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Lipopolysaccharide-induced multinuclear cells: Increased internalization of polystyrene beads and possible signals for cell fusion

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ABSTRACT

A murine macrophage-derived line, RAW264.7, becomes multinuclear on stimulation with lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria. These multinuclear cells internalized more polystyrene beads than mononuclear cells or osteoclasts (Nakanishi-Matsui, M., Yano, S., Matsumoto, N., and Futai, M., 2012). In this study, we analyzed the time courses of cell fusion in the presence of large beads. They were internalized into cells actively fusing to become multinuclear. However, the multinuclear cells once formed showed only low phagocytosis activity. These results suggest that formation of the multinuclear cells and bead internalization took place simultaneously. The formation of multinuclear cells was blocked by inhibitors for phosphoinositide 3-kinase, phospholipase C, calcineurin, and c-Jun N-terminal kinase. In addition, interleukin 6 and 10 also exhibited inhibitory effects. These signaling molecules and cytokines may play a crucial role in the LPS-induced multinuclear cell formation.

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1. Introduction

In response to cytokines and growth factors, macrophages differentiate into multinuclear cells, including osteoclasts, involved in bone homeostasis, and FBGC (foreign body giant cells), which internalize orthopedic implant wear debris [1–4]. Osteoclasts are formed from bone marrow macrophages after stimulation with RANKL (receptor activator of nuclear factor κB ligand), and FBGCs are formed with IL4 (interleukin 4) [1,5]. Murine macrophage-derived line RAW264.7 also differentiates into osteoclast-like cells upon stimulation with RANKL [6,7].

We found previously that the same cell line forms multinuclear cells through cell–cell fusion on addition of highly purified LPS (lipopolysaccharide) or synthetic lipid A [8]. The LPS-induced multinuclear cells (average 90 nuclei/cell), observed within 16 h after stimulation, did not express osteoclast-specific enzymes and showed no bone resorption [8]. However, these cells can internalize more polystyrene beads than mononuclear cells and osteoclasts [8].

Upon infection by Gram-negative bacteria, macrophages should recognize LPS in the bacterial outer membrane, which leads to inflammatory responses including phagocytosis. Macrophages may also form multinuclear cells when stimulated *in vivo* by the infection. In this regard, LPS-induced multinuclear cells could be a good model system for studying the role of macrophages in infection.

In this study, we analyzed the time courses of multinuclear cell formation in the presence of polystyrene beads, and found that large sized beads were internalized by cells actively fusing, suggesting that formation of the multinuclear cells and bead internalization took place simultaneously. We have also tested a series of inhibitors, and discuss possible signals involved in multinuclear cell formation with LPS.

2. Materials and methods

2.1. Materials

All chemicals used were of the highest grade commercially available: NSC23766, Tocris Bioscience (Park Ellisville, MO); Y-27632, Sigma–Aldrich (St. Louis, MO); PD98059, Cayman Chemical Company (Ann Arbor, MI); and cyclosporine A, Wako (Osaka, Japan). Cycloheximide, SP600125, SB203580, wortmannin, and 21U-73122 were from Calbiochem (La Jolla, CA). Recombinant

Abbreviations: LPS, lipopolysaccharide; TLR4, toll-like receptor-4; RANKL, receptor activator of nuclear factor κB ligand; RANK, receptor activator of nuclear factor κB; FBGC, foreign body giant cell; MEMκ, minimum essential medium alpha medium; PBS, phosphate-buffered saline; TRAP, tartrate-resistant acid phosphatase; FITC, fluorescein isothiocyanate; IL6, interleukin 6; IL10, interleukin 10; TNFα, tumor necrosis factor α; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK1/2, extracellular signal-regulated kinase 1/2; PI3K, phosphoinositide 3-kinase; TRAF6, tumor necrosis factor receptor associated factor 6; PLCγ, phospholipase C-γ; NFκB, nuclear factor κB; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1.

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murine IL6, IL10 and TNFα were obtained from Pepro Tech Inc. (Rocky Hill, NJ). Fetal bovine serum was from Sigma (St Louis, MO), and other reagents for cell culture were purchased from Life Technologies (NY, USA). Polystyrene beads of 6 and 15 μm in diameter were from Polyscience (Warrington, PA).

2.2. Cell culture, induction of multinuclear cells and determination of cytokines

RAW264.7 cells obtained from the European Collection of Cell Culture (ECACC Cat. 91062702) were grown in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. Multinuclear cells were induced with LPS as previously described [8]. Briefly, cells were seeded at 8×10^5 cells per well (1.77 cm^2), and then incubated with 100 ng/ml highly purified LPS from *E. coli*, O55:B5 (TLR grade., Alexis Biochemicals, San Diego, CA), in the presence of 6.8 mM $CaCl_2$ for 16 h. They were then washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS, and stained with Hoechst 33343 (Life Technologies) for nuclei and FITC-conjugated phalloidin (Fluka Biochemika, Buchs, Switzerland) for actin. Stained cells were observed under a LSM510 microscope (Carl Zeiss, Oberkochen, Germany). For determination of cytokine concentrations, culture medium was collected, and IL6, IL10, or TNFα was detected using an ELISA kit from Thermo Scientific (Rockford, IL, USA).

