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Phagocytosis of serum- and IgG-opsonized zymosan particles induces apoptosis through superoxide but not nitric oxide in macrophage J774A.1

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Abbreviations: CR, complement receptor; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; FcγR, Fcγ receptor; IOZ, IgG-opsonized zymosan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NOZ, non-opsonized zymosan; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; ROS, reactive oxygen species; SOD, Superoxide dismutase

Abstract

Phagocytosis of serum- and IgG-opsonized zymosan (SOZ and IOZ, respectively) particles into J774A.1 macrophages induced apoptosis of the cells, accompanied by the expression of $p21^{WAF1}$, one of cyclin-dependent protein kinase (CDK) inhibitors. Furthermore, phagocytosis of SOZ and IOZ particles into macophages induced superoxide formation. Tat-superoxide dismutase (SOD), which is readily transduced into the cells using Tat-domain, protected the cells from the apoptosis induced by phagocytosis of SOZ and IOZ particles. lipopolysaccharide (LPS) /interferon-γ (IFN-γ) also caused the apoptosis of the cells. However, Tat-SOD could not protect the cells from LPS/IFN-γ induced apoptosis, suggesting that apoptosis mechanisms involved are different from each other. In the present study, we determined the amounts of nitric oxide (NO) produced by SOZ, IOZ, and LPS/IFN-γ, and found that SOZ and IOZ did not induce the generation of NO in macrophages, whereas LPS/IFN-γ did. The apoptosis due to phagocytosis was accompanied with the release of cytochrome *c* from mitochondrial membrane to cytosolic fraction. Furthermore, SOZ and IOZ induced the cleavage of procasapase-3 (35 kDa) to give rise to an active caspase-3 (20 kDa), which was blocked by Tat- SOD but not by 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), a scavenger of NO. On the other hand, LPS/IFN-γ caused the activation of procaspase-3, which was blocked by PTIO but not by Tat-SOD. Taken together, phagocytosis of SOZ and IOZ particles induced apoptosis through superoxide but not NO in macrophages, accompanied with the release of cytochrome *c* and the activation of caspase-3.

Keywords: apoptosis; caspase-3; macrophages; nitric oxide; phagocytosis; superoxides; zymosan

Introduction

Phagocytosis is the process of recognition and engulfment of microorganisms and tissue debris that accumulate during infection, inflammation and wound repair. It is initiated by the recognition of ligands on the phagocytic target by specific receptors on the ingesting cell. Fcγ receptors (FcγRs) mediate phagocytosis of IgG-coated particles; complement receptors (CRs) can recognize the particles opsonized with C3b/C3bi; and mannose-receptor or β-glucan receptor can bind to saccharide moiety of particles (Aderem and Underhill, 1999).

Generation of superoxide anions by the NADPH oxidase complex of macrophages follows the phagocytosis in the macrophage (Babior, 2000) and is indispensable to the host defense response, since it is essential for killing of invading microorganisms. NADPH oxidase is a multisubunit complex that generates superoxide anions in one-electron reduction of O_2 using electrons supplied by NADPH (Babior, 2000), and consists of several components: the heterodimeric membrane-associated flavocytochrome b558 protein, which is composed of gp91superscript and p22superscript (Volpp *et al.*, 1988), cytosolic components p40superscript, p47superscript, and p67superscript (Nunoi *et*

al., 1988; Wientjes *et al.*, 1993), and small GTPase Rap1 and Rac1 or Rac2 (Kwong *et al.*, 1993).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are constantly generated in aerobic metabolism (Curtin *et al.*, 2002). However, apoptosis is induced by moderate amount of ROS in many types of cells (Gardner *et al.*, 1997), and by RNS in HL-60 (Lin *et al.*, 1995), PC12 (Estevez *et al.*, 1995), macrophages and smooth muscle cells (Szabo *et al.*, 1996). Furthermore, it is well known that two key events occur in the induced apoptosis; the cytochrome *c* release from mitochondria and the activation of caspase-3 (Green and Reed, 1998).

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), and factors to induce macrophages apoptosis 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 immunosuppressive agent (Suen *et al.*, 2001), cationic liposome (Aramaki *et al.*, 2001), and silica (Shen *et al.*, 2001). On the other hand, lipopolysaccharide (LPS), tumor necrosis factor-α (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). However, these studies have not dealt the apoptosis with phagocytosis mechanisms through receptors. Until now, there have been only a few reports on phagocytotic apoptosis induced through CR and FcR ligation (Coxon *et al.*, 1996; Gamberale *et al.*, 1998). Fcγ of IgG is recognized by FcγRs, and complement C3bi by CRs. It was found that Cdc42/Rac regulated the phagocytosis mediated through FcR, whereas Rho regulated the phagocytosis mediated through CR3, also referred as integrin $\alpha_M\beta_2$, CD11b/CD18 and Mac-1 (Caron and Hall, 1998; Massol *et al.*, 1998). Using oligonucleotide microarrays and FACS analysis (Kobayashi *et al.*, 2002), induction of apoptosis during phagocytosis has been recently confirmed again. Although these studies strongly suggest that CR- and FcR-ligation with ligands induce apoptosis in neutrophils, it was not well known that phagocytosis of serum- (SOZ) and IgG-opsonized zymosan (IOZ) particles into macrophage J774 induces apoptosis through superoxide formation including typical apoptotic processes like cytochrome *c* release and caspase-3 activation.

