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Phagocytosis induces superoxide formation and apoptosis in macrophages

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Abbreviations: FcR, Fc receptor; CR, complement receptor; fMLP, formyl-methionyl-leucyl-phenylalanine; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GM-CSF, granulocyte macrophage colony-stimulating factor; GXM, glucuronoxylmannan; IOZ, IgG-opsonized zymosan; COZ, complement-opsonized zymosan; IFN-γ, interferon-γ; IgG, imunoglobulin G; ITAMs, immunoglobulin gene family tyrosine activation motifs; JNK, c-jun N-terminal kinase; LIMK, LIM kinase; LPS, lipopolysaccharide; MLCK, myosin light chain kinase; NO, nitric oxide; NSF, N-ethylmaleimide-sensitive factor; OPMN, PMN in the oral cavity; PAF, platelet activating factor; PH, pleckstrin homology; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PAK, p21-activated kinase; PI3K, phosphatidylinositol-3 kinase; PIP3, phosphatidylinositol-3,4,5-triphosphate; PLD, phospholipase D; PMN, polymorphonuclear leukocytes; PS, phosphatidylserine; SHIP, src homology 2 domain-containing inositol 5'-phosphatase; RhoGDI, Rho GDP dissociation inhibitor; ROK, Rho-dependent protein kinase; ROS, reactive oxygen species, TNFR, tumor necrosis factor receptor; SAPK, stress activating protein kinase; SH2, Src homology 2; SNARE, soluble NSF attachment protein receptor; TNF-α, tumor necorsis factor-α; VAMP, Vesicle-associated membrane protein; WASP, Wiskott-Aldrich syndrome protein

Abstract

Phagocytosis by inflammatory cells is an essential step and a part of innate immunity for protection against foreign pathogens, microorganism or dead cells. Phagocytosis, endocytotic events sequel to binding particle ligands to the specific receptors on phagocyte cell surface such as Fcγ recptor (FcγR), complement receptor (CR), β-glucan receptor, and phosphatidylserine (PS) receptor, require actin assembly, pseudopod extension and phagosome closure. Rho GTPases (RhoA, Cdc42, and Rac1) are critically involved in these processes. Abrupt superoxide formation, called as oxidative burst, occurs through NADPH oxidase complex in leukocytes following phagocytosis. NADPH oxidase complex is composed of membrane proteins, $p22^{PHOX}$ and $gp91^{PHOX}$, and cytosolic proteins, $p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$. The cytosolic subunits and Rac-GTP are translocated to the membrane, forming complete NADPH oxidase complex with membrane part subunits. Binding of imunoglobulin G (IgG)- and complement-opsonized particles to FcγR and CR of leukocytes induces apoptosis of the cells, which may be due to oxidative burst and accompanying cytochrome c release and casapase-3 activation.

Keywords: apoptosis; NADPH oxidase; phagocytosis; Rho GTPases; superoxide

Introduction

In this review, a series of phagocytosis, superoxide formation and apoptosis of phagocyte cells will be discussed. The phagocytosis is regulated by a variety of participants including receptors, protein kinases, effector proteins, cytoskeletal rearrangement and Rho GTPases. Especially, NADPH oxidase is a key enzyme in oxidative respiratory burst, which follow the phagocytosis. Excessive superoxide could induce apoptosis of phagocytes themselves. Each step is controlled by various regulators.

Phagocytosis in macrophages

Phagocytosis is an essential mechanism that inflammatory leukocytes utilize in engulfing and killing or clearing pathogenic microorganisms or dead cells. It is well established that invading pathogens were initially neutralized by battery of circulating host defensive proteins and quickly cleared from circulation or body fluid by inflammatory leukocytes *via* phagocytosis. The recognition and engulfing of pathogen complex is triggered by interaction with specific receptors on the leukocytes, and followed by cellular actin assembly, pseudopod extension and phagosome closure (Aderem and Underhill, 1999). Rho family GTPase proteins known to be involved in many physiological functions like rearrangement of cytoskeletons, regulation of cellular morphology, chemotaxis, and regulation of transcription (Hall, 1998), are essential in actin dynamics

necessary for phagocytosis and engulfment (Chimini and Chavrier, 2000). Ligands interaction with Fcγ receptors (FcγRs) and complement receptors (CRs) that are linked to distinct signaling pathways, induce both morphologically and mechanistically distinct phagocytic processes.

