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Circulating complexes of the vitamin D binding protein with G-actin induce lung inflammation by targeting endothelial cells

Lingyin Ge[a], Glenda Trujillo[a], Edmund J. Miller[b], Richard R. Kew[a,*]

[a] Department of Pathology, Stony Brook University School of Medicine, Stony Brook, NY 11794-8691, USA
[b] Center for Heart and Lung Research, The Feinstein Institute for Medical Research, Manhasset, NY 11030-3816, USA

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ABSTRACT

This study investigated the actin scavenger function of the vitamin D binding protein (DBP) *in vivo* using DBP null (−/−) mice. Intravenous injection of G-actin into wild-type (DBP+/+) and DBP−/− mice showed that contrary to expectations, DBP+/+ mice developed more severe acute lung inflammation. Inflammation was restricted to the lung and pathological changes were clearly evident at 1.5 and 4 h post-injection but were largely resolved by 24 h. Histology of DBP+/+ lungs revealed noticeably more vascular leakage, hemorrhage and thickening of the alveolar wall. Flow cytometry analysis of whole lung homogenates showed significantly increased neutrophil infiltration into DBP+/+ mouse lungs at 1.5 and 4 h. Increased amounts of protein and leukocytes were also noted in bronchoalveolar lavage fluid from DBP+/+ mice 4 h after actin injection. *In vitro*, purified DBP-actin complexes did not activate complement or neutrophils but induced injury and death of cultured human lung microvascular endothelial cells (HLMVEC) and human umbilical vein endothelial cells (HUVEC). Cells treated with DBP-actin showed a significant reduction in viability at 4 h, this effect was reversible if cells were cultured in fresh media for another 24 h. However, a 24-h treatment with DBP-actin complexes showed a significant increase in cell death (95% for HLMVEC, 45% for HUVEC). The mechanism of endothelial cell death was via both caspase-3 dependent (HUVEC) and independent (HLMVEC) pathways. These results demonstrate that elevated levels and/or prolonged exposure to DBP-actin complexes may induce endothelial cell injury and death, particularly in the lung microvasculature.

Introduction

Actin is the most abundant and highly conserved protein inside all eukaryotic cells and exists in two forms: monomeric globular actin (G-actin) and polymerized filamentous actin (F-actin) (Rottner and Stradal 2011). During tissue injury large quantities of actin can be released into extracellular fluids where the ionic conditions and lack of regulators favor spontaneous generation of F-actin filaments (Janmey and Lind 1987). Circulating F-actin potentially is injurious and previous animal studies have shown that intravascular actin filaments can trigger angiopathic consequences in the microcirculation similar to fibrin (Haddad et al. 1990; Meier et al. 2006). Accordingly, higher organisms have evolved a robust extracellular actin scavenger system (EASS) consisting of two plasma proteins: gelsolin that caps and severs F-actin filaments, and the vitamin D binding protein (DBP) that binds G-actin monomers tightly for subsequent clearance from the blood (Meier et al. 2006). Circulating DBP-actin complexes have been observed in both humans and animals following traumatic injury, and the plasma concentration of actin-free DBP has been shown to be an effective but indirect marker of tissue injury in cases of severe trauma (Antoniades et al. 2007; Meier et al. 2006; Schiodt et al. 2007). Plasma levels of actin-free DBP below 3.5 μM (200 μg/ml) have been shown to significantly correlate with poor prognosis in human cases of sepsis, multiple trauma and acetaminophen-induced liver failure (Antoniades et al. 2007; Dahl et al. 2003; Meier et al. 2006; Schiodt et al. 2007). Clinical outcome and decreased plasma levels of DBP in trauma have a statistical correlation similar to the APACHE II score, Kings College criteria and the TRISS-like method (Antoniades et al. 2007; Dahl et al. 2003; Meier et al. 2006; Schiodt et al. 2007). Thus, the capacity to scavenge extracellular actin is a physiologically important role for this multifunctional plasma protein.

