Cellular Immunology 270 (2011) 103–113

Contents lists available at ScienceDirect

Cellular Immunology

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

RahU: An inducible and functionally pleiotropic protein in *Pseudomonas aeruginosa* modulates innate immunity and inflammation in host cells

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ARTICLE INFO

Article history:
Received 31 January 2011
Accepted 18 May 2011
Available online 24 May 2011

Keywords:
Innate immunity
Nitric oxide
Chemotaxis
Inflammation
Inflammation resolution

ABSTRACT

The aim of this study was to define the functional role of a recently identified RahU protein from *Pseudomonas aeruginosa* in macrophages and its role in bacterial defense. Recombinant (r)-RahU had no significant effect on cell apoptosis or cell viability in human monocytic THP-1 cells. Gene expression array of murine macrophage cells (RAW 264.7) stimulated with LPS showed modulation of common transcripts (by r-RahU and predisone) involved in inflammation. Functional cellular analysis showed RAW cells incubated with r-RahU at 1.0–10 μg/ml (0.06–0.6 μM) inhibited accumulation of nitric oxide (NO) in the presence of LPS by 10–50%. The IC_{50} of r-RahU (0.6 μM) was distinct from the known inhibitors of NO production: prednisone (50 μM) and L-NMMA (100 μM). r-RahU also significantly inhibited chemotactic activity of THP-1 cells toward CCL2 or chemotactic supernatants from apoptotic T-cells. These reports show previously unknown pleiotropic properties of RahU in modulating both microbial physiology and host innate immunity.

1. Introduction

Aegerolysins (PF06355), which have been previously discovered in fungi, bacteria and plants, are highly similar proteins with different biological properties. These include ostreolysin (*Pleurotus ostreatus*), Asp-hemolysin (*Aspergillus fumigatus*), hemolysins from the basidiomycete (*Moniliophthora perniciosa*), and terrelysin from *Aspergillus terreus* [1–8]. Although the putative biological role of fungal aegerolysins is suggested to be similar to bacterial aegerolysins [1,8,9], the functional role of microbial aegerolysin-like proteins in eukaryotic systems remains elusive [1]. A gene (PA0122) product of *Pseudomonas aeruginosa* belongs to the aegerolysin family of proteins [10] and is designated as RahU in this and adjoining manuscript. The present reports further investigated the role of recombinant (r)-RahU to understand host–bacteria interactions at the functional genomics and cellular level. The rationales of these studies include: (a) surface plasmon resonance revealed that r-PA0122 (r-RahU) binds with high affinity 1.36×10^{-9} M to oxidized-low density lipoprotein (Ox-LDL) and to synthetic C:6 lysophosphatidylcholine (lysoPC) at 2.94×10^{-5} M, a major subcomponent of Ox-LDL, but not to the LDL [10]; (b) oxidation of LDL is promoted mainly by macrophages and endothelial cells within the subendothelial extracellular matrix; (c) RahU protein was associated with inner membrane and also secreted into the extracellular medium [10], and we believe that it may form a modified ligand after combining with free or cellular bound oxidized phospholipids (Ox-phospholipids) and may affect the cellular event; and (d) RahU or the modified ligands may bind and signal *via* scavenger and/or pattern recognition receptors including TLRs on macrophages. In the accompanying report we show that RahU functions as a sensing apparatus in *P. aeruginosa* to distinguish different forms of host-derived Ox-phospholipids, which may also

Abbreviations: LysoPC, lysophosphatidylcholine, 1-hexanoyl-2-hydroxy-sn-glycero-3-phosphocholine; Ox-LDL, oxidized-low density lipoprotein; LDL, low density lipoprotein; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemotactic protein-1; MCAF, monocyte chemotactic and activating factor; CCL2, chemokine (C–C motif) ligand 2; IMAC, immobilized metal affinity chromatography; PK, proteinase K; L-NMMA, NG-Monomethyl-arginine; NO, nitric oxide; LB, Luria–Bertani medium; RahU, a sanskrit word for "the seizer"; Asp-hemolysin, *Aspergillus*-hemolysin protein; VEGF, vascular endothelial growth factor; PGF, placental growth factor; PHSCA, *Pseudomonas*/host signal and communication apparatus.

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doi:10.1016/j.cellimm.2011.05.012

interact with host cells during host–bacterial interactions in the microenvironment. Together, these observations and other reports provide several examples of ligands or classes of ligands and bacterial products that share seemingly dissimilar components of the innate immune system, and they raise intriguing questions about functional interactions between these molecules. In the present report we focused on the functional impact of r-RahU on model cell lines of macrophages and human monocytes.

Host innate immune systems are well conserved in evolution [11]. They consist of intracellular and extracellular signaling mechanisms that identify, respond to, and defend the host from a broad range of infections. The major functions of the vertebrate innate immune system include, but are not limited to: (a) activation of inflammatory cells and complement; production of cytokines, chemokines, nitric oxide, and other reactive oxygen molecules; promotion of clearance of dead and apoptotic cells [12]; and (b) removal of foreign substances or pathogens; chemotaxis of inflammatory cells; and activation of adaptive immune system [13]. *P. aeruginosa* is an opportunistic pathogen causing chronic lung infections in cystic fibrosis (CF) patients, those hospitalized with urinary tract infections, in wounded or immune compromised patients, and in burn victims [14–17]. *P. aeruginosa* expresses numerous virulence factors, such as flagellum, pilus, LPS and secretory factors including extracellular products such as type III secretary proteins, quorum sensing molecules and alginate [18].

