Thrombosis Research 132 (2013) 69–76

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Histone induced platelet aggregation is inhibited by normal albumin ☆

Fong W. Lam [a,b,*], Miguel A. Cruz [c], Hon-Chiu E. Leung [d], Kathan S. Parikh [a], C. Wayne Smith [b], Rolando E. Rumbaut [b,c,e]

[a] Department of Pediatrics, Critical Care Section, Baylor College of Medicine, Houston, TX, USA
[b] Department of Pediatrics, Division of Leukocyte Biology, Baylor College of Medicine, Houston, TX, USA
[c] Department of Medicine, Division of Thrombosis Research, Baylor College of Medicine, Houston, TX, USA
[d] Department of Pediatrics, Department of Molecular and Cellular Biology, Dan Duncan Cancer Center, Mass Spectrometry-Proteomics Core Facility, Baylor College of Medicine, Houston, TX, USA
[e] Center for Translational Research on Inflammatory Diseases, Michael E. DeBakey Veterans Affairs Medical Center, Houston, TX, USA

ARTICLE INFO

Article history:
Received 14 November 2012
Received in revised form 11 April 2013
Accepted 16 April 2013
Available online 11 May 2013

Keywords:
Albumin
Aggregation
Histone
Platelet
Sepsis
Thrombosis

ABSTRACT

Introduction: Histones are small, nuclear proteins that serve to package DNA. Recent reports suggest that extracellular histones, including histone H4, may contribute to the pathogenesis of sepsis; they promote platelet aggregation and thrombosis when released into the circulation during inflammation or cell death. The mechanisms by which the body minimizes the deleterious effects of circulating histones are unclear. Because histones can bind to plasma proteins, including albumin, we hypothesized that normal albumin can prevent histones from activating platelets.

Materials and Methods: Platelets and platelet-free plasma were obtained from healthy, adult subjects. The dose-dependent effects of histone H4 on platelet aggregation were studied by optical aggregometry. The effects of native and albumin-depleted plasma (prepared by affinity chromatography) on histone-induced platelet aggregation were also assessed. The effects of normal and surface-neutralized albumin (through modification of carboxyl groups) on histone-induced platelet activation and aggregation were evaluated using flow cytometry and aggregometry.

Results: Histone H4 induced platelet aggregation in a dose-dependent manner. This histone-induced platelet aggregation was inhibited by both plasma and human serum albumin in a dose-dependent fashion. Furthermore, depletion of albumin from plasma reduced its ability to inhibit aggregation. Finally, surface neutralization of albumin decreased its ability to inhibit histone-induced activation and aggregation.

Discussion: These data suggest that normal albumin serves a role in preventing histone-induced platelet aggregation in a charge-dependent manner.

Introduction

Histones are small, positively charged (*p*I ~ 11), nuclear proteins that serve to package DNA in the nucleus of cells [1]. Normally, histones are contained within cells, but they may be released from apoptotic and necrotic cells as well as from activated neutrophils as a component of neutrophil extracellular traps (NETs) [2]. Recently, extracellular histones released during inflammation have been shown to induce platelet aggregation and thrombocytopenia, exacerbate stroke, and result in death in animal models of sepsis [3–6]. Circulating histones released during inflammation may also play a role in human sepsis. Elevated levels of circulating nucleosomes (histone-DNA complexes) are associated with both severity of illness [7] and mortality [8] in patients with sepsis. Sepsis, a systemic response to infection, continues to be a major cause of death [9–11]; septic patients who develop coagulation or thrombotic disorders, such as disseminated intravascular coagulation (DIC), have the highest mortality [12–14]. More recently, levels of circulating nucleosomes have been associated with disease activity in patients with thrombotic microangiopathies [15]. These observations suggest that extracellular histones may be associated with thrombocytopenia in critically ill patients, and its associated risk of mortality [16]. Additionally, activation of thrombosis via extracellular histones could initiate widespread activation of coagulation as well, leading to microvascular thrombosis and disseminated intravascular coagulation (DIC), which are strongly associated with mortality [12–14].

The body is subjected to cell turnover and inflammation continuously, thereby producing extracellular histones with their potential deleterious effects. The mechanisms by which the body limits the pathologic effects of histones are unclear. Xu et al. have demonstrated

☆ Financial Support: This study was supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development and by institutional departmental funds from Baylor College of Medicine.

* Corresponding author at: Texas Children's Hospital, Baylor College of Medicine, 6621 Fannin St, WT 6-006, Houston, TX 77030, USA. Tel.: +1 832 826 6202; fax: +1 832 825 6229.
E-mail address: flam@bcm.edu (F.W. Lam).

0049-3848/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.thromres.2013.04.018

that administration of activated protein C, a serine protease involved in the anticoagulation pathway, is protective in mice given lethal doses of histones [6]. Another potential mechanism the body utilizes to minimize the deleterious effects of histones is by binding to plasma proteins; in one study, histones were shown to bind to 36 distinct plasma proteins, including albumin [17]. Albumin is the most abundant circulating protein in the body and has the ability to interact and bind with a large variety of compounds in the circulation [18,19]. These observations are consistent with the notion that albumin represents an alternative mechanism utilized by the body to inhibit the pathologic effects of circulating histones.

In this study, we tested the hypothesis that binding of histones to albumin serves a protective role against histone-induced platelet aggregation. We therefore assessed the effects of albumin, plasma, and albumin-depleted plasma on histone-induced platelet aggregation. Further, we examined the charge-dependent effects of albumin on these responses.