Abbreviations: AO/EB, acridine orange/ethidium bromide; BAL, bronchoalveolar lavage; CTB, cell-titer blue viability assay; DBP, vitamin D binding protein; DCFDA, 2′,7′-dichlorofluorescein diacetate; EA, antibody-coated sheep erythrocytes; EASS, extracellular actin scavenger system; EGM-2, endothelial growth medium-2; HLMVEC, human lung microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; PARP, poly ADP ribose polymerase; ROS, reactive oxygen species.

* Corresponding author. Tel.: +1 6314443941.
E-mail address: richard.kew@stonybrook.edu (R.R. Kew).

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DBP, also referred to as Gc-globulin, is an abundant (6–7 μM) 56 kDa plasma protein that is part of the albumin gene family and shares the multiple disulfide linked triple domain structure of albumin (Chun 2012). As its name implies, it is the primary extracellular transport protein for all vitamin D metabolites. Besides the vitamin D and actin binding functions, DBP can serve as a neutrophil chemotactic cofactor, and a deglycosylated form of DBP acts as a macrophage activating factor (Chun 2012). There are no known natural deficiencies of DBP in any vertebrate species but a DBP null (−/−) mouse, fully backcrossed on a C57BL/6 background, has been generated. These mice are healthy and develop and reproduce similar to their wild-type counterparts when fed a vitamin D sufficient mouse chow diet (Safadi et al. 1999; White et al. 2002). Studies using DBP−/− mice have shown that the primary role of DBP is to maintain circulating vitamin D levels within a physiological range to protect against transient vitamin deficiencies (Zella et al. 2008). More recently, our lab has shown that DBP−/− mice have significantly reduced (∼50%) neutrophil recruitment to the lungs compared to their wild-type DBP+/+ counterparts in three different alveolitis models, two acute and one chronic (Trujillo et al. 2013). However, the actin scavenger function of DBP has not been investigated *in vivo* using DBP null mice, and the objective of this study was to characterize how mice with a systemic deficiency of DBP respond to actin in the circulation. Contrary to expectations, results show that DBP−/− mice developed much less acute lung inflammation than their wild-type DBP+/+ counterparts when actin was injected intravenously. Moreover, *in vitro* studies showed that DBP-actin complexes induce endothelial cell injury and death. These studies suggest that elevated levels and/or prolonged exposure to DBP-actin complexes *in vivo* may contribute to the sequelae of traumatic tissue injury.

Materials and methods

Reagents

Purified human DBP was obtained from Athens Research & Technology (Athens, GA). The IgG fraction of goat polyclonal anti-human DBP was purchased from DiaSorin (Stillwater, MN) and then affinity-purified in our lab using immobilized DBP. Highly purified rabbit skeletal muscle actin was obtained from Cytoskeleton, Inc. (Denver, CO). Goat polyclonal anti-actin (I-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and monoclonal pan anti-actin mAb (ACTN05) was purchased from Thermo Scientific/Lab Vision (Fremont, CA). Polyclonal anti-gelsolin (ab74420) was obtained from Abcam (Cambridge, MA). Sterile, endotoxin-free HBSS, DPBS and RPMI 1640 were purchase from Mediatech, Inc. (Manassas, VA). EDTA solution was purchased from Life Technologies-Gibco (Grand Island, NY). Collagenase and DNase I were purchased from Roche (Indianapolis, IN). Rat anti-mouse monoclonal antibodies for flow cytometry and their corresponding labeled isotype controls, were all purchased from Biolegend (San Diego, CA): PE-labeled anti Gr-1 (RB6-8C5), FITC-labeled anti-F4/80 (BM8). Purified cobra venom factor (CVF) and sheep erythrocytes were obtained from Complement Technology, Inc. (Tyler, TX). Rabbit anti-sheep erythrocyte antibody was purchased from Dako (Accurate Scientific Westbury, NY). Mouse monoclonal anti-human factor B (clone D33/3) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Purified human TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN). Bio-Plex 23-mouse cytokine panel was obtained from BioRad (Hercules, CA).