FcγR-mediated phagocytes

FcγR can recognize Fc domain of immunoglobulin G (IgG). FcγRs that mediate phagocytosis in human macrophages include FcγRI, IIA, and FcγRIII (Ravetch, 1997). The human FcγRIIA is a monomeric protein which has an extracellular Fc binding domain, a transmembrane domain, and a cytoplasmic tail domain containing two immunoglobulin gene family tyrosine activation motifs (ITAMs), which is phosphorylated by tyrosine kinase of the Src family activated upon FcγR aggregation. FcγRI and FcγIIIA have also extracellular Fc binding domains similar to FcγRIIA, but lack ITAMs on their cytoplasmic tails. For proper signaling, these receptors must interact with a dimmer of or γ subunit, which is small transmembrane proteins that contain the ITAMs needed for signal transduction (Aderem and Underhill, 1999). Other protein tyrosine kinase, Syk, is then recruited through Src homology 2 (SH2) domains to the phosphorylated ITAMs, and undergoes autophosphorylation and activation (Chimini and Chavrier, 2000). The activation of Syk kinase triggers many pathways leading to transcriptional activation, cytoskeletal rearrangement, and the release of inflammatory mediators (Andrem and Underhill, 1999).

It was found that Cdc42/Rac regulated the phagocytosis mediated through FcγR in macrophages (Cox *et al*., 1997; Caron and Hall, 1998; Massol *et al*., 1998). The expression of dominant negative inhibitory mutant forms of Cdc42 and Rac1 in phagocytic cells inhibits particle uptake into phagocytes. Rac1 inhibition prevented pseudopod fusion and phagosome closure. In addition, in response to ligation of FcγRs for IgG, the guanine nucleotide exchange factor (GEF) Vav translocates to nascent phagosomes and catalyzes GTP loading on Rac, but not Cdc42. The Vav-induced Rac activation proceeds independently of Cdc42 function, suggesting distinct roles for each GTPases during engulfment (Patel *et al*., 2002). On the other hand, Vav is stimulated by both tyrosine phosphorylation and phosphoinositide product of the PI3K that binds to the pleckstrin/homology (PH) domain (Crespo *et al*., 1997) (Figure 1).

Complement receptor-mediated phagocytosis

Another receptor through which phagocytosis occurs is the CRs, which recognize the C3b/C3bi fragments. The complement fragments are generated by cleavage of C3 to active C3b and subsequently to inactive C3b (C3bi). CR1 is thought to participate mainly in particle binding. CR3 (CD11b/ CD18; Mac-1) and CR4 (CD11c/CD18) are heterodimers of integrin members, $\alpha_M\beta_2$ and $\alpha_X\beta_2$, respectively, which are responsible for particle internalization (Aderem and Underhill, 1999).

Rho appeared to be involved in the regulation of phagocytosis that is mediated through CR3 (Caron and Hall, 1998). The inhibition of RhoA induced by C3 exoenzyme results in the block of CR3-mediated particle uptake. On the contrary, C3 exoenzyme does not inhibit the uptake of IgG-opsonized particles and expression of dominant negative forms of Cdc42 and Rac1 does not affect CR3-mediated phagocytosis (Caron and Hall, 1998). However, Rho appeared to be also involved in FcγR-mediated phagocytosis; the inactivation of Rho by C3 exoenzyme resulted in the complete abrogation of FcγR-mediated phagocytosis (Hackam *et al*., 1997). In addition, FcγR-mediated phagocytosis is abrogated by C3 exoenzyme in COS cells, which become phagocytic upon transfection of the FcγRIIA receptor (Hackam *et al*., 1997). The clustering of receptors in response to opsonin, an essential step in Fcγ-induced signaling, is the earliest event to be inhibited by C3 exoenzyme (Hackam *et al*., 1997). Taken together, Rho is also required for the initiation of phagocytosis by FcγRs in macrophages. The reason of discrepancy between Rho-involvement in FcγR- and CR-mediated phagocytosis is presently unclear, although question was raised in regards to the difference of initial binding of IgG-coated particles (Caron and Hall, 1998) (Figure 1).

The precise mechanism of RhoA of how it regulates the formation of actin rich forci during CR3-mediated phagocytosis remains yet to be resolved.

Another small GTPase, constitutively active Rap1 is sufficient for functional activation of CR3 allowing phagocytosis of C3bi-opsonized target, and inhibition of Rap1 abolishes activation of CR3 induced by phobol esters, lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α or platelet activating factor (PAF). Rap1 may link the signaling through FcγR and that through CR3. IgG-coated particles induce a rapid and transient Rap1 activation. However, Rap1 is activated independently of respiratory burst induction (M'Rabet, 1998). In turn, Rap1 activation specifically controls the binding properties of CR3 towards its physiological ligand, the complement-opsonized phagocytic target (Caron *et al*., 2000).

CR3 requires additional stimuli such as phorbol 12-myristate 13-acetate (PMA), chemokines, TNF-α or adhesion to fibronectin-coated surface, which results in activation of protein kinase C (PKC) and increased expression of CR3 at the surface (Chimini and Chavrier, 2000). In some case, CR3 and CR4 are also involved in antibody-mediated complement-independent phagocytosis (Taborda and Casadevall, 2002).

