Developmental and Comparative Immunology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

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

Shrimp hemocytes release extracellular traps that kill bacteria

Tze Hann Ng, Sheng-Hsiung Chang, Miao-Hsien Wu, Han-Ching Wang *

Institute of Biotechnology, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan 701, Taiwan

ARTICLE INFO

Article history:
Received 3 June 2013
Revised 21 June 2013
Accepted 22 June 2013
Available online xxxx

Keywords:
Shrimp
Hemocytes
Extracellular traps

ABSTRACT

Extracellular traps (ETs) are formed from the DNA, histones and cytoplasmic antimicrobial proteins that are released from a range of vertebrate immune-cells in response to pathogenic stimulation. This novel defense mechanism has not been demonstrated in invertebrates. In this study, we investigated the formation of ETs in the crustacean *Litopenaeus vannamei*. We found that stimulation of shrimp hemocytes with phorbol myristate acetate (PMA), lipopolysaccharide (LPS) and live *Escherichia coli* all led to the formation of the characteristic ET fibers made from host cell DNA. After *E. coli* stimulation, we found that histone proteins were co-localized with these extracellular DNA fibers. The results further showed that *E. coli* were trapped by these ET-like fibers and that some of the trapped bacteria were permeabilized. All of these results are characteristic of the ETs that are seen in vertebrates and we therefore conclude that shrimp are also capable of forming extracellular traps.

1. Introduction

As part of the innate mammalian immune system, neutrophils were originally thought to use two strategies against pathogenic invaders: phagocytosis and release of antimicrobial components via degranulation. However, in 2004, Brinkmann et al. (2004) showed that neutrophils release extracellular traps (ETs) and that these neutrophil extracellular traps (NETs) are a novel form of immune response. Subsequently, in addition to neutrophils, various other vertebrate cell types from a range of animals have been shown to form ETs in response to immuno-stimulation (Chow et al., 2010; Goldmann and Medina, 2012; von Köckritz-Blickwede et al., 2008; Yousefi et al., 2008). This mechanism is now thought to be a general immune response, although the ETs that arise from different cell types each have their own unique characteristics.

ETs are composed of DNA and antimicrobial proteins (Brinkmann et al., 2004), regardless of whether they are produced by neutrophils or other cell types. In most cases, the source of the released DNA is decondensed chromatin DNA from the nucleus (Goldmann and Medina, 2012; Guimarães-Costa et al., 2012; Papayannopoulos and Zychlinsky, 2009). In general, once ET formation is triggered, the activated cells enter a unique cell-death program, namely ETosis, which is distinct from necrosis or apoptosis (Fuchs et al., 2007). According to Fuchs et al. (2007), when ETosis is initiated, the membranes of the nucleus and cytoplasmic granules dissolve, which allows the contents of the nucleus to associate with the granular antimicrobial proteins. Eventually, after the disruption of the cell membrane, fibers formed from the chromatin DNA and granular proteins are released into the extracellular regions. Alternatively, in some cases, mitochondrial DNA can be used instead of nuclear DNA in a pathway that does not necessarily lead to cell death (Yousefi et al., 2008). The released ETs then entrap the invading microbes, prevent their dissemination, and kill the invaders with various ET-associated antimicrobial proteins, such as myeloperoxidase (MPO), elastase, defensins, and histone proteins (Brinkmann et al., 2004; Kraemer et al., 2011; Li et al., 2007).

Extracellular traps are triggered not only by Gram-positive bacteria, Gram-negative bacteria, fungi, parasites and viruses, but also in response to a number of PAMPs (pathogen associated molecular pattern) including lipopolysaccharide (LPS) (Brinkmann et al., 2004; Guimarães-Costa et al., 2009; Jenne et al., 2013; Saitoh et al., 2012). Various pharmaceuticals and host molecules can also trigger the signal pathways that lead to ET formation, such as phorbol myristate acetate (PMA; Brinkmann et al., 2004), interlukin-8 (IL-8; Brinkmann et al., 2004), interferon (IFN; Yousefi et al., 2008) and tumor necrosis factor alpha (TNFα; Wang et al., 2009).