DBP+/+ and −/− mouse colonies

The DBP−/− mouse line has been fully backcrossed on a C57BL6/J background for 11 generations. A detailed description of the DBP−/− and the corresponding DBP+/+ mouse colonies, produced from DBP+/− hemizygotes, has been recently published (Trujillo et al. 2013). In some experiments, wild type C57BL6/J mice were purchased from Jackson Labs (Bar Harbor, ME). Only mature male age-matched mice (age ranged from 10 weeks to 10 months) were utilized and all animals were housed in a maximum isolation facility at Stony Brook University. Animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee at Stony Brook University.

Intravenous administration of G-actin

Highly purified rabbit skeletal muscle actin was reconstituted using sterile pyrogen-free DPBS at 1 mg/ml under aseptic conditions. DBP+/+ and −/− mice were injected with 100 μl of G-actin (1 mg/ml) via the tail vein to introduce an actin bolus into the circulation. The control group received 100 μl of DBPS via the tail vein. The mice were observed for signs of respiratory distress. After 1.5, 4 or 24 h, groups of mice were anesthetized by an i.p. injection (0.3 ml) of ketamine (90 mg/kg) and xylazine (10 mg/kg) solution. Following anesthesia, EDTA plasma was obtained by retro-orbital bleeding and mice were then euthanized by cervical dislocation and secured to a small animal surgery board (Kent Scientific, Torrington, CT). A small midline incision was made in the suprasternal region and the trachea was exposed by blunt dissection. A sterile BD angiocath (18 gauge needle stylet in a 1.3 mm × 44 mm long I.V. catheter) was inserted into the anterior portion of the exposed trachea between cartilage rings and the lungs were lavaged once and the BAL fluid was stored on ice until analyzed for total protein and cell count. BAL fluid was centrifuged at 200 × *g* for 5 min at 4 °C to pellet the cells. BAL cells pellets were resuspended in 1 ml of PBS-1% BSA and duplicate counts of total cell number were made using a BioRad TC10 automated cell counter. The protein concentration of the BAL supernatant was measured using a Lowry Protein Assay.

Preparation of organs for histology

In a separate group of mice that were not lavaged, the mouse lungs were inflated with 10% PBS-buffered formalin for 15 min at 25 cm water pressure to ensure the proper degree of lung inflation. The lungs were removed from the thoracic cavity en bloc with the heart and submerged in 10% PBS-buffered formalin for 24 h at 4 °C. The kidneys, and livers were also isolated and submerged into formalin. The fixed organs were transferred to the Stony Brook University Hospital Histology Laboratory for preparation of thin-sectioned slides stained with hematoxylin and eosin (H&E). Digital photos were taken using a Nikon Eclipse Ti inverted microscope with an Insight Spot2 digital camera using 20× and 100× objectives.

Preparation of lung homogenates

Single cell suspensions were prepared from whole lung homogenates at each time point after actin injection. Lungs were isolated and placed into a well in 24-well plate containing 1 ml of complete culture medium (RPMI 1640 + 10% FBS) and thoroughly minced into small pieces using surgical scissors. The minced tissue was transferred to a 15 ml conical tube containing 5 ml of fresh collagenase solution (1 mg/ml type IV collagenase, 25 U/ml DNase I, 5% FBS and the volume adjusted up to 5 ml with RPMI). The samples were then incubated at 37 °C for 45 min with shaking. After the incubation, the tissue was disaggregated into a cellular suspension by passing it through an 18-gauge needle 20 times. The samples were centrifuged at 500 × *g* for 5 min. The supernatant was aspirated and the pellet was resuspended in RBC lysis buffer and immediately centrifuged (50 × *g* for 5 min). The supernatant was saved and the pellet, containing large tissue aggregates,