In the present study, we showed that SOZ or IOZ induced the apoptosis of macrophages. In contrast to LPS/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 macrphages resulted in the release of cytochrome *c* as well as the activation of caspase-3 in an oxygen-dependent manner.

Materials and Methods

Materials

Zymosan A particles, fluorescein isothiocyanate (FITC), luminol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, annexin V-FITC, Griess reagent, LPS, IFN-γ, and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) were purchased from Sigma Chemicals (St. Louis, MO). Superoxide dismutase (SOD) and catalase were purchased from Calbiochem (SD, CA). 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate), opsonizing reagents were from Molecular Probe, Inc. (Eugene, OR), anti-cytochrome *c* antibody was from Pharmingen (San Jose, CA), anti-caspase 3 antibody was purchased from Cell Signaling Technology (Beverly, MA), and anti-$p21^{WAF1}$ and anti-IgG antibodies were from Santa Cruz (CA). Tat-SOD was prepared by the methods described by report of Kwon *et al.* (2000).

Cell culture

J774A.1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37°C in an atmosphere of 5% CO_2. Cells were seeded onto 24-well plates for the MTT, chemiluminescence, and nitrite assays, and onto 35-mm dishes for trypan blue dye exclusion assay and immunoblots.

Serum- and IgG-opsonization of zymosan

Zymosan A particles were labeled with FITC according to Gelfand *et al.* (1976) without adding gelatin. To prepare SOZ, zymosan particles were opsoinzed by incubating with 25% FBS for 1 h at 37°C, washed in KRG buffer (120 mM NaCl, 5 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, and 1 mM $CaCl_2$), and resuspended in KRG buffer to an appropriate concentration (Hed and Stendahl, 1982). IOZ was prepared by incubation of anti-zymosan antibody with zymosan particles according to the manufacturer's protocol (Molecular Probe). The aliquots were stored at -70°C and thawed immediately before use.

Phagocytosis assay

Macrophage cells were plated onto 35-mm dishes at a density of 2×10^5 cells and grown overnight, and the cells were then incubated in DMEM media without FBS for 16 h at 37°C in a CO_2 incubator. Subsequently, the cells were incubated with FITC-conjugated SOZ or IOZ particles for various periods of time. The cells were then washed three times with PBS to remove unbound zymosan particles, and were detached from dishes with 2 ml PBS. The phagocytosis was assayed by measuring the intensity of FITC engulfed into the cells, and fluorescence of FITC was fluoro spectrophotometrically measured by excitation at 490 nm and emission at 520 nm (Kontron SFM25). Fluorescence of adherent FITC-zymosan onto the surface of the cells was quenched by adding crystal violet to 10 μM final concentration (Hed *et al.*, 1987). The associated FITC-zymosan on the cell surface was calculated by subtracting the intensity value of the net-translocated fluorescence intensity from total fluorescence intensity of FITC-zymosan.

Cell growth and viability analysis

Cell viability was assessed by MTT assay. Briefly, cells plated in 24-well plates were incubated for 1 day before the addition of SOZ or IOZ. After incubation for 24 h, the cells were treated with MTT (1 mg/ml) for 60 min, the culture medium was removed, and the cells were suspended in dimethyl sulfoxide and shaken for 10 min. Absorbance at 570 nm was measured using a microplate reader. To analyse the growth of J774A.1 cells, cells were harvested by cold PBS at indicated time, and the viable cell number was measured by the trypan blue dye exclusion method.

Apoptosis analysis

Apoptotic death of macrophages was determined by examining DNA fragmentation and annexin binding using standard procedures. As for DNA fragmentation assay, cell pellets were resuspended in 750 μl of ice-cold lysis buffer (20 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 8.0) for 45 min with occasional shaking. DNA was extracted with phenol and precipitated with alcohol. The pellet was dried and resuspended in 100 μl of 20 mM Tris-HCl, pH 8.0. After digesting RNA with RNase (0.1 mg/ml) at 37°C for 1 h, the samples (5 μg) were electrophoresed on a 1% agarose gel in 45 mM Tris-acetate-EDTA buffer, pH 8.0. DNA was photographed under UV light. The binding of annexin V to phosphatidylserine (PS) was also determined, since it was an established biochemical marker of apoptosis. Briefly, cells were pretreated with or without 2 μM of Tat-SOD or 0.5 mM of PTIO for 30 min before treatment with SOZ, IOZ or 1 μg/ml of LPS plus 50 U/ml of IFN-γ (LPS/IFN-γ) for 24 h. And cells were then washed twice with PBS (pH 7.4) and resuspended in staining buffer containing 25 ng/ml annexinV-FITC, and the suspension was incubated at room temperature for 15 min in the dark before flow cytometric analysis (Koopman *et al.*, 1994).

Determination of superoxide

For the measurement of intracellular superoxide, J774 cells (2×10^3) were harvested by centrifugation, washed 3 times with PBS, and resuspended in 1 ml of KRG buffer containing 50 U/ml SOD, 2,000 U/ml catalase, and 0.05 mM luminol. Superoxide formation was started by the addition of either SOZ, IOZ, or LPS/IFN-γ, and the resulting chemiluminiscence was measured by luminometer (Lumat LB 9507, EG & G, Berthold, Germany). In some cases, the cells were pretreated with Tat-SOD for 30 min to confirm the production of superoxide (Dahlgren and Karlsson, 1999).

