Molecular Immunology 64 (2015) 55–62

Contents lists available at ScienceDirect

Molecular Immunology

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

Enhanced recognition of plasma proteins in a non-native state by complement C3b. A possible clearance mechanism for damaged proteins in blood

Mahalakshmi Ramadass[a], Berhane Ghebrehiwet[a,b], Richard R. Kew[a,*]

[a] Department of Pathology, Stony Brook University School of Medicine, Stony Brook, NY 11794, USA
[b] Department of Medicine, Stony Brook University School of Medicine, Stony Brook, NY 11794, USA

ARTICLE INFO

Article history:
Received 15 October 2014
Accepted 26 October 2014
Available online 15 November 2014

Keywords:
Alternative pathway activation
Complement C3
Extracellular chaperone
Plasma proteins
Denatured proteins
Inflammation

ABSTRACT

Complement C3 is a key fluid-phase protein of the immune system that covalently tags pathogenic cells and molecules for subsequent clearance. Previously, we reported that complement activation results in the formation of multiple C3b:plasma protein complexes in serum. However, it is not known if C3b attaches to any plasma protein in close proximity or preferentially binds damaged proteins. The objective of this study was to determine if C3b couples to plasma proteins in a non-native state and if this could be a potential mechanism to detect and clear damaged proteins from the blood. Using a purified in vitro system with alternative pathway proteins C3, factors B and D it was observed that guanidinium-HCl denaturation of three purified plasma proteins (albumin, alpha-1 proteinase inhibitor, vitamin D binding protein) greatly increased their capacity to form covalent complexes with C3b. However, native vitamin D binding protein, covalently attached to C3b, still retained the ability to bind its natural ligand G-actin, indicating that C3b links to plasma proteins in their native configuration but denaturation substantially increases this interaction. Serum complement activation generated a large number of C3b:plasma protein complexes that bound red blood cell membranes, suggesting a CR1-mediated clearance mechanism. Thermally denatured (60 °C) serum activated the alternative pathway when added to fresh serum as evidenced by factor B cleavage and iC3b generation, but this heat-treated serum could not generate the pro-inflammatory peptide C5a. These results show that C3 recognizes and tags damaged plasma proteins for subsequent removal from the blood without triggering proinflammatory functions.

1. Introduction

The third component of complement (C3) is the most abundant and essential protein of the complement system in all vertebrates (Mastellos et al., 2013; Zarkadis et al., 2001). High concentrations of C3 circulate in blood plasma (adult human range: 0.7 to 1.6 mg/ml) and can increase almost two-fold during the acute phase response of inflammation. C3 is required for full activation of the classical and lectin recognition pathways and is central for the initiation and activation of the alternative pathway (Ricklin et al., 2010). During complement activation, proteolytic cleavage of C3 to C3b exposes the unstable thioester bond, which then reacts extremely fast with either a target surface or surrounding water molecules to generate surface bound or soluble C3b (Janssen et al., 2006; Pangburn and Muller-Eberhard, 1980). It is well established that C3 plays a major role in the clearance of microbial pathogens, immune complexes, apoptotic cells and tissue debris by covalently tagging targets with C3 cleavage products (C3b and iC3b) for subsequent removal via phagocytic cells expressing C3b/iC3b receptors (He et al., 2008).

Extracellular protein misfolding underlies most of the serious conditions of pathological protein deposition including systemic amyloidosis, Alzheimer's disease, diabetes and spongiform encephalitis (Aguzzi and O'Connor, 2010; Naiki and Nagai, 2009). Both the intra and extracellular compartments can impose strains on protein structure due to fluctuations in pH, temperature and oxidative stress. Thus, it is critical to maintain correct protein structure and function in living systems, a process that is collectively known as protein homeostasis or proteostasis (Aguzzi and O'Connor, 2010; Hipp et al., 2014; Naiki and Nagai, 2009). The extracellular space is a more oxidizing environment than the

Abbreviations: α_1PI, alpha 1 proteinase inhibitor; CVF, cobra venom factor; DBP, vitamin D binding protein; HAGG, heat-aggregated human IgG; HSA, human serum albumin; NHS, normal human serum; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate fluorescent dye; Δ serum, NHS heated at 60 °C for 60 min.

* Corresponding author. Tel.: +01 631 444 3941.
E-mail address: richard.kew@stonybrook.edu (R.R. Kew).

http://dx.doi.org/10.1016/j.molimm.2014.10.022
0161-5890/

intracellular compartment, and there is an additional challenge posed to protein stability in the blood due to shear stress of circulating fluids. Chaperones play a key role in protecting against such stresses either by binding and preventing aggregation of the unfolded/misfolded protein or by facilitating protein refolding or clearance from circulation. Excessive misfolding/unfolding can cause disease due to loss of normal function, gain of pathological function and/or dysfunctional accumulation in tissues (Aguzzi and O'Connor, 2010; Naiki and Nagai, 2009). Though the process of protein homeostasis has been studied extensively in the intracellular context (Hipp et al., 2014), extracellular chaperones are not as well recognized or understood and characterization of these proteins and their functions has only recently gained more attention (Wyatt et al., 2013). The number of extracellular chaperones continues to grow; currently at least seven plasma proteins have been shown to function as chaperones including clusterin, α_2-macroglobulin, haptoglobin, apolipoprotein E, serum amyloid P (SAP), caseins and fibrinogen (Wyatt et al., 2013).