IgM and IgA-mediated phagocytosis of *C. neoformans* is dependent on CR3 expression, and is inhibited by soluble glucuronoxylmannan (GXM), which binds CD18. Since CD18 can bind GXM, it is considered that IgM- and IgA-mediated phagocytosis reflected facilitated binding of exposed capsular polysaccharide by CR3 and CR4 as a consequence of antibody binding to capsule of microorganism (Taborda and Casadevall, 2002).

Mannose receptor-mediated phagocytosis

The third type of receptor involved in phagocytosis is the mannose receptor that recognizes mannose and fucose saccharides in the capsule on the lipopolysaccharide of invading bacteria (Brown, 1995). Cellular recognition of nonopsonized zymosan is mediated by mannose and β-glucan receptors (Giaimis *et al*., 1993). Nonopsonized zymosan can be also ingested through CR3 (Le Cabec *et al*., 2000), the lectin domain of which binds to soluble β-glucan and mediates phagocytosis of particles containing β-glucan, such as zymosan (Ross *et al*., 1985). Thereby, it is proposed that CR3 be β-glucan receptor (Ross, 2000). However, it was recently reported that dectin-1 is a major β-glucan receptor on macrophages (Brown *et al*., 2002).

Cytoskeleton rearrangements during phagocytosis

Localized actin polymerization provides the driving force for engulfment of particles. A variety of signals can converge to locally reorganize the actin cytoskeleton at a phagosome (May and Machesky, 2001). Celluar content of F-actin increases transiently during phagocytosis. Immunofluorescence localization of myosins in macrophages fixed at various times during FcγR-mediated phagocytosis indicates that myosin II and IXb are concentrated in early phagosomes, myosin IC increased later, and myosin V appeared after phagosome closure (Diakonova *et al*., 2002). The morphology of internalization and the molecular pathways involved in both FcγR- and CR-mediated phagocytosis differ between the two receptors (Allen and Aderem, 1996). During ingestion of complement-opsonized zymosan (COZ), the particles sink into the cells, punctuate structures rich in F-actin, vinculin, α-actinin, paxillin and phosphotyrosin-containing proteins are distributed over the phagosome surface. Moreover, CR-mediated internalization requires intact microtubules and is accompanied by the accumulation of vesicles beneath the forming phagosome, suggesting membrane trafficking plays a key role in CR-mediated phagocytosis (Allen and Aderem, 1996). By contrast, during FcγR-mediated phagocytosis, all proteins examined are uniformly distributed on or near phagosome surface. Local polymerization of actin filaments supports the protrusion of pseudopodia that eventually engulf the particles upon engagement of FcγRs in macrophages. Cdc42 and Rac1 control actin filament assembly through proteins of the Wiskot-Aldrich Syndrome protein (WASP) that interact with Arp2/3 (Machesky and Insall, 1998). Arp2/3 complex, a multifunctional actin organizer, is involved in actin remodeling during phagocytosis. This complex is required for both FcγR- and CR3-mediated phagocytosis, although the upstream signals that recruit the Arp2/3 complex to phagosomes differ for the two receptors (May *et al*., 2000). In addition, N-WASP accumulates at the nascent phagosome (May *et al*., 2000), indicating that WASP is an important effector protein that link Cdc42 and Rac to cytoskeleton regulation during phagocytosis. p21-activated kinase (PAK-1) is also localized to the phagosome, and the activity of PAK-1 is controlled by Cdc42/Rac and has been shown to regulate actinomyosin cytoskeleton contractility (Sanders *et al*., 1999).

On the other hand, LIM kinase-1 (LIMK1) regulates the macrophage-like U937 cells through phosphorylation of cofilin and enhances the formation of filamentous actin. This results lead to the increase of phagocytosis, superoxide production, and translocation of LIMK1 to plasma membrane, when the cells are activated with opsonized zymosan particles (Matsui *et al*., 2002).

Membrane mobilization during phagocytosis

Lipid product of phosphatidylinositol-3 kinase (PI3K), phosphatidylinositol-3,4,5-triphophate (PIP3) is required for pseudopod extension and phagosome closure. PIP3 is rapidly accumulated in phagocytic cups after ligation of FcγRs in macrophages (Cox *et al*., 1999; Marshall *et al*., 2001). Moreover, inhibition of PI3K activity blocks pseudopod extension and phgocytosis without affecting actin polymerization (Cox *et al*., 1999). Moreover, over-expression of Src homology 2 domain-containing inositol 5'-phosphatase (SHIP) in macrophages led to an inhibition of phagocytosis mediated by FcγR and CR3. SHIP is localized to FcγR- and CR3-containing phagocytic cups and was recruited to the cytoskeleton upon clustering of CR3. PI3K and SHIP regulate multiple forms of phagocytosis and endogenous SHIP plays a role in modulating β2 integrin outside-in signaling (Cox *et al*., 2001). However, it is not clear whether SHIP is essential for regulation of membrane mobilization.

