate greater than 20 breaths/minute or hyperventilation, as indicated by a $PaCO_2$ of less than 32 mmHg; 4) an alteration in the white blood cell count, such as a count greater than 12.000/mm^3, a count less than 4.000/mm^3, or the presence of more than 10% immature neutrophils. Patients transferred from other ICU's, receiving antibiotics >48 hours prior to ICU admission and/or with an expected length of ICU stay <24 hours were excluded. Dedicated and trained physicians prospectively collected the following data from all patients: demographics, chronic comorbidities, medication use, ICU admission characteristics (including the Acute Physiology and Chronic Health Evaluation (APACHE) IV score [20]), and daily physiological measurements, vital signs, severity scores (including Sequential Organ Failure Assessment (SOFA) scores [21]), antibiotic use, and culture results. Severe sepsis was defined as the presence of at least one sign of organ hypoperfusion or organ dysfunction: areas of mottled skin, capillary refilling time ≥3 seconds, RIFLE-criterium "at risk", "injury" or "failure" [22], lactate >2 mmol/L, abrupt change in mental status or abnormal electroencephalogram, platelet counts <100 000/mL or disseminated intravascular coagulation, acute lung injury or acute respiratory distress syndrome or cardiac dysfunction, as defined by echocardiography or direct measurement of the cardiac index. Shock was defined as the use of vasopressors (noradrenaline) for hypotension in a dose of 0.1 mcg/kg/min during at least 50% of the ICU day.

Within 24 hours of ICU admission arterial blood from each patient was withdrawn in vacutainer tubes containing 3.2% citrate (BD, Franklin Lakes, New Jersey) and immediately centrifuged for 15 min at 1500 g at 4 °C. Plasma samples were stored at −80 °C to perform supplemental analyses at later time points. All laboratory tests were performed in a blind manner, without knowledge of the patient status. The study was approved by the Medical Ethics Review Committee of the AMC Amsterdam (NL 34294.018.10) and all participants or their representatives signed informed consent before enrollment in the study.

2.2. Semi-quantitative H3 Determination

Ten times diluted samples of plasma in HNBSA buffer (25 mM Hepes 150 mM NaCl 5 mg/ml BSA pH 7.7), were separated via SDS-PAGE gel electrophoresis (6-15%) and transferred to PVDF membranes (Pall Corporation, Port Washington, NY) by semi-dry blotting. After blocking, the membranes were overnight incubated at 4 °C with a rabbit polyclonal H3 antibody directed against an epitope at the C-terminus (1:1000, sc-8654-R, Santa Cruz Biotechnology, Heidelberg, Germany), followed by incubation with a biotinylated goat anti-rabbit IgG antibody (1:1000, Vector Laboratories, Burlingame, CA) for 30 minutes at RT and finally with StreptABComplex/AP solution (1:3000, Dako, Glostrup, Denmark) for 30 minutes at RT. Protein bands were detected by BCIP/NBT (Sigma-Aldrich, St. Louis, Missouri) and density of all H3 bands was quantified by ImageJ software (x86, Wayne Rasband). The densities of H3 bands in patient plasma samples were compared with the density of known concentrations of purified H3 (Roche, Basel, Switzerland) that were transferred to each of the blotting membranes onto which patient samples were transferred and these were used as a reference to calculate H3 concentrations in patient samples, see also Supplemental Fig. 1. The detection limit of the semi-quantitative assay was 5 ng/ml histone H3, whereas in a pooled normal plasma (prepared from the platelet poor plasma of 34 healthy volunteers) or in the individual plasmas of 15 healthy volunteers, we could not detect any extracellular histone H3 (data not shown).

2.3. Calibrated Automated Thrombinography (CAT)

Thrombin generation in clotting plasma was monitored by calibrated automated thrombinography (CAT), where conversion of a low affinity fluorogenic substrate (Z-Gly-Gly-Arg-7-amido-4-methylcoumarin (Bachem, Bubendorf, Switzerland)) by thrombin was followed over time in a Fluoroskan Ascent microtiter plate reader (Thermo Labsystems, Helsinki, Finland) with wavelengths for excitation at 390 nm and at 460 nm for emission [23]. CAT thrombin generation curves and the endogenous thrombin potential (ETP), were calculated using Thrombinoscope software (Thrombinoscope B.V., Maastricht, The Netherlands). Thrombin formation was routinely initiated by the addition of 5 pM of recombinant tissue factor, 16 mM $CaCl_2$, 30 μM phospholipid vesicles (20:60:20 DOPS:DOPC:DOPE (mol:mol:mol)) and 50 μg/ml corn trypsin inhibitor. Pooled normal human plasma (34 donors: 14 females, 20 males), which was collected in the same way as the sepsis plasma samples, was used for normalization of measured values of the sepsis patients.

Patients who had received anticoagulant medication (acenocoumarol, heparin) before ICU admittance (n = 6), patients who received heparin at ICU before blood sampling (n = 5) and patients who received both anticoagulant medication before ICU admittance and heparin at ICU before blood sampling (n = 4) were excluded from thrombin generation measurements.

2.4. Lactate Dehydrogenase (LDH) Activity Assay

Plasma samples were 25 times diluted in HNBSA buffer and a LDH activity assay kit (Sigma-Aldrich, St. Louis, Missouri) was used to measure LDH activity.

2.5. Antithrombin Determination

For the quantitative determination of antithrombin in plasma samples of sepsis patients the ACTICHROME® Antithrombin chromogenic activity kit (American Diagnostica, Stamford, CT) was used. As described before [24], thrombin was added to a plasma dilution containing antithrombin in the presence of excess heparin. After an initial incubation, residual thrombin was determined with a thrombin-specific chromogenic substrate. The residual thrombin activity was inversely proportional to the antithrombin concentration. Pooled normal human plasma (34 donors: 14 females, 20 males), which was collected following a same protocol as for the sepsis plasma samples, was used as an antithrombin reference.

2.6. Prothrombin Determination

Prothrombin concentrations in plasma samples of sepsis patients were determined using Ecarin (Pentapharm, Basel, Switzerland), a metalloprotease isolated from the venom of the saw-scaled viper (Echis carinatus), that specifically activates prothrombin independent of calcium, phospholipids and FXa/FV. Plasma samples were diluted in buffer (25 mm HEPES buffer, 150 mm NaCl, 5 mg/ml bovine serum albumin, 5 mM $CaCl_2$, pH 7.7), Ecarin was added (final concentration