Nitrite assay

Accumulation of nitrite in the medium was determined colorimetrically with Griess reagent. Briefly, the cells were cultured in phenol red free DMEM medium containing 10% fetal bovine serum, and treated either with SOZ, IOZ, or LPS/IFN-γ for 12 h. Aliquots of conditioned media were mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 5% phosphoric acid], and nitrite concentrations were determined by absorbance at 550 nm, using standard sodium nitrite solutions prepared in cell culture medium (Grisham *et al.*, 1996).

Detection of intracellular NO

Intracellular NO produced by cultured cells was bioassayed by the previously described (Lo'pez-Figueroa *et al.*, 2001) DAF-FM diacetate detection system. Briefly, after cells were stimulated with SOZ, IOZ, or LPS/IFN-γ for 12 h, the cells were washed in PBS and then treated with phenol red- and serum-free DMEM medium containing 10 μM DAF-FM diacetate for 30 min at 37°C. Intracellular NO formation was visualized by green fluorescence of DAF-FM in live macrophage cells by using confocal microscope.

Cytochrome *c* release

Macrophage J774A.1 cells were incubated with various concentrations of SOZ or IOZ for 12 h, then harvested and washed in cold PBS. For preparation of mitochondrial and cytosolic fractions, cells were resuspended in ice-cold buffer A (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM

EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 8 μg/ml aprotinin and 2 μg/ml leupeptin), and were passed through a needle 10 times. Debris and nuclei were pelleted by centrifugation of the suspension for 10 min at 750 rpm. The supernatant was then centrifuged at 100,000 *g* for 15 min. The pellet was resuspended in buffer A and designated as the mitochondrial fraction. The supernatant was again centrifuged at 100,000 *g* for 60 min (Yao and Tabas, 2001), and the obtained supernatant was used as cytosolic fraction. For assay of cytochrome *c* release, the cytosolic fraction was electrophoresed on 10% SDS-polyacryl-