Myosin X is also recruited to the forming phagosome through the interaction of its PH domains with membrane PIP3. The motor head domain of Myosin X interacts with actin filament and moves towards the tip of filaments with growing pseudopod. Consequently, by this dual interaction, Myosin X would be

able to couple actin polymerization and pseudopod extension (Chavrier, 2002; Cox *et al*., 2002).

The internalization of the surface during phagocytosis is predicted to reduce the surface area of the phagocytes. Contrary to this prediction, phagocytosis is associated exocytosis, which requires soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptor (SNARE) proteins and ATPase NSF (Hackman *et al*., 1998). In addition, membranes required for pseudopod extension are also provided by insertion of recycling endosome membranes enriched in the SNARE VAMP3 at the site of phagocytosis (Bajno *et al*., 2000). ARF6, one of Ras-related GTPase, is required in Fcγ receptor-mediated phagocytosis in macrophages (Zhang *et al*., 1998).

Phagocytosis of apoptotic cells

Phagocytosis of cells dying by apoptosis differs in many ways from the phagocytic events of IgG- and CR-mediated phagocytosis. One of its peculiarities is the active silencing of the inflammatory responses that are normally the sequel to phagocyte activation. The interaction between the apoptotic cell surface and phagocytotic receptor is rather unclear, and the molecular dissection of the process is still limited (Chimini and Chavrier, 2000). Abnormal exposure of phosphatidylserine (PS) on the outermonolayer has been reported as an essential determinant for recognition by phagocytes. Recently, stereospecific PS receptor has been identified, although the molecular details of its action are still to be answered (Fadok *et al*., 2000). Integrin receptors are important for the phagocytosis of apoptotic cells. It was documented that integrin αvβ5 receptor mediates both binding and internalization of apoptotic cells. Internalization is dependent upon signaling through β5 cytoplasmic tail, and engagement of the αvβ5 heterodimer results in recruitment of the p130cas-CrkII-Dock180 molecular complex, which in turn triggers Rac1 activation and phagosome formation (Albert *et al*., 2000).

Superoxide formation through NADPH oxidase

NADPH oxidase complex

Subsequent to phagocytosis in macrophage, there is an abrupt increase of superoxide formation known as the oxidative burst, which is catalyzed by an NADPH oxidase enzyme complex. The NADPH oxidase is a membrane-associated enzyme complex that generates superoxide (O_2^-) by the one-electron reduction of oxygen, using NADPH as the electron donor (Babior,

