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Human platelet FcγRIIA and phagocytes in immune-complex clearance

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ABSTRACT

In addition to their primary role in hemostasis and wound healing, platelets play important roles in a multitude of physiological functions including immune and inflammatory responses. We present data that platelets, by virtue of their expression of the human specific FcγR, FcγRIIA, bind IgG complexes *in vivo* and that circulating phagocytes from healthy individuals internalize platelets *in vivo*. Human platelets, as a consequence of their expression of FcγRIIA, may thus, contribute to the clearance of IgG-containing complexes from the circulation.

1. Introduction

Although platelets are best known as the primary mediators of hemostasis in humans and other mammals, they also play important roles in a multitude of other physiological functions (Klinger and Jelkmann, 2002). Platelets are rapidly deployed to sites of injury or infection and release proteins that kill certain bacteria and fungi. Platelets also modulate inflammatory processes by interacting with leukocytes and by secreting cytokines, chemokines, and other inflammatory mediators (Elzey et al., 2005; Semple and Freedman, 2010). In addition, their function has been linked with various pathological conditions, including atherosclerosis, arthritis and immune thrombocytopenic purpura (ITP) (Semple and Freedman, 2010; Cines and Blanchette, 2002; Gasparyan et al., 2010). Our demonstration that human platelets can bind and endocytose IgG complexes *in vitro* (Worth et al., 2006) suggested that platelets also participate in the clearance of IgG-containing complexes.

Immune complexes (IC) are present in the circulation of healthy individuals and the formation of such complexes is part of a normal immune process. Efficient clearance of IgG complexes can be critical because their deposition in tissues and organs can set off reactions that lead to inflammation and tissue damage (Jancar and Sánchez Crespo, 2005; Mayadas et al., 2009). For example, in some pathological conditions, including autoimmune diseases such as systemic lupus erythematosus and autoimmune glomerulonephritis significant amounts of immune complexes are formed and deposited in the kidney and other tissues, causing severe injury (Niederer et al., 2010; Bagavant and Fu, 2009).

FcγRIIA is the only Fcγ receptor expressed on platelets (Cassel et al., 1993; King et al., 1990). On professional human phagocytes such as monocytes and neutrophils, FcγRIIA plays an important role in the clearance of IgG immune complexes and the phagocytosis of IgG coated particles (McKenzie and Schreiber, 1994). Apart from the recognition that FcγRIIA is important for platelet activation by von Willebrand factor (Canobbio et al., 2001), the role of FcγRIIA in platelet function has not been well defined.

It is known that platelets from patients suffering from certain autoimmune and thrombocytopenic disorders have high levels of surface bound IgG. Platelets from normal donors also bind IgG immune complexes *in vivo*, albeit at lower levels (Romero-Guzmán et al., 2000; George, 1990; Christopoulos et al., 1993; Court et al., 1987). We present evidence that platelets of normal individuals can serve as vehicles for delivery of immune complexes for destruction by phagocytes and that circulating neutrophils/monocytes play a role in this process. We also demonstrate that in addition to the ability to ingest small IgG particulates (heat aggregated IgG, Worth

Abbreviations: HA-IgG, heat aggregated IgG; Mono-IgG, monomeric IgG; MF, mean fluorescence; TG, transgenic; WT, wild type.

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et al., 2006), human platelets can internalize larger IgG coated particles by a process similar in some respects to phagocytosis in leukocytes.

2. Materials and methods

2.1. Reagents

PE labeled $F(ab)'_2$ fractions of goat anti-rat IgG, goat anti-human IgG, goat anti-mouse IgG and FITC labeled human IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Mouse anti-human CD61 was obtained from BD Biosciences (San Jose, CA). Fab fractions of mAb anti-human FcγRII (IV.3) were prepared in our laboratory. Monomeric human IgG (mono-IgG) was prepared by ultracentrifugation of human IgG (MP Biomedicals, OH) at 80 K for 15 min. Heat aggregated human IgG (HA-IgG) was prepared by heating human IgG (10 mg/ml) in PBS at 62 °C for 20 min, followed by centrifugation at 12,000 rpm for 10 min to remove insoluble aggregates. The final IgG complex was used at 100 μg/ml as assessed by absorbance at 280 nm.

2.2. Animals and cell lines

FcγRIIA transgenic mice were provided by Dr. Steven E. McKenzie (Thomas Jefferson University, Philadelphia, PA). All protocols were performed in accordance with National Institutes of Health guidelines and with the approval by the University of Pennsylvania Animal Use Committee. A COS cell line stably expressing FcγRIIA (COSIIA) was constructed in our laboratory as previously described (Huang et al., 2004).

2.3. Isolation of human and mouse platelets

Platelet-rich plasma was prepared from heparinized venous blood of healthy volunteers by centrifugation of the blood at 900 rpm (175 × *g*) at room temperature. Platelets were isolated by gel filtration on Sepharose 2B. A similar protocol was used to isolate platelets from WT and FcγRIIA TG mice.