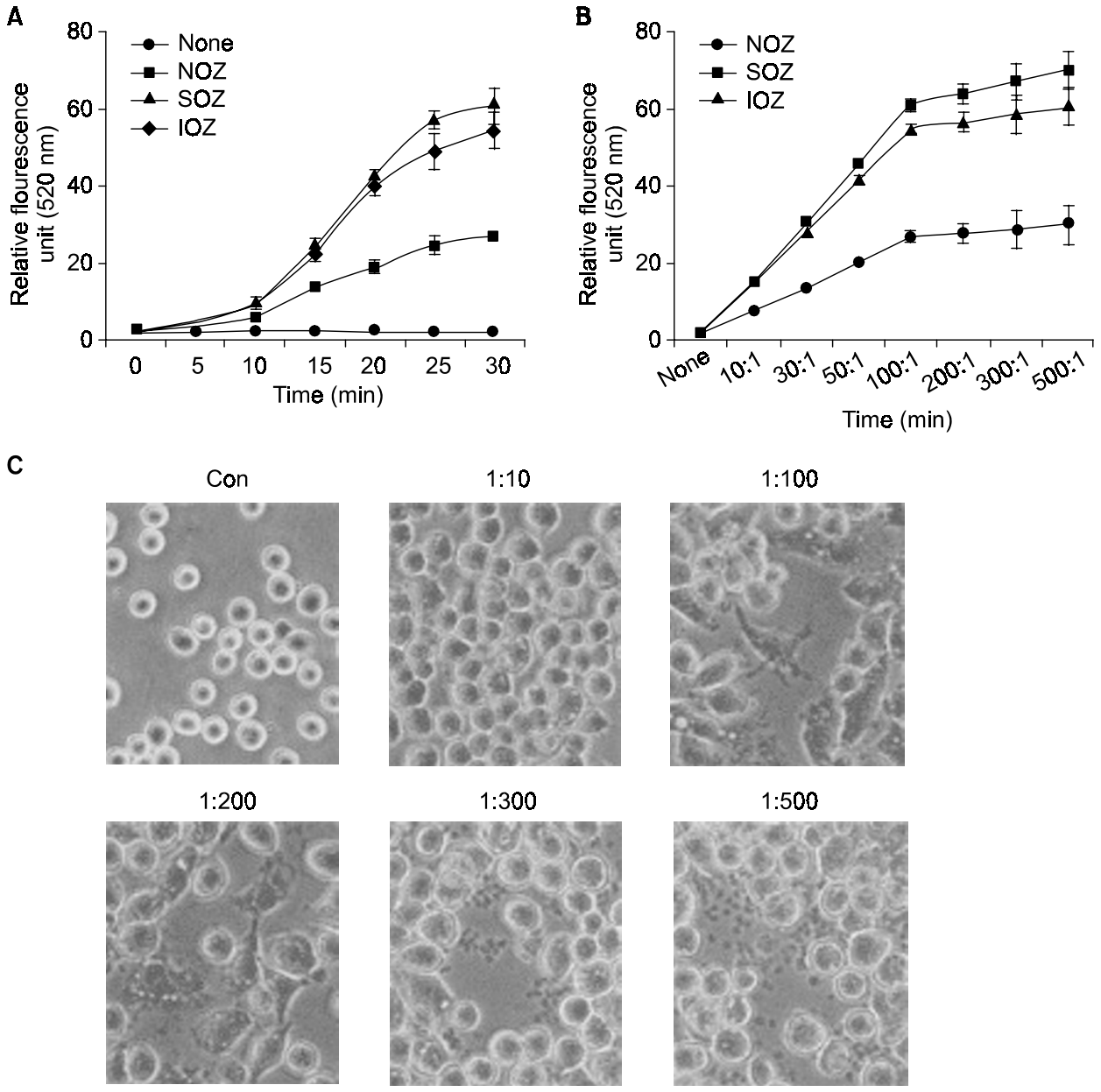

Figure 1 Phagocytosis of SOZ, IOZ, and NOZ particles. Cells were incubated with serum free DMEM for 16 h. For the phagocytosis, the cells were incubated with FITC-zymosan particles (ratio of 100:1; NOZ/, SOZ/, or IOZ/macrophages) for indicated periods of time. The phagocytosis was assayed by measuring the intensity of FITC fluorescence bound to zymosan by using a fluorescence spectrophotometer (A). For the phagocytosis, the cells were incubated with FITC-zymosan particles (indicated ratio; NOZ/, SOZ/, or IOZ/macrophages) for 30 min. The phagocytosis was assayed by measuring the intensity of FITC fluorescence bound to zymosan by using a fluorescence spectrophotometer (B). Cells were incubated with the indicated ratio of SOZ for 12 h. And then the cells were visualized using phase contrast with 200-fold magnification under microscope (C). The values were presented as the mean±SE of at least three independent experiments (A and B), and a representative of at least three experiments (C).

amide gels, and Western blot was then performed using an anti-cytochrome *c* antibody.

Immunoblotting analysis

To perform the general Western blot of some proteins including $p21^{WAF1}$ and caspase-3, cells were rinsed twice with ice-cold PBS, and 100 μl of whole-cell lysis buffer (20 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) were added to each well. The cell lysates were then centrifuged. Equal amount of the soluble protein was denatured in SDS, electrophoresed on 14% SDS-polyacrylamide gel, and transferred to a PVDF membrane. Nonspecific binding was blocked with 5% skim milk containing TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for overnight at 4°C.

Results

Phagocytosis of SOZ and IOZ particles

To determine whether the phagocytosis of SOZ particles by J774A.1 cells induced cell proliferation or apoptosis, the cells were challenged with various amount of SOZ. Complement C3b in serum was found rapidly fixing onto zymosan, and close to 80% of the C3b was converted to C3bi, when zymosan was opsonized with fresh FBS at 37°C (Newman and Mikus, 1985). Engulfing of non-opsonized zymosan (NOZ) particles by the cells occurred but phagocytosis of SOZ and IOZ particles were far enhanced and peaked in about 30 min (Figure 1A). Phagocytosis of SOZ, IOZ, and NOZ particles were increased as the particles to macrophage ratio increased, and reached a plateau at ratio of 100:1 (Figure 1B). The morphological shape of macrophage in a round form becoming slightly spreading form as the cells exposed to low ratio of SOZ/macrophage (10:1). When the phagocytosis was allowed for 12 h in the intermediate high ratio of SOZ/macrophage (100:1-200:1), the cells became a round form and remain differentiated. The cells exposed to the high ratio of SOZ/macrophage (300:1-500:1) were found not attached on the plates but suspended in the medium as a round form, which was believed to be due to cell death (Figure 1C).

Phagocytosis inhibits cell viability and proliferation

The viable state of macrophages that have been exposed to high concentration of SOZ, was assayed using MTT. As the ratio of SOZ/macrophage increased, the cell viability was significantly decreased