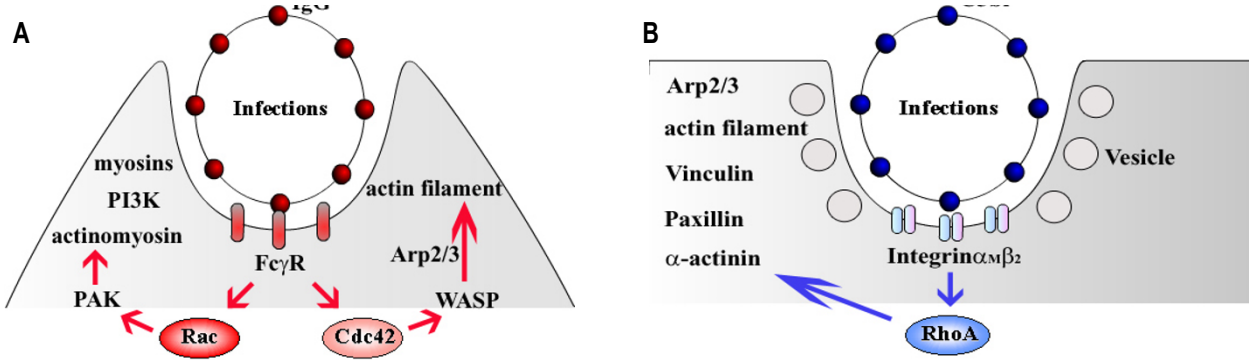

Figure 1. A model for FcR- and CR3-mediated phagocytosis. (A) FcR-mediated phagocytosis. IgG coated on the particle including pathogens binds to FcγR at the surface of the macrophage and triggers FcγR aggregation, and tyrosine kinase is activated and phosphorylates the FcγR. Other protein tyrosine kinase, Syk, is recruited at the phosphorylated site, and undergoes autophosphorylation and activation. Cdc42 activation by a guanine-nucleotide exchange factor (GEF) leads to the recruitment of WASP. In turn, WASP activates the Arp2/3 complex that induces actin polymerization to produce the protrusive force for pseudopod extension. Activation of a Rac1 GEF, possibly Vav, which is translocated to phagosome and stimulated by both tyrosine phosphorylation and phosphoinositide product of PI3K, catalyzes GDP/GTP exchange on Rac1. GTP-Rac1 interacts with and activates the Pak1, which may trigger the actinomyosin contractility involved in phagosome closure (Chimini and Chavrier, 2000). However, it is not clear how the signal pathway is coupled between FcγR and the activation of Cdc42 and Rac1. PI3K is also involved in pseudopod extension (Cox *et al*., 1999) and myosin X is able to couple actin polymerization and pseudopod extension (Cox *et al*., 2002). (B) CR3-mediated phagocytosis. Integrin αMβ2 (Mac-1, CD11b/CD18) is the complement receptor (CR3), which binds complement C3bi on the surface of complement-opsonized particles. Binding of integrin αMβ2 to CR3 activates RhoA by unknown mechanism, which triggers CR3-mediated phagocytosis. In turn, these may induce integrin αMβ2 clustering, assembly of actin filaments, and recruitment of cytoskeletal proteins, including vinculin, paxillin and α-actinin to generate F-actin-rich foci. RhoA-mediated actin assembly may require the activation of the nucleating activity of the Arp2/3 complex that is recruited to the F-actin foci. Activated RhoA may promote myosin filament formation and contractility resulting in the particle sinking into the cytosol (Chimini and Chavrier, 2000). Small vesicles are located below phagosomal plasma membrane at which complement opsonized particles are bound to CR3. These vesicles may be fused to the plasma membrane leading to the augmentation of phagosomal plasma membranes and forming phagosomes.

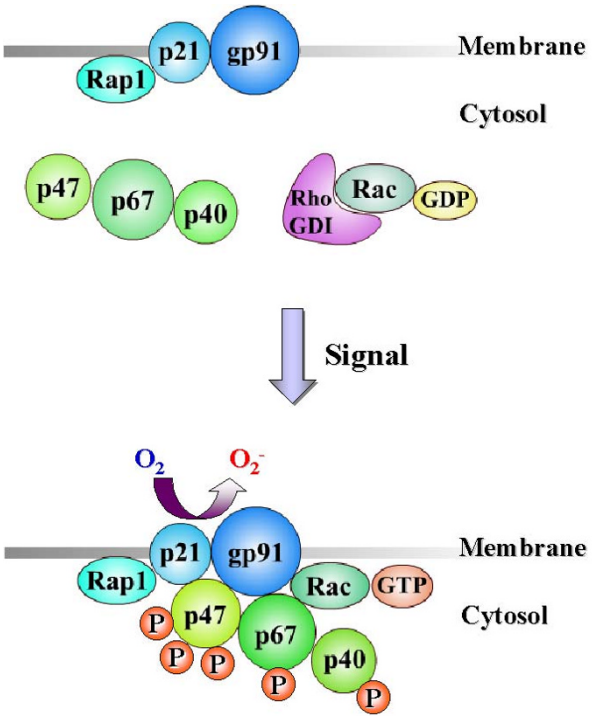

Figure 2. Activation of NADPH oxidase complex. When phagocytes are activated, some signals induce the activation of cytosolic factors ($p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$) like phosphorylation of $p47^{PHOX}$, and $p67^{PHOX}$ and GTP-binding to Rac, and translocation of cytosolic factors to membrane fractions to form a complex of NADPH oxidase with $p22^{PHOX}$ and $gp91^{PHOX}$ membrane protein (cytochrome b_{558}). Inactive GDP-bound Rac exists in complex form with Rho GDP dissociation inhibitor (RhoGDI), and the complex is broken to the active GTP-bound Rac that translocates to NADPH oxidase complex when the cells are stimulated.

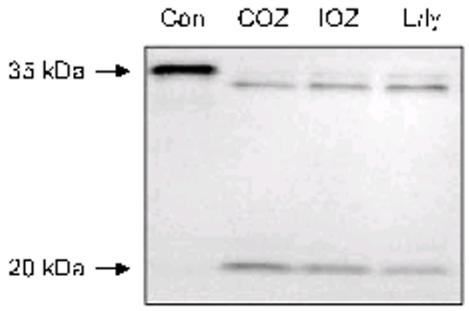

Figure 3. Activation of caspase-3 by IgG-, C3bi-opsonized zymosan particles, and lysopolysaccharide (LPS) and interferon γ (IFNγ). Procaspase-3 (35 kDa) was cleaved to active caspase-3 (20 kDa) by the stimulation with IgG-(IOZ), C3bi-opsonized zymosan (COZ) particles, and 1 g/ml lysopolysaccharide (LPS) plus interferon-γ (IFN-γ) in macrophage J774A.1 cells. Phagocytosis of IOZ and COZ particles induce ROS formation, which in turn lead to apoptosis of J774 macrophage cells through activation of caspase-3 and release of cytochrome c (Kim *et al*., 2003).

