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Vitellogenin mediates phagocytosis through interaction with FcγR

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ABSTRACT

Vitellogenin (Vg), once reported to be a female-specific protein, has been identified in both male and juvenile fishes. However, the biological significance of the production of Vg in the male and juvenile fishes is elusive. Our previous studies showed that Vg is an opsonin capable of enhancing phagocytosis, but the mechanism by which Vg mediates phagocytosis is unknown. In this study we demonstrated that Vg-opsonized phagocytosis was characterized by pseudopod extension and depended upon tyrosine kinase. In contrast, inhibition of Rho family proteins and microtubule depolymerization had little effects on Vg-opsonized phagocytosis. Besides, Vg-opsonized phagocytosis was substantially blocked by monoclonal antibodies against FcγRs but not by CR3 antibody. Moreover, theoretical prediction analysis further revealed that Vg had the potency to interact with Fcγ receptors. Finally, the expression of proinflammatory cytokine genes *tnf-α* and *il-1β* was significantly up-regulated by Vg, and this up-regulation was inhibited by selective inhibitors of FcR signaling pathways, wortmannin and piceatannol. Taken together, these results suggest that Vg plays an IgG-like role in that it activates FcγR-mediated phagocytosis, thus establishing an antibody-like function for Vg for the first time.

1. Introduction

Phagocytosis is the process by which cells recognize and engulf large particles (usually >0.5 μm in diameter) and plays a crucial role in host defense against invading microorganisms. It was first identified in starfish larvae by Elite Metchnikoff over a century ago (May and Machesky, 2001), and is found nowadays to be highly conserved in organisms ranging from unicellular eukaryotes to higher animals. Phagocytosis in higher organisms is fulfilled by a specialized subset of cells, named "professional" phagocytes including macrophages, neutrophils and monocytes (Rabinovitch, 1995).

Phagocytosis is initiated by the interaction of opsonins, which coat the particles to be engulfed, with specific receptors on the surface of the phagocyte. Two of the best characterized phagocytic receptors in macrophages are the Fc gamma receptors (FcγRs) and the complement receptor 3 (CR3), which recognize immunoglobulin G (IgG)- and C3bi-coated targets, respectively. Phagocytosis by both types of receptors is driven by the reorganization of filamentous actin, leading to particle internalization, but the mechanisms of internalization appear to be different, resulting in several differences in morphology, signaling requirements, and inflammatory responses (Allen and Aderem, 1996a,b; Newman et al., 1991). Broadly speaking, FcγR-mediated phagocytosis, known as type I phagocytosis, is characterized by pseudopod extension, tyrosine kinase dependence, activation of respiratory burst (to produce reactive oxygen species) and release of cytokines such as TNF-α, while CR3-mediated phagocytosis, known as type II phagocytosis, is characterized by sinking of particles into the cytoplasm, protein kinase C (PKC) dependence, requirement for intact microtubules and the absence of an inflammatory response (Kwiatkowska and Sobota, 1999). The requirements for guanosine triphosphatases (GTPases) have also been shown to be different: FcγR-mediated phagocytosis is a Rac- and Cdc42-dependent process, whereas CR3-mediated phagocytosis is dependent upon Rho and Rap1 (Caron and Hall, 1998).

Recently, accumulating data demonstrate that some soluble innate immune proteins such as ficolins and collectins structurally and functionally resemble the antibodies of the adaptive immune system (Litvack and Palaniyar, 2010; Palaniyar, 2010). The pentraxins, C-reactive protein (CRP) and serum amyloid P component (SAP) are soluble innate immune proteins involved in acute phase response in mammalian species. Both CRP and SAP have been shown to mediate type I phagocytosis through interactions with

Abbreviations: CR3, the complement receptor 3; CRP, C-reactive protein; FcγRs, Fc gamma receptors; FITC, fluorescein isothiocyanate; GTPases, guanosine triphosphatases; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PA, phagocytic ability; PGN, peptidoglycan; PI, phagocytic index; PKC, protein kinase C; PTK, protein tyrosine kinase; qRT-PCR, quantative real-time PCR; SAP, serum amyloid P component; TEM, transmission electron microscopy; Vg, vitellogenin.

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FcγRs (Bharadwaj et al., 2001; Mold and Du Clos, 2006; Mold et al., 2001), playing antibody-like functions in the FcγR pathway (Lu et al., 2008).

Vitellogenin (Vg), the precursor of yolk proteins in all oviparous organisms including fish, is traditionally regarded as the yolk reserve of nutrients essential for growth and development via cleaving into the yolk proteins phosvitin and lipovitellin, stored in eggs (Finn and Fyhn, 2010). Recently, Vg has been shown to participate in immune defense of different hosts such as fish (Garcia et al., 2010; Shi et al., 2006; Tong et al., 2010), amphioxus (Zhang et al., 2005) and mosquito (Rono et al., 2010). It has been revealed to be a multivalent pattern recognition molecule capable of identifying non-self components including lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA) and glucan, and to act as an opsonin that can enhance macrophage phagocytosis (Li et al., 2008, 2009; Liu et al., 2009). However, the mechanisms through which Vg mediates phagocytosis remain to be determined. Moreover, the receptors with which Vg interacts and the signals involved in Vg-mediated phagocytosis are unknown. We therefore sought to address these issues in this study.

2. Materials and methods

2.1. Purification and characterization of Vg

Vg was purified from E_2-induced greenling *Hexagrammos otakii* and characterized as described previously (Li et al., 2008). The purity of purified Vg was determined by 7.5% native-PAGE, and its molecular weight measured by 4%, 6%, 7.5%, 10% and 12% native-PAGE, using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (66 kDa) as molecular weight standards. The purified protein was characterized by MALDI/TOF MS analysis.

