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Annexin A3 plays a role in cytoplasmic calcium oscillation by extracellular calcium in the human promyelocytic leukemia HL-60 cells differentiated by phorbol-12-myristate-13-acetate

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ABSTRACT

The roles of annexin A3 (ANXA3) in macrophages are not fully understood. In contrast to C5a, we have demonstrated that C-terminal ribosomal protein S19 (RP S19)-tagged S-tagged C5a (S-tagged C5a/RP S19) raises an alternative cytoplasmic calcium oscillation by extracellular calcium during macrophage migration into apoptotic cells. We here differentiated human promyelocytic leukemia HL-60 cells bearing with either control sense RNA and shRNA for ANXA3 mRNA or a vector cDNA with or without ANXA3 cDNA into macrophage-like cells by phorbol-12-myristate-13-acetate and found that a fluorescence ratio (340 nm/380 nm) upon the S-tagged C5a/RP S19-induced alternative cytoplasmic calcium oscillation by extracellular calcium was an equilateral association with a dose of ANXA3. Moreover, the ANXA3-dependent modification was partially reflected upon the S-tagged C5a-induced classical cytoplasmic calcium oscillation by both intracellular calcium and extracellular calcium. ANXA3 seems to extend the C5aR-mediated cytoplasmic calcium oscillation by extracellular calcium at least in the HL-60 macrophage-like cells.

1. Introduction

Annexins (ANXs) function not only as an inhibitor for calcium-dependent cytosolic phospholipase A2 (cPLA2) but also as an organizer between calcium signaling and lipid signaling during remodeling of phospholipid bilayer in cell migration (Bandorowicz-Pikula et al., 2012). ANXA2 expressed in osteoblasts of the bone marrow interacts with stromal cell-derived factor for localization and homing of hematopoietic stem cells via CXCR4 belongs to Gi protein-coupled receptor (GiPCR) (Jung et al., 2011). A tetramer form of ANXA2 attracts human monocyte-derived macrophages via toll-like receptor 4 (Maratheftis et al., 2007). Conversely, ANXA3 is mainly expressed in macrophages (Le Cabec et al., 1992). However, a role of ANXA3 in macrophage migration is unclear.

The *C5a receptor* (*C5aR*) gene, which generates no mRNA splice variants, encodes the C5aR protein, which belongs to GiPCR (Gerard et al., 1993). In the case of C5a, phospholipase Cβ2 (PLCβ2) classically interacts with the Gβγ subunits in a calcium-dependent manner for making phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphates (IP3) and diacylglycerol (Cathcart, 2009). However, the source of calcium to interact with PLCβ2 is still unclear. After the binding of IP3 to the IP3R expressed on endoplasmic reticules intracellular calcium is released and calcium-released activation of the calcium channel expressed on the plasma membrane is opened for activation of protein kinase C (Mollapour et al., 2001). Both phosphatidylinositol 3-kinase signal and extracellular-signal-regulated kinase 1/2 (ERK1/2) signal are transmitted through small GTP binding protein Ras and mitogen-activated protein kinase (MAPK) kinase Raf. In the interval, cPLA2 activated by extracellular calcium through extracellular calcium through calcium-released activation of the calcium channel hydrolyses arachidonic acid for extension of cytoplasmic calcium oscillation by extracellular calcium. The above classical cytoplasmic calcium oscillation is believed to be crucial for the C5a-induced macrophage migration via the monocyte C5aR.

We have demonstrated that a ribosomal protein S19 (RP S19) polymer produced by apoptotic cells functions either as the monocyte C5aR agonist for an induction of macrophage migration or as the neutrophil C5aR antagonist for an inhibition of neutrophil migration (Nishiura et al., 2011b). The diametrically opposite mechanisms are suggested to be one of the phagocytic cell clearance systems in cell homeostasis (Lauber et al., 2004). To understand the nature of the RP S19 polymer,

Abbreviations: ANXA3, annexin A3; BEL, 4-bromoenol lactone; C5aR, C5a receptor; iPLA2, calcium-independent phospholipase A2; cPLA2, calcium-dependent cytoplasmic phospholipase A2; GPCR, G protein-coupled receptor; HL-60 macrophage-like cells, differentiated from human promyelocytic leukemia HL-60 cells by the addition of phorbol-12-myristate-13-acetate; ACA, N-(p-amylcinnamoyl) anthranilic acid; RP S19, ribosomal protein S19.