1999). NADPH oxidase is composed of multiple subunits from membrane and cytosolic fractions. The core enzyme of NADPH oxidase is composed of five components. Among them, $p22^{PHOX}$ and $gp91^{PHOX}$ exist in the membranes of secretory granular vesicles that fuse with the plasma membrane upon phagocytosis, and $p22^{PHOX}$ and $gp91^{PHOX}$ form a heterodimeric flavohemoprotein known as cytochrome b_{558} (Rotrosen *et al*., 1993). The other components, $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$ are located in the cytosol as a complex (Wientjes *et al*., 1996). When the cells are activated, $p47^{PHOX}$ becomes highly phosphorylated by protein kinases (Park and Babior, 1997), and the entire cytosolic complex of $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$ translocates to the membrane, where it associates with cytochrome b_{558} to assemble the active NADPH oxidase (Heyworth *et al*., 1991). In addition to $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$, two Ras-related small GTP-binding proteins, Rap1 and Rac1 (or Rac2) are required for the activation of NADPH oxidase. Ras-related small GTP-binding proteins, Rac1 (Kreck *et al*., 1994) and Rac2 (Mizuno *et al*., 1992) are essential regulators of the activation of NADPH oxidase. Rac2 is the major homologue found in human phagocytes, while Rac1 is the major form in mouse (Kim and Dinauer, 2001). In the resting state of phagocytosis, Rac is localized in the cytoplasm in a dimeric complex with Rho GDP dissociation inhibitor (GDI), while GTP-bound Rac translocates to the membrane independently of $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$ cytosolic complex translocation during activation of the phagocyte (Bokoch, 1994). Rap1 physically associated with and co-purifies with cytochrome b558 in the membranes (Quinn *et al*., 1989). Rap1 is phosphorylated by protein kinase A and the phosphorylated Rap1 is inhibited from binding to cytochrome b558 (Bokoch *et al*., 1991), suggesting that a kinase(s) may regulate the interaction between cytochrome b558 and Rap1. In addition, IgG-coated particles induce a rapid and transient Rap1 activation. In neutrophil, Rap1 is activated independently of respiratory induction (M'Rabet, 1998). The NADPH oxidase is activated on phagosomes and generates superoxide to aid in the killing of phagocytosed microorganisms. It is important that superoxide is primarily released into phagosomes to prevent damage to surrounding cells (Ridley, 2001).

Regulatory proteins involved in superoxide formation

The subsequent superoxide formation mainly occurs through FcR activation. However, the stimulation of CR3 by anti-CR3 antibody-coated particles (Serrander *et al*., 1999), by *Staphylococcus* particles coated with anti-CD18 antibodies (Lofgren *et al*., 1999), and by nonopsonized zymosan (Le Cabec *et al*., 2000) in-

duces superoxide production. Superoxide production by stimulation of serum-opsonized zymosan (SOZ), is attenuated by p38 MAPK inhibitor, SB203580. SOZ stimulation induces the translocation of $p47^{PHOX}$ and Rac to the plasma membrane, and SB203580 completely blocks the translocation of Rac, but only partially blocks that of $p47^{PHOX}$. PI3K inhibitors, wortmanin and LY294002, blocks not only p38 MAPK activation but also Rac activation. However, SB203580 shows no effect on the PI3K activity. Thus it was proposed that PI3K/p38 MAPK/Rac pathway is operative in the activation of NADPH oxidase in neutrophils (Yamamori *et al*., 2002). While SB203580 and PI3K inhibitor attenuates phagocytosis, GF109203X, PKC inhibitor, facilitated it. However, GF109203X significantly attenuates SOZ-induced superoxide formation. These results suggest that p38 MAPK and PI3K participate in both signaling pathways of phagocytosis and superoxide formation, and PKC participates in the signaling pathway of NADPH oxidase activation alone (Yamamori *et al*., 2000).

On the other hand, inhibition of ERK1/2 by PD98059, a specific inhibitor of ERK kinase, inhibited fornyl Met/Leu/Pro (fMLP)-induced phosphorylation of $p47^{PHOX}$, but weakly affected PMA-induced $p47^{PHOX}$ phosphorylation. Moreover, SB203580 did not inhibit the phosphorylation of $p47^{PHOX}$ induced either by fMLP or by PMA in human neutrophil (Dewas *et al*., 2000). This suggests that signal pathways for superoxide formation are different each other depending on kinds of stimulation to the cells.

