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Short communication

Carp thrombocyte phagocytosis requires activation factors secreted from other leukocytes

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ABSTRACT

Thrombocytes are nucleated blood cells in non-mammalian vertebrates, which were recently focused on not only as hemostatic cells but also as immune cells with potent phagocytic activities. We have analyzed the phagocytic activation mechanisms in common carp (*Cyprinus carpio*) thrombocytes. MACS-sorted mAb$^+$ thrombocytes showed no phagocytic activity even in the presence of several stimulants. However, remixing these thrombocytes with other anti-thrombocyte mAb$^-$ leukocyte populations restored their phagocytic activities, indicating that carp thrombocyte phagocytosis requires an appropriate exogenous stimulation. Culture supernatant from anti-thrombocyte mAb$^-$ leukocytes harvested after PMA or LPS stimulation, but not culture supernatant from unstimulated leukocytes, could activate thrombocyte phagocytosis. This proposed mechanism of thrombocyte phagocytosis activation involving soluble factors produced by activated leukocytes suggests that thrombocyte activation is restricted to areas proximal to injured tissues, ensuring suppression of excessive thrombocyte activation and a balance between inflammation and tissue repair.

1. Introduction

Thrombocytes are nucleated blood cells in non-mammalian vertebrates that are considered functional homologs of mammalian platelets. Platelets and thrombocytes share characteristics involved in hemostasis; in brief, they aggregate in response to several damage signals, including collagen, to initiate blood clotting (Belamarich et al., 1966; Nakayasu et al., 1997). In addition to their primary functions, platelets and thrombocytes are also important mediators of both the innate and adaptive immune systems (Elzey et al., 2003; Ferdous and Scott, 2015). Particularly, nucleated thrombocytes have a potential to become powerful immune cells owing to their phagocytic activities that kill internalized bacteria (Hill and Rowley, 1998; Nagasawa et al., 2014; Stosik et al., 2002). Many studies reported that mammalian platelets may have phagocytic activity; however, their ability to engulf microbes remains controversial (Antczak et al., 2011; White, 2006). On the other hand, nucleated thrombocytes express MHC class II molecules (Fink et al., 2015; Köllner et al., 2004; Nagasawa et al., 2014; St Paul et al., 2012), suggesting that they have the potential to become antigen-presenting cells (e.g., macrophages and dendritic cells), which present extracellular antigens. Elucidation of these phagocytic thrombocytes and platelets may be important for understanding immune systems, particularly in lower vertebrates.

Phagocytosis is the process of engulfing large particles into intracellular vacuoles, contributing to the elimination of pathogenic microbes. In professional phagocytes such as neutrophils and macrophages, phagocytosis is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) and opsonins such as immunoglobulins and complement components bound to the microbes, which are recognized by phagocytosis-promoting receptors (Jutras and Desjardins, 2005). These phagocytic abilities are also enhanced by several inflammatory cytokines such as interferon gamma (IFNγ) and tumor necrosis factor (TNF) produced by several immune cells (Shalaby et al., 1985). Similar regulatory mechanisms are also conserved in fish (Grayfer and Belosevic, 2009).

Platelet activation for hemostasis has been well studied and is induced by various factors, including collagen, adenosine diphosphate (ADP), and thrombin. Upon activation, platelets morph into a stellate form and secrete various factors to trigger coagulation and inflammation (Weyrich and Zimmerman, 2004). Furthermore, although nucleated thrombocytes are activated by collagen and other molecules to facilitate coagulation, fish thrombocytes responded differently, including being unresponsive to ADP (Belamarich et al., 1966; Matsushita et al., 2004). However, whether phagocytosis

Abbreviations: ADP, adenosine diphosphate; IFNγ, interferon gamma; LPS, lipopolysaccharide; mAb, monoclonal antibody; MACS, magnetic-activated cell sorting; PBL, peripheral blood leukocyte; PMA, phorbol 12-myristate 13-acetate.

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activation pathway is similar to typical hemostasis pathway remains unknown. For understanding the immune functions of thrombocytes, the activation mechanism for their phagocytic activity is required. In the present study, we evaluated activation mechanism of fish thrombocyte phagocytosis, including cell exposure to several stimulants and the influence of other leukocytes. Thrombocyte phagocytic activity was not induced by typical platelet stimulants, but was dramatically triggered by the presence of other leukocytes and by activated leukocyte cell culture supernatants. These results reveal mechanisms regulating thrombocyte phagocytosis in immune system and tissue maintenance.

2. Materials and methods

2.1. Fish

Common carp (*Cyprinus carpio*, approximately 100 g) were maintained in our laboratory at 25 °C and fed with commercial pellets. All animal experiments were performed in accordance with the guidelines of the Animal Experiments Committee at Kyushu University.