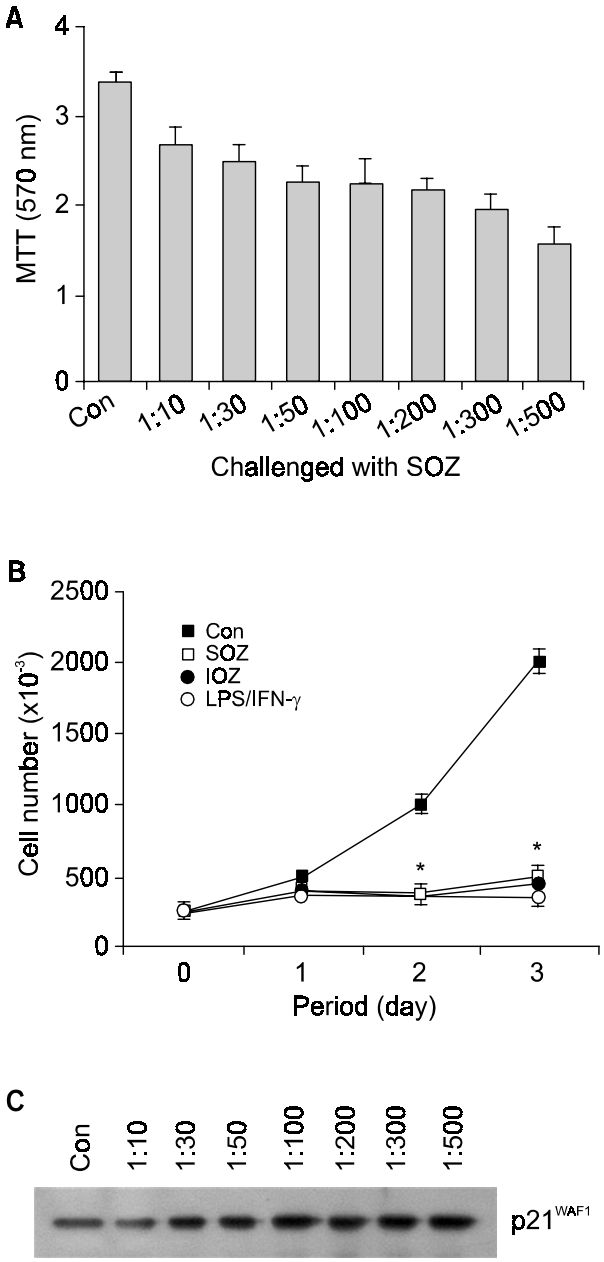

Figure 2. Cell viability and growth by phagocytosis. Cells were incubated with serum free DMEM for 16 h, and were stimulated with the indicated ratios of SOZ for 24 h. MTT (1 mg/ml) was added to the cells for 60 min, the culture medium was then removed, and the cells were dissolved in dimethyl sulfoxide and shaken for 10 min. Absorbance at 570 nm was measured using a microplate reader. Cell viability was significantly decreased in the cells treated with SOZ ($P < 0.01$) (A). The cells were stimulated with SOZ, IOZ (the ratio of 300:1), or 1 μg/ml LPS plus 50 U/ml IFN-γ, harvested by cold PBS at indicated times, and the viable cell number was measured by microscopic examination with trypan blue dye exclusion. SOZ, IOZ and 1 μg/ml LPS plus 50 U/ml IFN-γ significantly inhibited cell proliferation ($^*P < 0.01$) (B). The cells were stimulated with the indicated ratios of SOZ for 24 h. Expression of $p21^{WAF1}$ was then immunoblotted with anti-$p21^{WAF1}$ antibody (C). The values were presented as the mean ± SE of at least three independent experiments (A and B), and a representative of at least three experiments (C).

(Figure 2A). Furthermore, the cells treated with SOZ or IOZ particles, at the ratio of 300:1 (SOZ/macrophags) did not proliferate as indicated with a tryphan blue exclusion assay. LPS/IFN-γ also inhibited the proliferation of macrophages (Figure 2B) agreeable with the previous findings of Albina *et al.* (1993). To determine whether cell cycle-related growth arrest was involved in this apoptotic event, the expression of the $p21^{WAF1}$, a cell cycle kinase (CDK) inhibitor, was tested. Expression of $p21^{WAF1}$ was increased in a SOZ/macrophage ratio-dependent manner (Figure 2C).

Phagocytosis of SOZ and IOZ particles induces apoptosis of the cells

To explore a possible mechanism(s) of apoptotic state of macrophage cells that have been in phagocytosis of SOZ or IOZ in a high ratio of SOZ/ or IOZ/macrophages (300:1) environment for 12 h, the exposure of PS on the cell surface was measured by the binding of FITC-conjugated annexin V to PS. Flow cytometric analysis of FITC-conjuagted annexin V bound to the cell surface showed that the high concentration of SOZ and IOZ particles that have likely caused the apoptosis of macrophages, and NOZ did not induce apoptosis of the cells (Figure 3A). However, Tat-SOD, which has a penetrating 'Tat' to facilitate the transduction of SOD into the cells, prevented apoptosis of the cells treated with SOZ and IOZ. Similarly, PTIO, which is NO scavenger, also inhibited apoptosis of the cells treated with LPS/IFN-γ. On the contrary, apoptosis of the cells due to LPS/IFN-γ was not blocked by Tat-SOD, whereas PTIO had significant effect on blocking the apoptosis (Figure 3A). This suggested that the apoptosis mechanisms of SOZ and IOZ, and LPS/IFN-γ were different from each other. LPS/IFN-γ has been shown to induce apoptosis of macrophage (Forman and Torres, 2001), and we also confirmed apoptosis of macrophage by SOZ, IOZ and LPS/IFN-γ which induced DNA fragmentation, another well-known apoptosis maker (Figure 3B).