Recently, we have shown that upon activation of complement, C3 binds covalently via the thioester to a wide range of plasma proteins of varying abundance, molecular weight and isoelectric points (Ramadass et al., 2014). A significant proportion of C3b is complexed to plasma proteins, even with the large disparity in the percentage of water molecules to proteins in plasma (91.5% of plasma is water, 55 M, and only 7.5% is protein). We speculated that this could be a mechanism to neutralize the C3 thioester and limit deposition on host cells at sites of complement activation (Ramadass et al., 2014). In the present study we investigated if C3 tagging of plasma proteins facilitates their clearance, and if so does this process have a preference for non-native or unfolded plasma proteins, thus suggesting an extracellular chaperoning function for C3.

2. Materials and methods

2.1. Reagents

Purified cobra venom factor (CVF), human serum depleted of individual complement components (C3, factor B and factor D), and purified human complement proteins (C3, factor B and factor D) were all purchased from Complement Technology, Inc. (Tyler, TX). The following purified human proteins were all obtained from Athens Research and Technology (Athens, GA): alpha-1 proteinase inhibitor (α_1PI), vitamin D binding protein (DBP), human serum albumin (HSA) and neutrophil elastase. The following reagents were purchased from Sigma-Aldrich (St. Louis, MO): 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (MeOSuc-AAPV-pNA) and MeOSuc-Ala-Ala-Pro-Val chloromethyl ketone (MeOSuc-AAPV-CMK). Bio-Gel P30 matrix with medium polyacrylamide beads (90–180 μm wet bead size) for size exclusion chromatography and Lowry protein assay based DC protein assay kit both were purchased from Bio-Rad (Hercules, CA). Pre-diluted bovine serum albumin (BSA) protein standards were purchased from Thermo-Fisher Scientific (Pittsburgh, PA). Purified actin was purchased from Cytoskeleton, Inc. (Denver, CO). Protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Blood was obtained from several healthy donors as previously described using a protocol approved by Stony Brook University Institutional Review Board in accordance with the Declaration of Helsinki (Ramadass et al., 2014). Normal human serum (NHS) was collected and pooled from several donors.

The IgG fraction of goat polyclonal anti-human DBP was purchased from DiaSorin (Stillwater, MN) and then affinity-purified in our laboratory using immobilized DBP. Chicken polyclonal anti-α_1PI antibody was obtained from ProSci, Inc. (Poway, CA). Mouse monoclonal anti-human factor B (clone D33/3) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-albumin antibody was a generous gift from Dr. Berhane Ghebrehiwet, Stony Brook University. Chicken polyclonal anti-human C3 was obtained from Gallus Immunotech (Cary, NC).

2.2. Gel electrophoresis and immunoblotting

For denaturing and reducing gels, all samples were separated using 8% polyacrylamide gels with SDS at 80 V for the stacking gel and 100 V for the resolving gel. Resolved gels then were transferred onto an Immobilon PVDF membrane (Millipore, Bedford, MA) at 100 V for 75 min. The PVDF membrane was blocked with 5% non-fat dry milk (NFDM) in tris-buffered saline with 0.1% Tween 20 (TBS-T) for 30 min, followed by primary and HRP-labeled secondary antibody incubations in 5% NFDM. Finally, blots were developed using HyGLO Quick Spray chemiluminescent detection reagent (Denville Scientific, Denville, NJ) and X-ray film. For native gels, the same procedure was carried out using 8% native polyacrylamide gel but without SDS.

2.3. In vitro complex formation

To evaluate the role of activated C3 in complex formation, the alternative pathway was assembled in vitro using the purified proteins C3 (1.3 mg/ml), factor B (200 μg/ml) factor D (1 μg/ml) and DBP (400 μg/ml) or α_1PI (1 mg/ml) with 0.5 mM Mg^{2+} and incubated at 37 °C for the specified amount of time. In control experiments, a mixture containing either (20% of the original C3 concentration (0.26 mg/ml) or no C3 was used to test C3 dependence of the complex formation. The molar ratios of the various components were maintained at physiological levels even when the exact concentrations could not be maintained due to dilution effects.

2.4. Denaturation of proteins

The protein of interest was denatured using 5 M guanidinium-HCl (GuHCl), 30 mM DTT, 10 mM EDTA, 100 mM tris–HCl (pH 8.3) at room temperature for the time indicated on a rotary shaker. Upon denaturation, the denaturing reagents were removed by gel filtration using a Bio-Gel P-30 column. The protein concentration of denatured proteins was quantified using the Lowry assay and then tested for denaturation using functional assays.

2.5. TNS assay

Purified DBP or purified HSA (both native or GuHCl-denatured at 0.5 μM) were incubated with 30 nM TNS at room temperature for 20 min to allow dye binding, fluorescence then was measured at an excitation wavelength of 322 nm and an emission wavelength of 465 nm.

2.6. Elastase assay

Purified elastase (60 nM) was incubated at 37 °C for 30 min with either 60 nM native α_1PI, 60 nM GuHCl α_1PI or 100 μM of the synthetic elastase inhibitor MeOSuc-AAPV-CMK (positive control) in assay buffer (0.1 M HEPES, pH 7.25 with 0.5 M NaCl, 0.05% Tween 20) to allow binding. The *p*-nitroaniline substrate MeOSuc-AAPV-pNA (100 μM) was added and the mixture was incubated at 37 °C for 30 min. Absorbance was read at 405 nm to assess elastase activity.