2.2. Thrombocyte isolation and purification

Carp peripheral blood samples were collected from caudal veins using heparinized syringes, diluted with medium (RPMI-1640; Nissui Pharmaceutical), and overlaid onto Percoll adjusted to a concentration of 1.08 g/ml (BD Biosciences). Samples were then centrifuged at $500 \times g$ for 30 min at 4 °C to isolate peripheral blood leukocytes (PBLs). PBLs were harvested from the top of the Percoll layer, washed twice with the medium by centrifugation at $500 \times g$ for 10 min at 4 °C, and adjusted to a concentration of 1×10^7 cells/ml with medium. The PBLs were then incubated with an HB8 monoclonal antibody (mAb) specific to carp thrombocytes (Nakayasu et al., 1997) for 30 min on ice. After washing with medium twice, the PBLs were incubated with MACS microbeads coupled to a goat anti-mouse IgG antibody (Miltenyi Biotec). After washing with medium twice, the PBLs were resuspended in RPMI-1640 containing 2 mM EDTA and 5% fetal bovine serum (FBS), and then loaded on a mini MACS column (Miltenyi) to purify the thrombocytes. Samples were passed through the column twice to obtain thrombocytes at high purity. Purified thrombocytes were stained with phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody (Sigma–Aldrich) for 30 min at 4 °C.

2.3. Thrombocyte stimulation

The purified thrombocytes (1×10^7 cells/ml) were incubated for 30 min at 25 °C with medium containing lipopolysaccharides (LPS from *Escherichia coli* O55, 10 μg/ml; Wako Pure Chemical), phorbol 12-myristate 13-acetate (PMA, 1 μg/ml; Sigma), collagen type I (1 μg/ml; Wako), ADP (1 μg/ml; Tokyo Chemical Industry), or thrombin (1 unit/ml; from bovine, Sigma). Next, after washing twice, the cells were resuspended in medium at the same concentration.

2.4. Preparation of leukocyte culture supernatants

HB8 mAb-negative ($HB8^-$) leukocytes purified with MACS microbeads were stimulated with either LPS or PMA in the same manner as described earlier, washed 3 times, adjusted to 5×10^7 cells/ml with medium, and then incubated for 60 min at 25 °C. The culture supernatants were collected by centrifugation at $1000 \times g$ for 10 min at 4 °C 3 separate times to remove all leukocytes.

2.5. Phagocytosis assay

Stimulated thrombocytes (1×10^7 cells/ml) were incubated with 1 μm fluorescent latex beads (Fluoresbrite Yellow Green Microspheres; Polysciences, Warrington, PA, USA) at a cell-to-bead ratio of 1:5 for 3 h at 25 °C in 100 μl of medium. To evaluate the effect of other leukocytes on activation, thrombocytes and $HB8^-$ leukocytes were mixed at 5×10^6 cells/ml each and incubated as previously described. To evaluate the effect of soluble factors produced by leukocytes, thrombocytes were resuspended in 50% $HB8^-$ leukocyte culture supernatant diluted in medium and incubated with fluorescent beads as previously described. Flow cytometry (Epics XL, Beckman Coulter) was used to measure the percentage of phagocytic thrombocytes in each experimental condition.

2.6. Bacterial phagocytosis

FITC-conjugated *E. coli* (DH5α strain) were incubated with 10% normal carp serum in PBS in the presence of 2 mM Mg^{2+} and 2 mM Ca^{2+} for 30 min at 25 °C. As a control, bacteria were incubated with heat-inactivated (20 min, 50 °C) carp serum containing 10 mM EDTA under the same conditions. After washing with PBS 3 times, the treated bacteria were adjusted to $OD_{600nm} = 0.5$ and incubated with the carp thrombocytes using the same conditions as described for the incubation with the beads. The percentage of phagocytic thrombocytes was measured by flow cytometry.

3. Results and discussion

3.1. Purified thrombocytes are not phagocytic

As previously described (Nagasawa et al., 2014), carp thrombocytes in peripheral leukocyte pool actively ingest latex beads. In the present study, purified thrombocytes were used to assess the direct influence of several different stimulants individually on thrombocyte phagocytic activity. Purified carp thrombocytes incubated with 1-μm beads in the same manner as PBL incubation rarely ingested the beads (2.1 ± 0.7%; Fig. 1A, *right*). The phagocytic capacity of thrombocytes in the blood leukocyte samples was verified just before cell sorting, indicating that the anti-thrombocyte mAb staining did not inhibit thrombocyte phagocytosis (23.9 ± 5.1%; Fig. 1A, *left*). Based on these findings, we hypothesized that thrombocyte phagocytosis may be triggered by activators derived from other leukocytes.

Next, we individually assessed the ability of several potential stimulants to enhance purified thrombocyte phagocytic activity. For this assessment, thrombocytes were preincubated with different chemicals before mixing with the beads. Although the thrombocytes were exposed to typical platelet stimulators such as collagen, ADP, bovine thrombin, LPS, and PMA, none of them enhanced the thrombocyte bead ingestion (Fig. 1B). Moreover, the effects of these stimulants on thrombocyte morphology were examined by microscopy. In the absence of these reagents, thrombocytes formed a typical spindle shape (see Fig. S1A). Upon incubation with collagen, thrombocytes immediately adhered to the culture plate (Fig. S1B). In the presence of PMA, known to induce platelet activation associated with CD62P expression (Baudouin-Brignole et al., 1997), thrombocytes assumed round or oval forms with many cells adhering to the culture plate (Fig. S1C). ADP only weakly induced similar morphological change of the thrombocytes (Fig. S1D). These results suggest that thrombocyte phagocytic activity is activated in a manner that is distinct from hemostatic aggregation. LPS and thrombin did not induce morphological changes in the thrombocyte (data not shown). Mammalian platelets express TLR4 to sense LPS, which does not induce a typical platelet activation and aggregation (Cognasse et al., 2005; Ward et al., 2005); however, LPS enhances the secretion of several cytokines in platelets and expression of them in chicken