Independence of plasma membrane blebbing from other biochemical and biological characteristics of apoptotic cells

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Independence of Plasma Membrane Blebbing from Other Biochemical and Biological Characteristics of Apoptotic Cells[1]

Akiko Shiratsuchi,*,† Tomoe Mori,† and Yoshinobu Nakanishi*,†,[2]

**Graduate School of Medical Science and †Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Ishikawa 920-0934*

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Plasma membrane blebs are observed in many types of apoptotic cells, but their physiological roles remain to be clarified. We examined whether there is a causative connection between membrane blebbing and other apoptotic changes in Jurkat cells induced to undergo apoptosis by doxorubicin in the presence or absence of Y-27632, an inhibitor of the Rho kinase ROCK-I. The inclusion of the drug made most membrane blebs disappear, while other changes, such as chromatin condensation, inactivation of mitochondrial enzymes, externalization of the membrane phospholipid phosphatidylserine, and removal of cell surface sialic acid, remained unaffected. Furthermore, these apoptotic cells were phagocytosed by macrophages as efficiently as normally apoptosing cells. These results indicate that blebbing of the plasma membrane occurs independently from other apoptotic changes and is not involved in the recognition and engulfment of apoptotic cells by macrophages.

Key words: apoptosis, membrane blebbing, phagocytosis, ROCK-I.

Apoptotic cells are rapidly and selectively eliminated from organisms, and this event is necessary for the maintenance of tissue homeostasis (*1–8*). The phagocytic removal of apoptotic cells is accomplished through an array of distinct biological reactions: migration of phagocytes to the place where apoptotic cells exist, selective recognition and engulfment of target cells by phagocytes, and digestion of engulfed apoptotic cells in phagocytes. Although the importance of this phenomenon is widely accepted, the underlying molecular bases remain mostly unknown.

Among the reactions listed above, the recognition step has been most intensively studied. The surface of apoptotic cells changes structurally and functionally in order for them to be efficiently and selectively recognized by phagocytes (*9–11*). The most obvious change occurs in the composition of phospholipids in the plasma membrane. Phospholipids are distributed asymmetrically in the two leaflets of the membrane bilayer in normal cells (*12, 13*), but this asymmetry is disrupted in apoptotic cells through a caspase-dependent mechanism, resulting in the equal distribution of phospholipids in the two leaflets (*14, 15*). This change leads to the surface exposure of phospholipids that are otherwise hidden within the cytoplasmic face; phosphatidylserine (PS) is one such phospholipid specifically present at the surface of apoptotic cells and serves as a phagocytosis marker that binds to phagocytes and induces engulfment (*14–16*). Phagocytosis-inducing PS receptors have been identified in various phagocytes such as vascular endothelial cells (*17*), testicular Sertoli cells (*18*), and inflammatory macrophages (*19*).

Another change commonly observed at the surface of apoptotic cells is membrane blebbing, a more obvious structural alteration of the plasma membrane. Distinct parts of the membrane of apoptotic cells begin to swell, and apoptotic cells eventually look like raspberries with many "blebs" on their surface (*20*). Plasma membrane blebs are thought to serve as the sites for binding of functional molecules such as antigens (*21–24*) and phagocytosis markers (*25*), and might thus be involved in the recognition and engulfment of apoptotic cells by phagocytes. Although the detailed molecular basis for the formation of blebs remains unknown, the involvement of caspases and a Rho protein kinase, ROCK-I (*26, 27*), has been reported (*28–30*). In the present study, we examined the relationship between plasma membrane blebbing and other biochemical and biological changes observed in apoptotic cells using the ROCK-I inhibitor Y-27632 (*31, 32*).

MATERIALS AND METHODS

Cell Culture and Apoptosis Analysis—Jurkat cells, human leukemia T-cells, were grown in RPMI 1640 (Nissui Pharmaceutical, Tokyo) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were treated with doxorubicin (Sigma, St. Louis, MO, USA) (1.5 μg/ml) to induce apoptosis as described previously (*33*). To inhibit apoptosis, the pan-caspase inhibitor z-VAD-fmk (Peptide Institute, Osaka, Japan) (20 μM) was added to the culture 1 h before doxorubicin treatment. The occurrence of membrane blebbing was determined morpho-

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[2]To whom correspondence should be addressed. Tel: +81-76-234-4481, Fax: +81-76-234-4480, E-mail: nakanaka@kenroku.kanazawa-u.ac.jp

Abbreviations: FITC, fluorescein isothiocyanate; MAM, *Maackia amurensis* lectin; PS, phosphatidylserine; SAS, *Sambucus sieboldiana* lectin; WGA, wheat germ agglutinin.