To date, however, ETs have only been studied in vertebrates, and they have not been reported in any arthropod. In the present study, using *Litopenaeus vannamei* as an animal model, we therefore investigate the formation of ETs by shrimp hemocytes. We show that they can be triggered by immuno-stimulators and live bacteria, and further demonstrate that the shrimp hemocyte ETs kill the bacteria that they entrap.

2. Materials and methods

2.1. Experimental animals

Adult *L. vannamei* (Pacific white shrimp) were purchased from a local market in Tainan, Taiwan. The shrimp were kept in a 60 liter

* Corresponding author. Tel.: +886 6 2757575x65603 810.
E-mail address: wanghc@mail.ncku.edu.tw (H.-C. Wang).

0145-305X/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.dci.2013.06.014

aerated water tank system with constant salinity of 35 ppm at 23–26 °C for 2 days before the experiments.

2.2. Bacteria strain and growth conditions

Enhanced green fluorescent protein (EGFP)-expressing *Escherichia coli* were grown overnight in Luria Bertani (LB) medium with kanamycin (1 μg/μl) at 37 °C. The *E. coli* were then subcultured, induced for 3 h with IPTG (Isopropyl β-D-1-thiogalactopyranoside), and aliquots were plated on LB agar with kanamycin for the determination of colony forming units (CFU).

2.3. Collection and seeding of shrimp hemocytes

Hemolymph was collected from adult white shrimp using a 23 G needle syringe that contained an equal volume of cooled anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 7.5, osmolality 780 mOsm kg^{-1}) (Sritunyalucksana et al., 2001). A coverglass was placed at the bottom of each well of a 24-well plate, and 800 μl of 2× L-15 culture medium (Invitrogen) (2× Leibovitz's 15 [L-15] medium with 10% fetal bovine serum [FBS], 1% glucose, 0.005% NaCl) was added. The hemocytes in the hemolymph (~100 μl) were then seeded directly into the wells and allowed to incubate for 20–30 min at 25 °C before various immune stimulators were added.

2.4. Immune stimulation and cytochemical staining

After incubation, the resulting hemocyte monolayers on the coverglasses were treated with PBS that contained phorbol myristate acetate (PMA; 10 and 100 nM, Sigma–Aldrich), lipopolysaccharide (LPS; 0.1, 1.0 and 10 μg/ml, Sigma–Aldrich), or EGFP-expressing *E. coli* (1×10^5 CFU, 2.5×10^5 CFU and 5×10^5 CFU). Unstimulated cells (PBS only; PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) were used as controls. All experiments were carried out in duplicate.

To observe the cells and ETs, we used fluorescence staining. Briefly, at various time points, the hemocyte monolayers were gently washed with PBST (0.2% Tween-20 in PBS) and fixed with 4% paraformaldehyde. The stimulated or unstimulated hemocyte monolayers were counterstained with 4′-6′-diamidino-2-phenylindole dihydrochloride (DAPI, Vector Laboratories Inc.), and after being washed three times with PBST, the specimens were analyzed using a fluorescence microscope. SYTOX Orange was also used to observe the nucleic acids. For this procedure, cells were stained with SYTOX Orange (2 μM) for 15 min before washing, and this was followed by paraformaldehyde fixation.

In an additional series of experiments to investigate the ability of the shrimp hemocyte ETs to kill extracellular bacteria and virus, the hemocyte monolayers were treated with *E. coli* (5×10^5 CFU) or purified WSSV (White spot syndrome virus, 0.03, 0.3, 1.5 and 3.0 μg, with 94,097 WSSV copies/μg as measured using a real-time PCR kit [IQ-Real]) virions for 2 h at room temperature. The hemocytes were then observed using immunofluorescence staining or Scanning electron microscopy (SEM) analysis.