Rac is involved in FcγR-mediated phagocytosis, and directly in the activation of NADPH oxidase. In addition, RhoA was also reported to be involved in the production of H_2O_2 in other cell lines such as Swiss 3T3 fibroblast (Koo *et al*., 1999) and Rat-2 fibroblast (Lee *et al*., 2000), when stimulated by TGF and EGF, respectively. RhoA has been known as an activator for Rho-dependent protein kinase (ROK), which phosphorylates myosin light chain kinase (MLCK) (Totsukawa *et al*., 2000), and myosin binding subunit of myosin phosphatase (Kawano *et al*., 2000), which results in the formation of stress fibers.

Cytoskeleton reorganization is also linked to superoxide formation: Inhibition of superoxide formation by cytochalasin B suggests a necessary role of the cytoskeleton in the signaling pathway that activates the oxidase (Serrander *et al*., 1999). ML-7, an inhibitor of MLC kinase, inhibited superoxide formation and phagocytosis in some laboratories (Kimura *et al*., 1996; Masfield *et al*., 2000), suggesting that cytoskeleton reorganization is important for the superoxide formation. In addition, the inhibition of superoxide formation by ML-7 may arise from the reduction of the phagocytosis, where ML-7 will likely directly inhibits the machinery to produce superoxide: ML-7 was shown to reduce the phosphorylation and the translocation of $p47^{PHOX}$ to the membranes (Heyworth *et al*., 1995).

It has been shown that $p22^{PHOX}$ can be phosphorylated through phospholipase D (PLD), suggesting that PLD activity is required for superoxide formation (Regier *et al*., 2000). Involvement of PLD was further evidenced by that the activation of PLD is tightly coupled to the phagocytosis of opsonized zymosan by human macrophages (Kusner *et al*., 1996) and RhoA is an activator of PLD activity (Malcolm *et al*., 1994). Interestingly, Rac/RhoGDI complex was disrupted in the presence of various lipids like arachidonic acid, phosphatidylinositol, and phosphatidic acid (PA) which can be produced by PLD (Chuang *et al*., 1993).

Recently it was demonstrated that RhoA is regulated by Rac through superoxide where the change in cellular redox state appeared to couple to the control of actin cytoskeleton rearrangement by Rho GTPase (Nimnual *et al*., 2003). Rac-mediated ROS production results in the down-regulation of Rho activity. The pathway linking generation of ROS to down-regulation of Rho involves inhibition of the low-molecular weight protein tyrosine phosphatase and then an increase in the tyrosine phosphorylation and activation of its target, p190Rho-GAP (Nimnual *et al*., 2003). Despite the multiple events associated with superoxide formation, it remains to be studied in detail how NADPH oxidase complex is activated.

Apoptosis of phagocytes by reactive oxygen species (ROS)

Neutrophil apoptosis is accelerated by at least three different routes. First, engagement of death-inducing receptors such as tumor necrosis factor receptor (TNFR) or Fas, induces apoptosis of neutrophils. Second, stress stimuli of physical and chemical agents lead to neutrophil apoptosis. Last, phagocytosis of IgG- or complement opsonized particles triggers apoptosis of neutrophils (Zhang *et al*., 2003).

Agents inducing apoptosis

As for the cell death and apoptosis of phagocytes including macrophages and neutrophils, studies have mainly been focused on conditions that lead to endogenous production of ROS or nitric oxide (NO). The factors to induce apoptosis of macrophages through the induction of ROS include azurin, copper-containing protein involved in electron transfer during denitrification (Yamada *et al*., 2002), residual oil fly ash, a pollutant dust (Huang *et al*., 2003), sulfasalazine, a drug used in inflammatory bowel disease (Salh *et al*., 2002), chromium (VI), a widely used industrial chemical (Bagchi *et al*., 2001), gliotoxin, an immuno-

suppressive agent (Suen *et al*., 2001), cationic liposome (Aramaki *et al*., 2001), and silica (Shen *et al*., 2001).