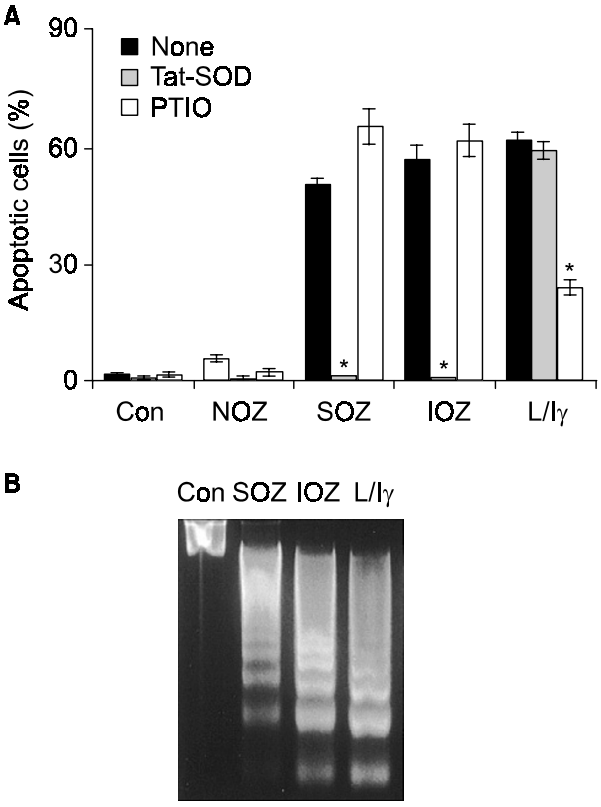

Figure 3 Apoptosis of macrophage cells by phagocytosis. Cells were pretreated with or without Tat-SOD or PTIO for 30 min before NOZ, SOZ, IOZ or 1 μg/ml LPS plus 50 U/ml IFN-γ treatment for 24 h. And the cells were then washed twice with PBS (pH 7.4) and resuspended in staining buffer containing 25 ng/ml annexin V-FITC. The labeling was carried out at room temperature for 15 min in the dark before flow cytometric analysis. Each experiment was performed in duplicate and repeated at least three times. The values were presented as the mean±SE of at least three independent experiments. Tat-SOD and PTIO significantly blocked apoptosis of the cells ($*P < 0.001$) (A). The cells were resuspended in 750 μl of ice-cold lysis buffer for 45 min with occasional shaking. DNA was extracted with phenol and precipitated with alcohol. The pellet was dried and resuspended in 100 μl of 20 mM Tris-HCl, pH 8.0. After digesting RNA with RNase (0.1 mg/ml) at 37°C for 1 h, the samples (5 μg) were electrophoresed through an 1% agarose gel in 45 mM Tris-acetate-EDTA buffer, pH 8.0. DNA was photographed under UV light (B). This is a representative of two independent experiments.

Phagocytosis of SOZ and IOZ particles produced superoxide, wheras LPS/IFN-γ produced NO

Formation of important mediators of apoptosis, superoxide and NO during the activation or phagocytosis in macrophages was reported (Forman and Tores, 2001). For the measurement of superoxide formation, macrophages were suspended in KRG buffer, then stimulated by SOZ/ and IOZ/macrophages (100:1) or LPS/IFN-γ for 30 min, and superoxide anions were measured by chemiluminescence assays using luminol and luminometer. It was known that 1 μg/ml LPS plus 50 U/ml IFNγ was enough to induce NO formation (Lakics and Vogel, 1998). As seen in Figure 4 superoxide anions were generated by all the indicated stimuli, and Tat-SOD seriously reduced the superoxide levels. Especially, IOZ induced superoxide formation much more than SOZ. It is believed that the superoxide was produced by NADPH oxidase during phagocytosis. LPS/IFN-γ could also induced small amounts of superoxide (about 15% compared to IOZ stimulus) in macrophages. For the measurement of NO generation in macrophages, the cells were cultured in phenol red-free DMEM media and then stimulated by SOZ/ and IOZ/macrophages (100:

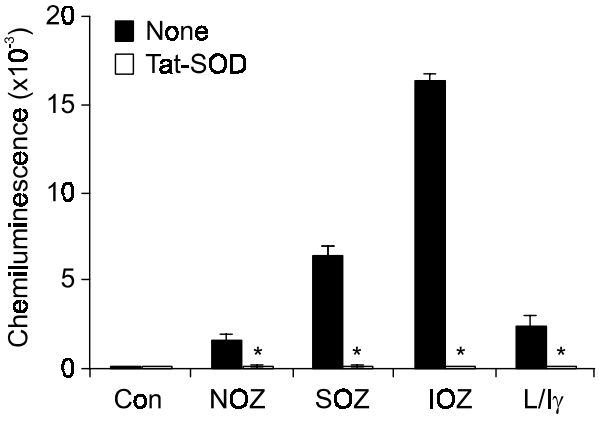

Figure 4. Superoxide formation during phagocytosis. Cells were harvested and washed 3 times with PBS, and resuspended in 1 μl of KRG buffer containing 50 U/ml SOD, 2,000 U/ml catalase and 0.05 mM luminol. The reaction of superoxide formation was started by addition of NOZ, SOZ and IOZ (300:1), or 1 μg/ml LPS plus 50 U/ml IFN-γ, and the subsequent chemiluminiscence was measured by luminometer. Macrophages were treated with Tat-SOD for 30 min to test the superoxide formation. The values were presented as the mean ± SE of at least three independent experiments. Tat-SOD significantly inhibited chemiluminescence intensity (*$P < 0.001$).

1) or LPS/IFN-γ for 12 h. Subsequently, Nitrite level in the culture supernatant was measured by Griess reagent assays, the expression of inducible NO synthase (iNOS) was detected by western blot of cell lysates, and intracellular NO was demonstrated by the confocal microscope using DAF staining. As shown in Figure 5A and B, a large amount of NO was generated in macrophage cells by LPS/IFN-γ, whereas a very little amount of NO was detected in the cells treated with SOZ and IOZ particles. Furthermore, the NO level induced by LPS/IFN-γ were significantly reduced by PITO (Figure 5A). Moreover, iNOS expression was detected in the cells stimulated with LPS/IFN-γ (Figure 5C), which was in accord with the production of NO. RhoA was indicated as a control protein, which did not alter its expression level irrespective of stimuli (Figure 5C).

Apoptosis of the cells accompanied with release of cytochrome *c* and activation of procaspase-3

To assess whether the release of cytochrome *c* was involved in apoptosis of macropahges during phagocytosis, the distribution of cytochrome *c* between mitochondrial membrane and cytosolic fractions of macro-