For the immunofluorescence staining, the hemocyte monolayers were permeabilized with cold acetone for 3 min after fixation. The hemocyte monolayers were blocked with 0.1% goat serum in PBST for 4 h at room temperature and then incubated overnight at 4 °C with primary anti-Histone H1 antibody (Millipore). After washing with PBST, the cells were reacted for 5 h at room temperature with carboxymethylindocyanine (Cy3)-conjugated goat anti-mouse IgG antibody (Sigma–Aldrich). The hemocyte monolayers were washed again with PBST and counterstained using DAPI for DNA detection. Specimens were analyzed using a Carl Zeiss LSM780 confocal laser scanning microscope.

2.5. Scanning electron microscopy (SEM)

For SEM analysis, the hemocyte monolayers treated with *E. coli* or WSSV were fixed with 2.5% glutaraldehyde for 2 h and then washed by putting the glass cover slips upside down on a drop of water. The hemocytes were then postfixed using repeated incubations with 0.5% osmium tetroxide/0.5–1% tannic acid for 30 min, dehydrated with a graded ethanol series, critical-point dried for 1 h, and coated with a 1–2 nm layer of platinum using a thin layer evaporator for 60–90 min. Specimens were analyzed in a high resolution field emission scanning electron microscope (HR FE-SEM, JEOL JSM6700F).

3. Results

3.1. Shrimp hemocytes treated with PMA formed extracellular traps

To determine if shrimp hemocytes can produce extracellular traps (ETs) similar to those produced by mammalian neutrophils (Behrendt et al., 2010; Brinkmann et al., 2004), we treated hemocytes with PMA, which strongly stimulates ET formation by activating the protein kinase Cs (PKCs) signal pathway. Fig. 1A shows that neutrophil extracellular trap-like (NET-like) structures were formed by shrimp hemocytes after PMA stimulation. SYTOX Orange staining confirmed that the fibers of these NET-like structures consisted mainly of DNA, which is the major structural component of the extracellular traps (Fig. 1A). PMA treatment stimulated the release of fibers in a dose-dependent manner, with more ETs being produced at the highest dose of PMA (100 nM). Fig. 1 also shows that the ETs were sometimes directionally aligned, but the reason for this alignment is not presently known.

3.2. Shrimp extracellular traps were also triggered by LPS

It is already known that NETs can be triggered by LPS (lipopolysaccharide), a PAMP associated with Gram-negative bacteria (McDonald et al., 2012), and here we found that LPS also stimulates the production of shrimp hemocyte ETs. As Fig. 1B shows, most of the healthy unstimulated hemocyte nuclei are round and there is no evidence of fibers, but stimulation with increasing doses of LPS for 1.5 h leads to increased ET formation. At the lower concentrations (0.1 and 1.0 μg/ml) ET formation also increased over time (i.e. for 1.5–3 h), but after 3 h at the highest dosage (10 μg/ml), the ETs were evidently degraded (Fig. 1B).

3.3. Shrimp extracellular traps catch bacteria

Neutrophils release NETs much faster and in larger quantities when treated with live parasites (Behrendt et al., 2010) or bacteria (Fuchs et al., 2007) than with PMA or an immune stimulator such as LPS. In the present study, we therefore investigated the effect of treating shrimp hemocytes with live EGFP-expressing *E. coli*. As Fig. 1C shows, stimulation with increasing CFU of EGFP-expressing *E. coli* for 1 h leads to increased ET formation. When hemocytes were exposed to the highest concentration of *E. coli* (5×10^5 CFU) they released delicate strands of fiber within 30 min, and increasing quantities of bacteria were trapped by these fibers at least through to 120 min (Fig. 1D). DAPI staining confirmed that the fibers were formed mainly from DNA.

Please cite this article in press as: Ng, T.H., et al. Shrimp hemocytes release extracellular traps that kill bacteria. Dev. Comp. Immunol. (2013), http://dx.doi.org/10.1016/j.dci.2013.06.014