Apoptosis by endogenous ROS

ROS endogenously produced inside the cells could induce apoptosis of the cells. Although apoptosis of neutrophils may be mediated by endogeneous oxidative products, the physiological roles of ROS in the apoptosis of neutrophil is unknown (Kasahara *et al*., 1997). ROS are also necessary for apoptosis in neuron, and NADPH oxidase subunits are shown in neurons. NADPH oxidase inhibitor, diphenyleneiodonium (DPI), and NADPH oxidase genetic deficiency inhibit apoptosis in NGF-deprived sympathetic neurons (Tammariello *et al*., 2000). Interestingly, unlike circulating polymorphonuclear leukocytes (PMN), PMN in the oral cavity (OPMN) spontaneously generate superoxide radical and NO in the absence of any stimuli, which results in apoptosis. However, herbimycin A, a protein tyrosine kinase inhibitor, suppressed the activation of caspase-3 and apoptosis of OPMN (Sato *et al*., 2002). On the other hand, LPS, TNF-α and the other cytokines induce apoptosis through the production of NO (Albina *et al*., 1993; Sarih *et al*., 1993; Tartaglia *et al*., 1993; Xaus *et al*., 2000). Interestingly, H_2O_2 at low concentration can induce survival pathway of cells, while that at high concentration induce cell apoptosis through activation of SAPK/JNK and caspase-3. This suggests that survival and apoptotic signal pathways through ROS can be competitively regulated (Um, 2001).

Apoptosis induced by activation of FcR and CR

Earlier studies showed that phagocytotic apoptosis was induced through CR and FcR ligation (Coxon *et al*., 1996; Gamberale *et al*., 1998) and Rho GTPases regulates the phagocytosis mediated through FcR and CR3 (Caron and Hall, 1998; Massol *et al*., 1998), which may be implicated to the superoxide formation. Using oligonucleotide microarrays and FACS analysis, it was confirmed that apoptosis can be induced during phagocytosis (Kobayashi *et al*., 2002).

Although these studies strongly suggest that CR- and FcR-ligation with ligands induce apoptosis in neutrophils, it was not clear on the downstream processes resulting in apoptosis of macrophages. Recently, it was shown that SOZ or IgG-opsonized zymosan (IOZ) induced the apoptosis of macrophages. In contrast to LPS/interferon-γ (IFNγ)-induced apoptosis, SOZ- and IOZ-induced apoptosis were strictly dependent on the ROS, which were generated during phagocytosis in the macrophage. In addition, the phagocytosis of macrophages resulted in the release of cytochrome *c* as well as the activation of caspase-3 in a superoxide-dependent manner (Kim *et al*., 2003). Recent study showed that CR3 mediated phagocytosis promotes apoptosis through a caspase8/3 dependent pathway that is modulated by NADPH-oxidase generated ROS and MAPK/ERK. Moreover, TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF), likely to be encountered by phagocytosing neutrophils at inflammatory sites, exploit pro-ROS and anti-ERK apoptotic signals triggered by phagocytosis to promote or suppress phagocytosis-induced cell death respectively, and thus modulate the fate of phagocytosing neutrophils (Zhang *et al*., 2003).

Activation of caspases induces apoptotic cell death. Many structural and regulatory proteins are inactivated by caspases, while other substrates can be activated. For instance, small GTPases and related proteins including Cdc42, Rabaptin-5, Rac, Ran-GAP, Ras-GAP, TIAM, Vav-1, and D4-GDI are cleaved by caspases (Kwon *et al*., 2002; Fischer *et al*., 2003).

On the other hand, overexpression of SHIP in macrophages leads to an inhibition of phagocytosis mediated by FcγR and CR3 (Cox *et al*., 2001). However, the activation of SHIP by NADPH oxidase-stimulated Lyn leads to enhanced apoptosis in neutrophils (Gardai *et al*., 2002).

ROS-induced apoptosis has been studied mostly by using drugs (Bagchi *et al*., 2001; Boggs *et al*., 2001; Shen *et al*., 2001) which are capable of generating intracellular ROS or H_2O_2, with respect to cytochrome *c* release and caspase activation. ROS has been shown directly or indirectly to target mitochondria and release cytochrome *c* from those organelles into the cytosol (Herrera *et al*., 2001). It is generally assumed that such release is required for activation of caspase-3. As yet, it is not clear whether the superoxide species generated by NADPH oxidase during phagocytosis, exogenous chemicals, or mitochondrial electron transport during respiration (Raha and Robinson, 2001) have the same mechanism to induce apoptosis of cells.

It was also observed that $p21^{WAF1}$ was induced, when macrophages were treated with SOZ particles (Kim *et al*., 2003). In early stage of the induction of apoptosis, $p21^{WAF1}$ may be increased to protect cells from apoptosis, since $p21^{WAF1}$ was known to have anti-apoptotic and cell cycle arrest functions (Seoane *et al*., 2002). but $p21^{WAF1}$ is cleaved by caspase3 at the onset of apoptosis, losing its apoptosis-suppressing activity (Zhang *et al*., 1999). Induction of $p21^{WAF1}$ in SOZ-treated macrophages is still need to be clarified for its role in apoptosis.

Conclusively, phagocytosis of SOZ and IOZ induces superoxide formation, and excessive superoxide causes the cells to die. Rho GTPases play important roles in specific steps.

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