2.4. Internalization of IgG coated beads

Streptavidin conjugated polystyrene beads (0.1, 0.5 and 1.0 μm) (Bangs Laboratories, Inc., Fishers, IN) were coated with biotin/anti-biotin mAb according to the manufacturer's instructions. The IgG-coated beads were allowed to bind (45 min) to platelets at 4 °C. One set of cells was maintained at 4 °C as a binding control. Other cells were warmed to 37 °C for 20 min to allow internalization of bound IgG-coated beads and then returned to 4 °C to stop internalization. Goat anti-mouse IgG conjugated to phycoerythrin (PE) was added to label remaining externally bound IC polystyrene beads and cell fluorescence was analyzed by flow cytometry. The cells maintained at 4 °C retain a large amount of IC on the surface and display bright fluorescence. However, cells incubated at 37 °C that have internalized some of the surface bound IgG-coated beads display less fluorescence. Internalization is defined as the % loss of surface expression (fluorescent label). For each condition, one set of cells was pre-incubated with cytochalesin D (CytD) and CytD was also present during the uptake assay.

2.5. Isolation of human monocytes and neutrophils

Heparinized venous blood collected from healthy volunteers was overlaid on Ficoll-Hypaque (LSM; Organon-Teknika, West Chester, PA) and centrifuged at 2200 rpm for 30 min. Pelleted granulocytes were cleared of residual erythrocytes by brief hypertonic lysis and resuspended in RPMI. Mononuclear cells from the interface were washed, suspended in RPMI medium (GIBCO, Grand Island, NY) plus 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), and incubated at 37 °C on serum-coated flasks for 45 min to adhere monocytes. Nonadherent cells were removed by washing with RPMI.

2.6. Internalization of human platelets by human monocytes and neutrophils: analysis by flow cytometry

Gel filtered human platelets were incubated with HA-IgG for 30 min at 0 °C. The IgG-coated platelets were washed and then incubated with human monocytes or neutrophils on ice for 45 min. One aliquot was maintained at 0 °C for an additional 30 min for use as a binding control. The other aliquot was rapidly warmed and incubated at 37 °C for 30 min to allow internalization of the HA-IgG platelet complexes. Both sets of cells were washed with ice-cold PBS and further incubated for 30 min on ice with 3.5 μg/ml phycoerythrin (PE) conjugated $F(ab)'_2$ goat anti-human IgG to label surface bound HA-IgG platelet complexes. Cells were washed again with ice-cold PBS before analysis by flow cytometry. The mean fluorescence (MF) of cells maintained on ice throughout the procedure was used as the binding reference (no phagocytosis) (Van de Winkel and Capel, 1993). In other experiments, monocytes were exposed to platelets labeled with FITC-conjugated HA-IgG. Under these conditions, FITC fluorescence represents both surface-bound and intracellular HA-IgG platelet complexes.

2.7. Electron microscopy

Monocytes, isolated as described above, were washed and fixed with 2% gluteraldehyde at 4 °C overnight. The samples were then washed extensively with cacodylate buffer, fixed with osmium tetroxide, dehydrated, embedded in Spurr's resin and sectioned (Worth et al., 2006). The sections were viewed at the EM core facility at University of Pennsylvania.

3. Results and discussion

3.1. Human platelets from healthy donors bind IgG in vivo

The binding of IgG to the surface of normal human platelets *in vivo* is here illustrated using flow cytometry (Fig. 1A). Gel filtered human platelets were treated with fluorescence-labeled antibody directed to human IgG [PE-labeled $F(ab)'_2$ anti-human IgG]. The large increase in fluorescence intensity of these platelets compared to platelets treated with control antibody (PE labeled $F(ab)'_2$ anti-rat IgG) or saline indicates the presence of surface bound human IgG.

It is known that FcγRIIA, the only FcγR expressed on platelets, binds IgG complexes (McKenzie and Schreiber, 1994). Since platelet FcγRIIA encounters circulating monomeric IgG as well as IgG complexes *in vivo*, we examined whether platelet FcγRIIA can bind IgG complexes in the presence of physiologic levels of monomeric IgG (Fig. 1B). The mean fluorescence intensity of platelets treated with monomeric IgG (10 mg/ml) before exposure to heat-aggregated IgG (HA-IgG) is equivalent to that of platelets pretreated with saline, indicating that monomeric IgG does not interfere with the binding of HA-IgG complexes to platelet FcγRIIA. Fig. 1B also demonstrates that incubation of the platelets with mAb IV.3 (which binds to the ligand binding site of FcγRIIA) before exposure to human HA-IgG diminishes fluorescence intensity, illustrating that access to FcγRIIA is required for IgG complex binding to platelets.

The inability of monomeric IgG to interfere with platelet FcγRIIA binding to IgG complexes is a consequence of the low affinity of