The host response to *P. aeruginosa* infections involves cells in the local environment, such as airway epithelial cells, macrophages and monocytes, neutrophils and lymphocytes, which release mediators that enable mounting of an attack on the invading bacteria [13,19,20]. These include macrophages that engulf and destroy the bacteria through the generation of a "respiratory burst", causing the release of reactive oxygen species such as nitric oxide and hydrogen peroxide [21,22]. However, Vishwanath et al. (1988), suggested that inhibition of phagocytosis by the leukocytes may be contributed by a defect in uptake and/or destruction of mucin-coated bacteria [23]. It should be noted that macrophages have roles in both the innate and adaptive immune response to infection [24]. This is well documented in depletion of lung macrophage in mice, delayed neutrophil recruitment and chemotactic production, and delayed bacterial clearance as compared to controls [25]. Furthermore, macrophages have been reported to restrict *P. aeruginosa* growth, regulate neutrophil influx and balance pro and anti-inflammatory cytokines in BALB/c [13]. Activation of macrophages promotes the recruitment of other cells such as T cells to the site of inflammation and/or infection [26].

The experiments in the present study show a global impact of RahU on macrophage gene expression that is shared with "oral anti-inflammatory compounds" such as prednisone. r-RahU from *P. aeruginosa* also interferes in innate immunity by inhibiting nitric oxide production and chemotaxis of monocytes and/or macrophages. Together, these studies demonstrate a dual role of RahU (in host and bacteria), which also bridges and positions itself to cross-communicate multiple functions in host–bacterial interactions.

2. Materials and methods

2.1. Cell lines and reagents

RAW 264.7 cells, a mouse macrophage cell line, human monocytic (THP-1) cells, and the human Jurkat T cell line (clone E6-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Isopropyl β-D-1-thiogalactopyranoside (IPTG), Griess kit, and CellTiter-Glo™ were procured from Promega (Madison, WI). BD Talon his-tag protein purification kit was purchased from BD Bioscience (Bedford, MA). iScript One-step-RT-PCR kit, Criterion SD-PAGE and blotting systems, iQ5 Real-Time system, Coomassie Brilliant Blue R-250, Immuno-Star HRP Chemiluminescence kit, and Bradford reagent were acquired from Bio-Rad (Hercules, CA). Lipopolysaccharide (LPS; L3024), which was extracted from *Escherichia Coli* 0111:B4 and purified by ion-exchange chromatography, was purchased from Sigma–Aldrich (St. Louis, MO). Arachdoinc acid, prednisone, sodium deoxycholate, proteinase K–acrylic beads, *Tritirachium album* and L-NMMA were purchased from Sigma–Aldrich. All the tissue culture media, fetal bovine serum (HyClone), L-glutamine solution, phosphate buffered saline (PBS) pH 7.4, Detoxi-Gel™ Endotoxin Removing Gel, tissue culture cellBIND coated multi-well plates and tissue culture antibiotics were procured from Lonza Biowhittaker/Costar from (Thermo-Fisher) (Pittsburgh, PA). Recombinant human MCP-1/MCAF (CCL2) was purchased from PeproTech, (Rocky Hill, NJ). NanoDrop was from Thermo-Fisher, and 1450 Microbeta Trilux luminometer was acquired from Wallac (Ramsey, Minnesota). The RNAeasy kit from Qiagen (Valencia, CA) was used for RNA isolation and purification.

2.2. Isolation and purification of recombinant-RahU protein (r-RahU) from E. coli

The cloning and expression of r-RahU protein in *E. coli* has been previously described [10]. *E. coli* was transformed with pET28b-RahU-his cloned plasmid to express r-RahU, with his-tag at C-terminus (data not shown). Briefly, a single colony of NovaBlue (DE3) containing pET28b-RahU-his was inoculated into Luria-broth (LB) and grown to OD_{600} = 0.5. The cells were induced with 1 mM IPTG, harvested after 4 h upon reaching OD_{600} = 1.0 and frozen at −80 °C until further use. Purification of his-tagged r-RahU protein was performed twice using a BD TALON immobilized metal affinity chromatography (IMAC) resin, as recommended by the manufacturer. Briefly, the cell lysate was mixed with IMAC, loaded onto a column and washed extensively with the buffer. The r-RahU-his protein was eluted with imidazole and each fraction was analyzed with Coomassie Brilliant Blue R-250 staining on SDS–PAGE gels. The 16 kDa purified r-RahU-his protein was quantified by Bradford assay. Fraction(s) having a single 16 kDa protein were further purified and reassessed by repeating the above affinity purification protocol and SDS–PAGE-Coomassie Brilliant Blue R-250 staining. All purified fractions representing a single 16 kDa r-RahU band were pooled and were also subjected to Western blot analysis using a specific anti-r-RahU (r-PA0122) antibody [10]. Pre-stained Kaleidoscope molecular weight markers were used in the SDS–PAGE gels and Western blot analysis.

2.3. Preparation of endotoxin-free r-RahU protein

Immobilized polymixin B-agarose (Detoxi gel) was used according to manufacturer's recommendation and of which removed 99.99% EU (endotoxin units). Briefly, one ml pre-loaded column was washed with five volumes of 1% sodium deoxycholate followed by 10 volumes of PBS (pH 7.4). One ml of affinity purified r-RahU at ~1 mg/ml was loaded onto a Detoxi column and incubated at room temperature for 1 h. The column was then eluted with PBS to exclude residual contaminated endotoxin. The fractions were analyzed by SDS–PAGE and stained with Coomassie Brilliant Blue R-250. Pooled fractions were quantified by Bradford assay. Only the fraction which showed a 16 kDa r-RahU band was pooled for further studies.

2.4. SDS–PAGE and Western blotting

Ten micrograms of r-RahU protein was loaded on 8–16% SDS–PAGE using the Criterion gel system from Bio-Rad. Separated strips