2.2. Isolation of macrophages

The macrophages were prepared from the head kidneys of sea bass *Lateolabrax japonicus*. The head kidneys were dissected out of *L. japonicus*, washed five times with 10 mM PBS with 150 mM NaCl (pH 7.4) and cut into ~1 mm^3 pieces. The tissue pieces were soaked with Leibovitz's L-15 medium (L-15, GIBCO) containing 10% newborn calf serum (NCS; GIBCO) and gently passed though an 80 μm sterile steel mesh. The cell suspensions were again gently passed through 15 μm sterile steel mesh and loaded onto a discontinuous gradient consisting of 45% (v/v) Percoll (Solarbio) overlaid with 31% (v/v) Percoll. After centrifugation at 400 × *g* for 30 min at 4 °C, the macrophage fraction was collected from 31 to 45% interface. The macrophage cells were washed three times with L-15 containing 10% NCS, harvested by centrifugation at 200 × *g* for 5 min at 4 °C and adjusted to a density of 5×10^6 cells/ml with L-15 containing 10% NCS. The cell viability was assessed by trypan blue exclusion.

2.3. Labeling of Pichia pastoris with fluorescein isothiocyanate (FITC)

The yeast *P. pastoris* cells were suspended in 10 mM PBS with 150 mM NaCl (pH 7.4) and killed by boiling for 15 min. Aliquots of 1 ml *P. pastoris* suspension (10^8 cells/ml) were mixed with 100 μl of 1 mg/ml FITC (Sigma) in DMSO and incubated in the dark, with gentle shaking, at room temperature for 30 min, followed by six washes with PBS. The FITC-labeled yeast cells were adjusted to 1.25×10^8 cells/ml before use.

2.4. Assay for kinetics of phagocytosis

To determine the phagocytic kinetics, aliquots of 200 μl FITC-labeled *P. pastoris* suspension (1.25×10^8 cells/ml) were mixed with an equal volume of macrophage suspension (5×10^6 cells/ml). The mixtures were incubated at 25 °C in the dark and sampled at 5, 10, 15, 20, 30, 60, 90 and 120 min, respectively. After centrifugation at 200 × *g* at 4 °C for 5 min, the macrophages were fixed in 2% glutaraldehyde (200 μl) in 10 mM PBS and each time about 60 μl of the macrophage suspension was taken to make smears for microscopic examination under fluorescence microscope (Olympus BX51). For each sample, at least 100 macrophages were examined. The number of macrophages with phagocytosed microbes and the number of microbes in each macrophage were recorded. The phagocytic ability (PA) was defined as the percentage of macrophages with one or more engulfed microbes within the total cell population, and the phagocytic index (PI) was defined as the average number of *Pichia* in the macrophages with engulfed microbes.

2.5. Assay for effects of Vg on phagocytosis

Aliquots of 200 μl FITC-labeled *P. pastoris* suspension (1.25×10^8 cells/ml) were added to each Eppendorf tube, and centrifuged at 3000 × *g* for 5 min. The pelleted yeast cells were re-suspended in 200 μl of 100 μg/ml Vg and incubated at room temperature in the dark on a shaker for 45 min. After centrifugation at 3000 × *g* at 4 °C for 5 min, the pelleted cells were re-suspended in 200 μl of 10 mM PBS and mixed with 200 μl of macrophage suspension (5×10^6 cells/ml). The mixtures were incubated at room temperature in the dark for 90 min, shaking every 5 min. After centrifugation at 200 × *g* at 4 °C for 5 min, the macrophages pelleted were fixed in 2% glutaraldehyde, and processed as above. For control, phagocytosis assay was similarly performed in the presence of BSA instead of Vg or Tris buffer (pH 7.4) alone.

2.6. Transmission electron microscopy (TEM)

A total of 200 μl yeast suspension (1.25×10^8 cells/ml) with FITC-labeled *P. pastoris* cells, that had been pre-incubated with Vg or with Tris buffer, was mixed with 200 μl of macrophage suspension (5×10^6 cells/ml). The mixtures were incubated at 25 °C in the dark for 5 min, and centrifuged at 200 × *g* at 4 °C for 5 min. The macrophages pelleted were fixed in 2% glutaraldehyde and processed for TEM observation under a JEM-1200 EX electron microscope.

2.7. Inhibition of phagocytosis by various inhibitors

Pilot experiments had been performed to test the effects of different concentrations of inhibitors on Vg-opsonized phagocytosis by sea bass macrophages. Inhibition assays were carried out according to the methods of Allen and Aderem (1996b) and Le Cabec et al. (2002). In brief, aliquots of 200 μl macrophage suspension (5×10^6 cells/ml) were incubated with 50 μM (final concentration) lavendustin (Alexis) and 15 μM PP1, respectively, to inhibit tyrosine kinases, for 8 h, with 0.5 μM staurosporine (Alexis) to inhibit protein kinase C, for 10 h, with 100 μg/ml C3 exoenzyme to inhibit Rho family proteins, for 10 h, and with 4 μg/ml nocodazole to depolymerize microtubules, for 15 min, at room temperature. After centrifugation at 200 × *g* for 5 min, the macrophages pelleted were washed, resuspended in 200 μl L-15 medium and mixed with 200 μl yeast suspension (1.25×10^8 cells/ml) with FITC-labeled *P. pastoris* cells that had been pre-incubated with Vg or BSA. After incubation for 90 min at room temperature, the macrophages pelleted were fixed in 2% glutaraldehyde. Phagocytosis was observed as described above.