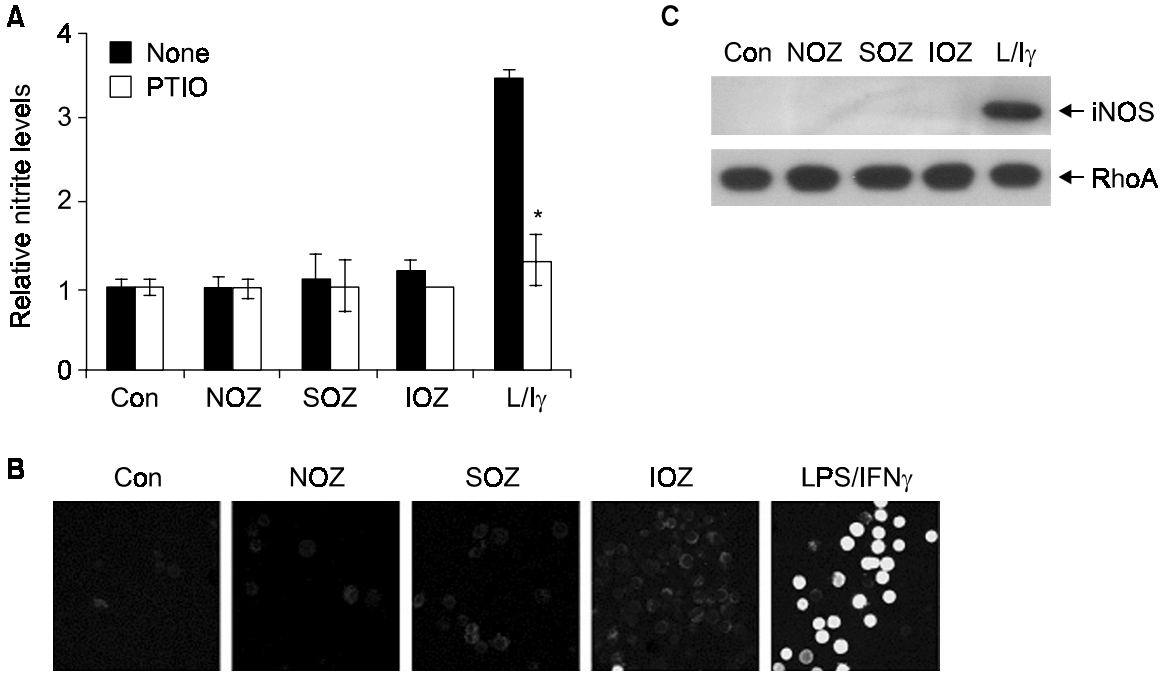

Figure 5. NO formation by phagocytosis. Cells were treated with NOZ, SOZ and IOZ (300:1), and 1 μg/ml LPS plus 50 U/ml IFN-γ for 12 h. Aliquots of conditioned media were mixed with an equal volume of Griess reagent, and nitrite concentrations were determined by comparison of absorbance at 550 nm, using standard sodium nitrite solutions prepared in cell culture medium The values were presented as the mean ± SE of at least three independent experiment. PTIO significantly inhibited the nitrite level of macrophages treated with 1 μg/ml LPS plus 50 U/ml IFN-γ (*$P < 0.001$) (A). Cells were washed in PBS and then treated with 10 μM DAF-FM diacetate for 30 min at 37°C. Intracellular NO formation was seen by green fluorescence of DAF-FM in live macrophage cells by conforcal microscope. This is a representative of three independent experiments (B). Expression of iNOS protein was detected by anti-iNOS antibody by western blot methods, and RhoA was compared as controls. This is a representative of three independent experiments (C).

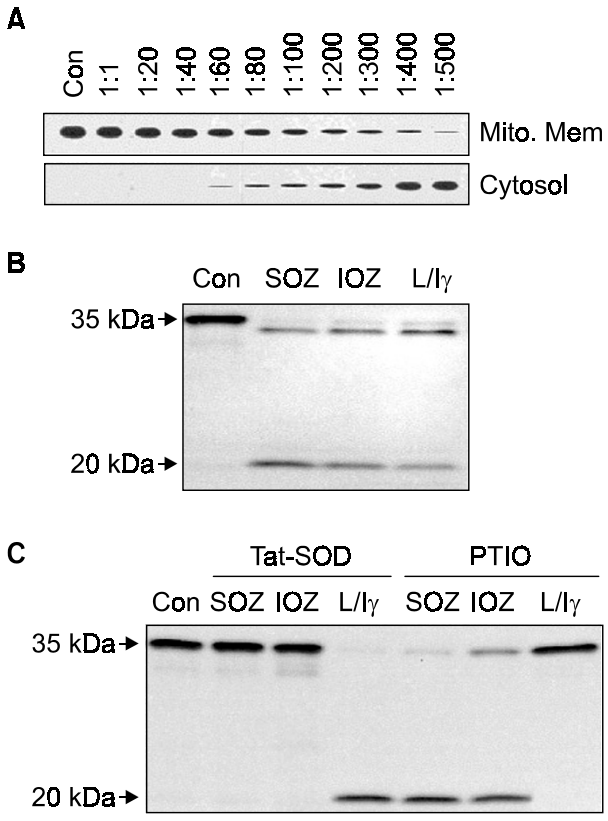

Figure 6. Cytochrome *c* release and activation of the caspase-3 by phagocytosis. Cells were incubated with various amounts of SOZ for 12 h, and cytochrome *c* released from mitochondria to cytosol was then immunobloted with anti-cytochrome *c* antibody (A). The cells were treated with SOZ, IOZ (300:1) or 1 μg/ml LPS plus 50 U/ml IFN-γ for 24 h. Procaspase-3 and claeved activated caspase-3 were then immunoblotted with anti-caspases-3 antibody (B). The cells were pretreated with or without Tat-SOD or PTIO for 30 min, and then treated with SOZ, IOZ (300:1) or 1 μg/ml LPS plus 50 U/ml IFN-γ for 24 h. Procaspase-3 and claeved activated caspase-3 were then immunoblotted with anti-caspases-3 antibody (C). These are representatives of at least three independent experiments.