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we have found that L_{131}DR and the C-terminus (I_{134}AGQVAAANKKH$_{145}$) moiety of RP S19 display binding and migration inhibitory properties against the neutrophil C5aR (Shibuya et al., 2001; Shrestha et al., 2003). Therefore, an S-tagged C5a/RP S19 complex harboring both the mutant Gly73Asp-C5a and the RP S19 C-terminus moiety results primarily in macrophage infiltration (Nishiura et al., 2010a; Oda et al., 2008).

By using S-tagged C5a/RP S19, we recently showed that the monocyte C5aR-mediated alternative cytoplasmic calcium oscillation by extracellular calcium was inhibited via the catalysis of the ADP-ribosylation of the Gα subunit (pertussis toxin), a p38MAPK inhibitor (SB203580), a non-specific PLA_2 inhibitor (N-(p-Amylcinnamoyl) anthranilic Acid (ACA)), non-specific calcium and/or magnesium channel inhibitors (2-aminoethoxydiphenyl borate (2-APB) and $MgCl_2$) in the human promyelocytic leukemia HL-60 cells differentiated by phorbol-12-myristate-13-acetate (the HL-60 macrophage-like cells) (Nishiura et al., 2011b). In this paper, we assessed a contribution of ANXA3 with cytoplasmic calcium oscillation by extracellular calcium during the C5aR-mediated macrophage migration.

2. Methods

2.1. Vectors and HL-60 transformations

ANXA3 cDNA was prepared by RT-PCR from total mRNA template isolated from human hepatocellular liver carcinoma HepG2 cells. The ANXA3 cDNA was inserted into a pAcGFP1-C1 vector (Clontech, CA, USA) and a CSII-EF-MCS vector (RIKEN, Tsukuba, Japan). For the ANXA3 mRNA knockdown, we prepared control sense RNA and shRNA oligoribonucleotides (Table 1). They were inserted into the pENTR4-H1 vector. H1-shcontrol and H1-shANXA3 cassettes were replaced with the RfA region in the pCS-RfA-EG vector by LR reaction (Invitrogen, CA, USA).

HL-60 cells were obtained from the RIKEN BioResource Center, Japan. To establish HL-60 transformed cells containing pAcGFP1-ANXA3 cDNA, HL-60 cells were suspended in DMEM at a density of 5×10^6 cells/mL and subjected to electroporation with 40 μg of linearized plasmid DNA using a Gene Pulsar II (Bio-Rad, CA, USA) in a 4-mm gap cuvette at 200 V and 950 μF. 24 positive clones were confirmed using 250 μM geneticin and western blotting and the best clone was selected based on the cell growth rate (data not shown).

To establish HL-60 transformed cells bearing CSIIE-ANXA3 cDNA, 293T cells (5×10^6 cells/mL) were maintained in DMEM medium containing 10% FBS in poly-lysine coated 10-cm tissue culture plates for 24 h after which fresh medium was added. A plasmid DNA (450 μL) solution containing 17 μg packaging plasmid and 10 μg VSV-G- and Rev-expressing plasmids was mixed with 50 μL 2.5 M $CaCl_2$ and 500 μL 2× HEBS (1.5 mM Na_2HPO_4, 280 mM NaCl, and 50 mM HEPES). The HIV-1-based lentivirus was recovered by ultracentrifugation and resuspended in 50 μL HBSS medium. We mixed the lentivirus with 100 μL HL-60 cells (1×10^6 cells/mL) at a multiplicity rate of infection of 10, and stable transfected clones were selected using 1 mM Zeocin. Successfully transformed cells were sometimes sorted using a BD FACSAria™II Cell Sorter (Tokyo, Japan).