Materials and Methods

Chemicals and Reagents

Prostacyclin (PGI_2), lyophilized human serum albumin (HSA), glycine methyl ester, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), thrombin and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO). Quickstart Bradford Protein Assay was purchased from Bio-Rad (Hercules, CA). Adenosine diphosphate (ADP) was purchased from Bio/Data Corporation (Horsham, PA). Histone H4 (provided in 1 mM DTT) was purchased from New England Biolabs, Inc., (Ipswich, MA). Human Enzyme-Linked Immunosorbent Assay (ELISA) Quantitation Set and horseradish peroxidase (HRP)-conjugated anti-human albumin antibodies were purchased from Bethyl Laboratories, Inc. (Montgomery, TX). Rabbit anti-histone H4 antibodies were purchased from Biolegend (San Diego, CA). HRP-conjugated goat anti-rabbit IgG antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Substrate Reagent Pack was purchased from R & D Systems (Minneapolis, MN). HiTrap Blue HP affinity chromatography columns were purchased from GE Healthcare (Waukesha, WI).

Blood Collection

After informed consent was obtained, blood was collected from healthy, non-smoking, adult volunteers who had not received aspirin or anti-platelet medications for the past two weeks and non-steroidal anti-inflammatory medications for the past two days. Subject enrollment and sample collection were approved by the Baylor College of Medicine Institutional Review Board.

Platelet and Plasma Isolation

To obtain washed platelets, citrated (1 part 3.2% sodium citrate to 9 parts blood) whole blood was centrifuged for 15 minutes at 150*g*. Platelet-rich plasma was collected and allowed to rest for one hour at 37 °C in 5% carbon dioxide and in the presence of 0.5 μM PGI_2. The platelet-rich plasma was then centrifuged for 10 minutes at 900*g* and resuspended in either Tyrode's buffer (138 mM sodium chloride, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, 6.36 mM sodium phosphate (dibasic), and 5.5 mM glucose; pH 7.35-7.45), plasma, or albumin. The final concentration of platelets used for aggregometry was 250×10^6 platelets/mL.

To obtain platelet-free plasma, citrated whole blood was centrifuged for 15 minutes at 1,800*g*. The supernatant, platelet-poor plasma, was centrifuged for 10 minutes at 2,500*g* to obtain platelet-free plasma, which was then immediately used or stored at −80C.

Platelet aggregation studies

Platelet aggregation was performed on a PAP-8E optical aggregometer (Bio/Data Corporation) in the presence of 1.7 mM calcium chloride and 0.4 mM magnesium chloride. All samples were incubated at 37C and stirred at 1,200 RPM for the duration of each test. Aggregation was measured in platelets suspended in either Tyrode's buffer, varying concentrations of plasma, and varying levels of albumin. In addition to albumin, we assessed the effect of fibrinogen (HYPHEN BioMed; Neuville-Sur-Oise, France) on histone-induced platelet aggregation, due to fibrinogen's ability to bind histones [17]. Aggregation was induced with histone H4 at differing concentrations, with thrombin (0.5 units/mL) as a positive control and vehicle control (1 mM DTT) as the negative control. Data collection was performed for 10 minutes, although all samples reached maximal aggregation by 5 minutes. All aggregation was performed in duplicate with the mean used for each sample.

Albumin Depletion from Plasma

To determine if albumin was necessary for plasma's inhibition against histone-induced platelet aggregation, we performed platelet aggregometry in platelets resuspended in either albumin-depleted or control plasma. Plasma from each subject was divided into two aliquots and perfused, in parallel, through affinity chromatography columns containing Sepharose Blue (HiTrap Blue HP, 5 mL capacity). Perfusion was performed using syringe pumps and according to the manufacturer's specifications. Each column was pretreated with either 5% albumin to occupy albumin-binding sites (control) or Tyrode's buffer (albumin-depleted). After the columns were pretreated and prior to perfusion with plasma, they were washed with Tyrode's buffer to remove excess, unbound albumin from the columns. Plasma (2 mL) was then perfused through each column at a rate of 5 mL/min. Tyrode's buffer (15 mL) was then perfused through each column at a rate of 5 mL/min. Eluent from each column was collected in 1 mL aliquots and total protein was measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The three consecutive aliquots containing the highest protein levels were collected from both the control and the albumin-depleted groups. Total protein concentration and albumin were measured using the Bradford assay and the Human ELISA Quantitation Set, respectively, on a SpectraMax Plus^{384} Absorbance Microplate Reader (Molecular Devices). Diluted samples were also compared using polyacrylamide gel electrophoresis with Coomassie brilliant blue stain.

Tandem Mass Spectrometry of Protein Samples

Plasma samples undergoing affinity chromatography were quantified, processed, and analyzed using tandem mass spectrometry as outlined in the Supplemental Material and Methods.

Surface Neutralization of Human Serum Albumin

To determine if albumin's inhibitory effect on histone-induced platelet aggregation was due to charge-based protein-protein interactions, we performed modification of the carboxyl groups on HSA using the procedure described by Lund et al. [20]. HSA (1.5 g) was dissolved in 15 mL 0.133 M glycine methyl ester at pH 4.75 at room temperature. 5 mL 0.04 EDC was then added to the solution to initiate the reaction. The pH was continuously maintained at 4.75 by adding 0.1 M sodium hydroxide. After 45 minutes, the reaction was quenched with an equal volume of 4 M acetate buffer (pH 4.75). The neutralized albumin (nHSA) was then dialyzed against two exchanges of distilled water.

Isoelectric focusing of HSA and nHSA was performed on Zoom IPG strips (*p*I 3-10; Invitrogen) to determine the *p*I. The isoelectric focusing gradients were set up as follows: 200V for 1 hour, 450V for 1 hour,