logically by phase-contrast microscopy. For the inhibition of membrane blebbing, cells were pre-treated with Y-27632 (a gift from Mitsubishi Welpharma, Tokyo) 1 h before the induction of apoptosis. Plasma membrane permeability, the activity of mitochondrial enzymes, chromatin condensation, and DNA fragmentation were determined by standard procedures: a dye exclusion assay with trypan blue, an enzyme assay using 3-(4,5-dimethyl-2-thyazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (Dojin, Kumamoto) (MTT assay), staining with Hoechst 33342, and agarose gel electrophoresis, respectively (*33*). PS externalization and the loss of cell surface sialic acid were examined by flow cytometry (EPICS-XL; Beckman Coulter, Miami, FL, USA) using fluorescence-labeled annexin V and sialic acid–recognizing lectins, respectively (*34, 35*). Briefly, cells were incubated with fluorescein isothiocyanate (FITC)–labeled annexin V (Bender MedSystems, Vienna, Austria) (*36, 37*) or FITC-labeled lectin [wheat germ agglutinin (WGA), *Maackia amurensis* lectin (MAM), or *Sambucus sieboldiana* lectin (SAS)] (Honen Corporation, Tokyo) in the presence of propidium iodide, and the cells that were less intensely stained with propidium iodide were gated and analyzed for the binding of FITC-annexin V or FITC-lectin.

Western Blotting—Jurkat cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.1 M NaCl, and a protease inhibitor cocktail (Sigma), incubated on ice for 30 min, and centrifuged at 18,000 ×*g* for 10 min at 4°C. The solubilized proteins were electrophoretically separated on a 10% SDS–polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA, USA). The membrane was blocked with 5% dry skim milk, reacted successively with anti-human ROCK-I antibody (C-17 or K-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and alkaline phosphatase-conjugated anti-goat IgG (Bio-Rad Laboratories, Hercules, CA, USA), and chemiluminescently analyzed for signals using Immun-Star substrates (Bio-Rad). To examine the specificity of the antibody reactions, the first antibody was pre-incubated with an excess amount of the corresponding antigen peptide (Santa Cruz Biotechnology).

Macrophage Preparation and Phagocytosis Assay—Macrophages were prepared from peritoneal fluids of thioglycollate-injected BDF_1 mice (female, 8–12 weeks old) and cultivated on coverslips until use as described previously (*33, 38*). The phagocytosis assay was performed essentially as described (*33, 38*). Briefly, apoptotic Jurkat cells were mixed with macrophages at a ratio of 10 target cells to one macrophage, and incubated at 37°C for various lengths of time. The mixture was treated with trypsin to remove target cells lightly attached to macrophages. The remaining macrophages were fixed with paraformaldehyde, permeabilized with methanol, and stained with hematoxylin. The numbers of macrophages with engulfed Jurkat cells and the number of engulfed cells present in each macrophage were determined by examination under a light microscope. The scores were expressed relative to the total number of macrophages (in percentage; the phagocytic index) and per 100 macrophages, respectively, as the mean and SD of a