phage cells was measured. Figure 6A shows that release of cytochrome *c* from mitochondrial membranefraction to cytosolic fraction was increased as the ratio of the SOZ/macrophage increased. To examine the involvement of caspase system in the apoptosis of the cells treated with SOZ and IOZ particles, the activation of caspase-3, which is a component of apoptosome to bring apoptosis was measured. SOZ, IOZ, and LPS/IFNγ induced activation of caspase-3 through cleavage of 35 kDa procaspase-3 to 20 kDa caspase-3 (Figure 6B). Tat-SOD inhibited the activation of caspase-3 induced by SOZ and IOZ, but did not inhibit the activation of caspase-3 when induced by LPS/IFNγ. Conversely, PTIO inhibited the activation of caspase-3 induced by LPS/IFN-γ, but did not inhibit it when induced by SOZ and IOZ (Figure 6C). Thus, these results showed that the superoxide, but not NO, produced during phagocytosis of SOZ and IOZ had signaling events of cytochrome *c* release and activation of caspase-3 for the cell death.

Disscussion

In the present study, we observed that high ratio of SOZ/ and IOZ/macrophage induced apoptosis of macrophages during phagocytosis (Figure 1, 2 and 3). Initial observation on the morphological state of the cells exposed to the high ratio of SOZ and IOZ particles (300:1-500:1) indicated healthy state of the cells (Figure 1). Tat (aa 49-57) is a transduction domain of human immunodeficiency virus-1 (HIV-1) and has been used to transduce many proteins into various types of cells (Kwon *et al.*, 2000; Yoon *et al.*, 2002). In the present study, Tat-SOD effectively blocked SOZ- and IOZ-induced, but not LPS/IFN-γ induced, apoptosis of macrophages (Figure 3A), indicating that superoxide but not NO generated during phagocytosis of SOZ and IOZ was a prerequisite for induction of apoptosis of the macrophages (Figure 3 and 4). In addition, it was confirmed that both SOZ and IOZ particles did not induce NO production in macrophages (Figure 5). On the other hand, LPS/IFN-γ induced iNOS and NO but not superoxide (Figure 4 and 5) and induced apoptosis in macrophage (Figure 3), which was blocked by PTIO, a NO scavenger. LPS is know to induce apoptosis in macrophage through production of TNF-α and NO (Xaus et al., 2000), and LPS plus IFN-γ enhanced induction of macrophage apoptosis through NO production (Lakios and Vogel, 1998), which are in accord with our results (Figure 3, 5 and 6). Thus, it was found that mechanisms of SOZ- and IOZ-induced and LPS plus IFN-γ induced apoptosis are different each other.

NOZ induced a little suproxide and apoptosis of the cells (Figure 3 and 4), implying that the stimulation of the cells by zymosan particles alone and the phagocytosis of NOZ did not produce much superoxide nor apoptosis. It remains to be elucidated whether FcγR-, CR-, and mannose receptor-mediated phagocytosis may have different mechanisms of phagosome formation and superoxide production, thus a complete complex formation of NADPH oxidase on the phagosome being different each other.

Despite evidence that ROS are common mediators of apoptosis in various types of cells, conflicting results have been reported regarding their role in neutrophil apoptosis. For example, the phagocytosis of bacteria by neutrophils, which is associated with production of ROS, has been shown to promote or inhibit apoptosis in these cells (Watson *et al.*, 1996; Mould-

ing *et al.*, 1999). Also, Fadeel *et al.* (1998) reported that the production of ROS in PMA-treated neutrophils led to inactivation of caspase-3. Such a discrepancy is most likely due to use of different stimuli to produce ROS. As suggested by the same group, mild oxidative stress can activate the caspase cascade and induce apoptosis, whereas prolonged or excessive oxidative stress (e.g. stimulated by PMA) prevents caspase activation (Hampton *et al.*, 1998). In the present study, the SOZ and IOZ induced the activation of caspase-3 and also cytochrome *c* release (Figure 6). In addition, LPS/IFN-γ, which induced NO production, also activated caspase-3 (Figure 6). NO can be transformed to more toxic peroxinitrite ($ONOO^-$) when NO reacts with O_2^-. H_2O_2 and NO act synergistically to induce apoptosis in which activation of caspase-3 (Wang *et al.*, 2003). In other systems, ROS has been shown to directly or indirectly 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.

Earlier studies have reported that neutrophil apoptosis is modulated by ROS produced through phagocytosis of CR3 (Coxon *et al.*, 1996) or FcR (Gamberale *et al.*, 1998). However, these earlier studies did not unravel concrete apoptosis pathway; they did not show any the relationship between superoxide formation by NADPH-oxidase in phagosome and cytochrome *c* release or caspase activation, which are key points of apoptosis. Nevertheless, a question still remains to be elucidated how the superoxide formation during phagocytosis, which is believed to occur through NADPH oxidase in phagosome, induced the cytochrome *c* release and capase-3 activation. On the other hand, 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. 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. We also observed that $p21^{WAF1}$ was induced, when macrophages were treated with SOZ particles (Figure 2C). However, it remains to be answered how $p21^{WAF1}$ is induced in SOZ-treated macrophages and what is its concrete function in apoptosis. 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). On the other hand, $p21^{WAF1}$ is cleaved by caspase3 at the onset of apoptosis, losing its apoptosis-suppressing activity (Zhang *et al.*, 1999).

In conclusion, we showed in the present study that superoxide but not NO, which was produced during phagocytosis and believed to be catalyzed by NADPH oxidase, induced the apoptosis of macrophages, accompanied by cytochrome *c* release and caspase-3 activation.

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