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cells were suspended in lysis buffer (1×10^6 cells/100 μL) (7 M urea, 2 M thiourea, 2% Triton X-100, 18 mM dithiothreitol and 0.1% bromothymol blue) and applied to a 12% SDS polyacrylamide gel. The proteins were electrophoretically transferred from the SDS polyacrylamide gel to an Immobilon Transfer Membrane™ (Millipore, MA, USA) using a Semi Dry Electroblotter (Sartorius, Goettingen, Germany) for 90 min at an electric current of 15 V. The membrane was treated with 4% Block Ace™ (Dainippon, Kyoto, Japan) for 30 min at 22 °C. The first reaction was performed with anti-p38MAPK rabbit IgG (200 ng/mL, Cell Signaling Technology®, Boston, USA) or anti-ANXA3 rabbit IgG (200 ng/mL, Proteintech Group, Inc., CA, USA) in PBS containing 0.03% Tween 20 for 1 h at 22 °C. After washing, the second reaction was performed with HRP-conjugated anti-rabbit IgG goat IgG (20 ng/mL, Santa Cruz Biotechnology, CA, USA) for 30 min at 22 °C. The enhanced chemiluminescence reaction was performed using the ECL Plus Western Blotting Detection System™ (GE).

2.3. Calcium imaging

Cells (2×10^6 cells/mL) were loaded with calcium-sensitive Fura 2-AM (1 μM) in the calcium buffer (HBSS containing 20 mM HEPES and 3% BSA, pH. 7.4) for 30 min at 37 °C (Dojindo, Kumamoto, Japan). Samples were placed directly into the cell suspension in a cuvette after a 5-minute baseline recording. Recordings were made with an F-2500 calcium imaging system with FL Solutions (Hitachi, Tokyo, Japan) at intervals of 5 s that calculated the ratio of fluorescent signals obtained at 37 °C with excitation wavelengths at 340 and at 380 nm and with an emission wavelength at 510 nm.

2.4. Chemotaxis assay

Cells (2×10^6 cells/mL) in the calcium buffer were prepared for the chamber assay. The assay was performed for 90 min using a Nucleopore filter with a pore size of 5 μm (Falk et al., 1980). The total number of cells that migrated beyond the lower surface of the membrane was counted in five microscopic high-power fields.

2.5. Statistical analysis

The results of representative examinations were confirmed by multiple experiments at least with triplicate samples. Statistical significance was calculated by the non-parametric or parametric tests offered in the two way analysis of variance window, respectively. Values are expressed as mean ± SD. A P-value < 0.05 was considered statistically significant and shown as $P < 0.05$: * and $P < 0.01$: **.

3. Results

To examine a role of ANXA3 in the C5aR-mediated cytoplasmic calcium oscillation through plasma membrane during the C5aR-mediated macrophage migration, we prepared the HL-60 macrophage-like cells with either control sense RNA (Control) or shRNA (Knockdown) for ANXA3 mRNA (Fig. 1A). ANXA3 protein expression in the Control HL-60 macrophage-like cells was reduced to approximately 10% following ANXA3 knockdown. To confirm a sequential order of ANXA3 in the calcium signaling, the HL-60 macrophage-like cells were collected at several time points after a stimulation with S-tagged C5a/RP S19 (10^{-8} M) (Fig. 1B). Phosphorylation of p38MAPK in the HL-60 macrophage-like cells was slightly blocked by ANXA3 knockdown, indicating a position of ANXA3 downstream of p38MAPK. The fluorescence

Table 1
Oligoribonucleotides for ANXA3 mRNA.

shRNA	Sense	GATCTGGACATTGTGGACAGCATAAAATTCAAGAGATTTTATGCTGTCCACAATGTCCGCGCG
	Antisense	CGCGCGGACATTGTGGACAGCATAAAATCTCTTGAATTTTATGCTGTCCACAATGTCCA
Control sense RNA	Sense	GATCTGGACATTGTGGACAGCATAAAATTCAAGAGATTTTCGCGCG
	Antisense	CGCGCGCTCTTGAATTTTATGCTGTCCACAATGTCCA