2.3. Internalization of polystyrene beads

Internalization of beads was estimated as described previously [8]. Polystyrene beads of 6 or 15 μm in diameter were fed to cells ($2.5 \times 10^5/cm^2$) 6 h after the addition of LPS. They were further incubated for 10 h, fixed, and stained as described above. The 10 h incubation was required to obtain maximum internalization of 15-μm beads.

3. Results

3.1. Internalization of polystyrene beads by LPS-induced multinuclear cells

We observed previously that LPS-induced multinuclear cells could internalize more beads (6- or 10-μm diameter) than osteoclasts and mononuclear cells: LPS-induced multinuclear cells

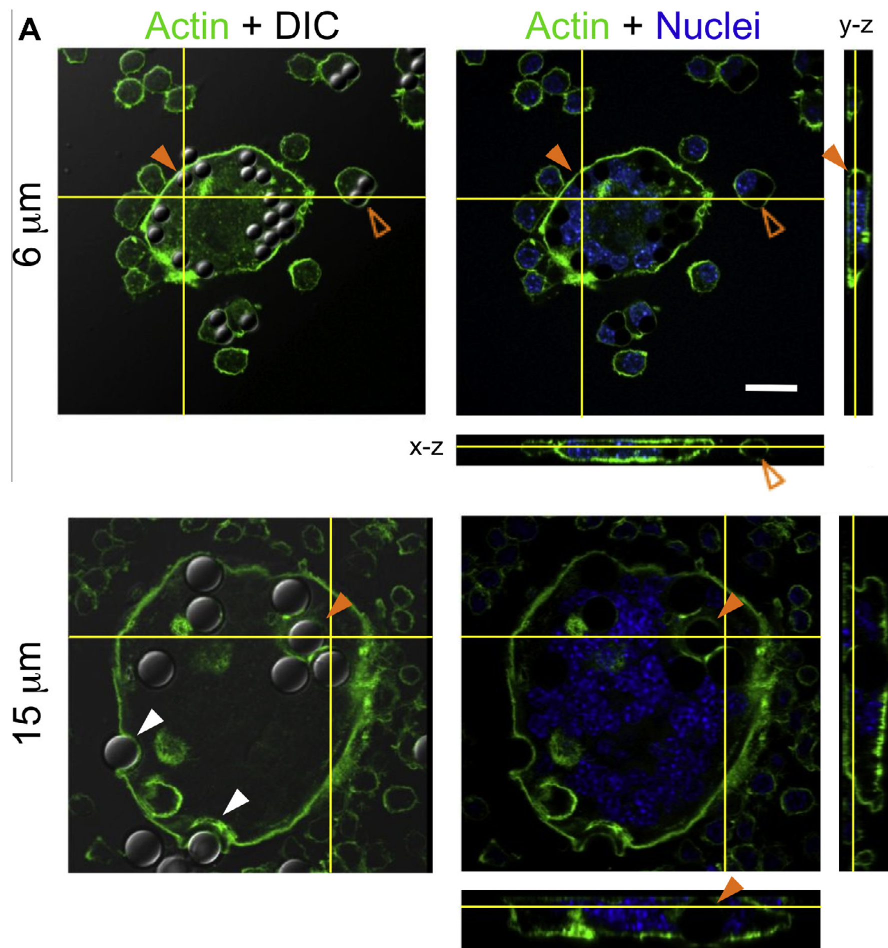

Fig. 1. Internalization of polystyrene beads by LPS-induced multinuclear cells. (A) Polystyrene beads internalized into multinuclear cells. Cells were grown with 100 ng/ml highly purified LPS, and fed with polystyrene beads ($2.5 \times 10^5/cm^2$) of two sizes (diameter 6 or 15 μm) after 6 h incubation. They were incubated for a further 10 h, fixed, and then stained for actin filaments (green) and nuclei (blue). Cells were observed under a confocal microscope. Actin staining is shown with differential interference contrast (DIC) (left) or with nuclei stained (right). Closed and open orange arrowheads indicate beads within multinuclear and mononuclear cells, respectively. White arrowheads indicate beads seem to be undergoing the internalization process. The positions of lateral images (x–z and y–z sections) are shown by yellow lines in horizontal views (x–y sections). Scale bar, 20 μm. (B) Mononuclear and small multinuclear cells attached to large beads. 15 μm-beads were introduced as in A, and cells were fixed 4 h later, and then stained for actin (green) and nuclei (blue). Six typical beads attached to mononuclear and small multinuclear cells are shown. In the merged field (*Merged*, right), beads (white circles) and cells with more than two nuclei (white arrowheads) are shown. Dotted lines indicate the shapes of cells based on DIC and the actin cytoskeleton. Scale bar, 20 μm.

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