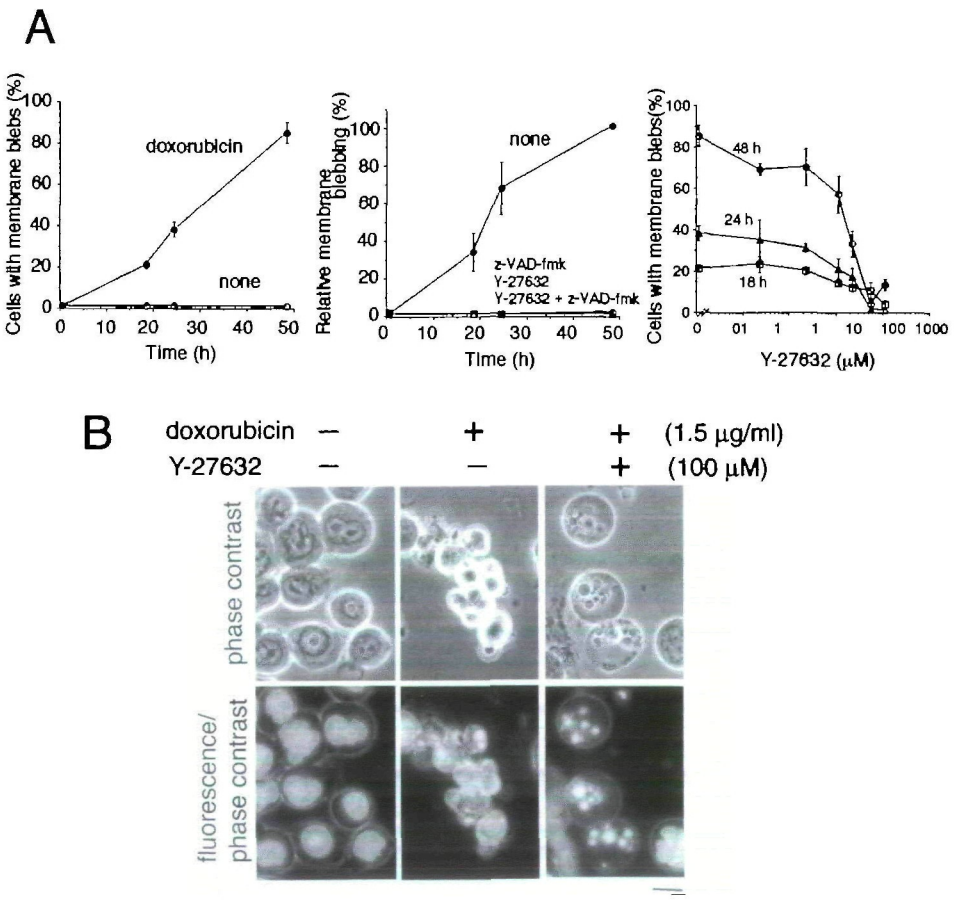

Fig. 1. **Inhibition of membrane blebbing by Y-27632 in doxorubicin-treated Jurkat cells.** A: Jurkat cells were induced to undergo apoptosis by doxorubicin with or without 1-h pre-treatment with z-VAD-fmk and/or Y-27632 (100 μM except for the right panel), and the number of cells containing plasma membrane blebs was determined by microscopic examination. In the middle panel, the number of cells with blebs is shown relative to the number of cells with blebs in the absence of inhibitor at time 50 h, taken as 100. In the right panel, cells were pre-incubated with Y-27632 at various concentrations and then treated with doxorubicin for the indicated periods. B: Jurkat cells treated or not treated with Y-27632 for 1 h were incubated in the presence or absence of doxorubicin for 24 h and stained with Hoechst 33342. Phase contrast and fluorescence/phase contrast views of the same fields are shown. Bar = 10 μm.

typical example from at least three independent experiments. Liposomes composed of a combination of phosphatidylcholine and PS at a ratio of 7:3 were prepared as described previously (*34*).

RESULTS

Inhibition of Membrane Blebbing by ROCK-I Inhibitor Y-27632—Jurkat cells were induced to undergo apoptosis by treatment with doxorubicin and examined for the occurrence of plasma membrane blebbing (Fig. 1). Distinct parts of the cell periphery protruded like balloons (Fig. 1B) which became evident 18 h after doxorubicin addition and continued to increase thereafter (left panel in Fig. 1A). These results indicate that treatment with doxorubicin effectively induces plasma membrane blebbing in Jurkat cells. This change in the structure of the plasma membrane almost completely disappeared when Jurkat cells were incubated with the pan-caspase inhibitor z-VAD-fmk prior to the addition of doxorubicin (middle panel in Fig. 1A). Furthermore, the ROCK-I inhibitor Y-27632 showed a similar inhibitory effect on membrane blebbing in a dose-dependent manner (middle and right panels in Fig. 1A and Fig. 1B). These results indicate that plasma membrane blebbing in apoptotic Jurkat cells depends on the activity of both caspases and ROCK-I, and this accords well with previous observations using other types of cells and apoptosis inducers (*29, 30, 39–41*).

ROCK-I is activated by the caspase-mediated cleavage of its C-terminal region in apoptotic cells (*29, 30*). In order to verify that this occurs in doxorubicin-treated Jurkat cells, whole-cell lysates were analyzed by Western blotting with anti-ROCK-I antibody (Fig. 2). A signal with a molecular mass of about 160 kDa was detected, and its intensity decreased during apoptosis (top panel in Fig. 2A). This signal resulted from the specific reaction with the antibody, since the inclusion of the corresponding antigen peptide in the antibody reaction completely eliminated the signal (bottom panel in Fig. 2A). These results indicate that the amount of the intact form of ROCK-I decreases during apoptosis, most probably due to cleavage by caspases. This change was abrogated, though incompletely, by the addition of z-VAD-fmk (top panel in Fig. 2B), but, unexpectedly, ROCK-I appeared to be cleaved after the induction of apoptosis even in cells pre-treated with Y-27632 (bottom panel in Fig. 2B). These results conflict with previous observations that ROCK-I does not undergo cleavage in the presence of Y-27632 (*29, 30*), and suggest that the inhibition of plasma membrane blebbing by Y-27632 is achieved through a mechanism other than interference with the activation of ROCK-I.

No Effect of Y-27632 on Other Biochemical Apoptotic Changes—We examined whether or not the presence of Y-27632 inhibits other biochemical apoptotic changes in doxorubicin-treated cells. The condensation of chromatin (Fig. 1B and Fig. 3A), inactivation of mitochondrial enzymes (Fig. 3B), and externalization of PS (Fig. 3C) all occurred normally even in the presence of Y-27632. In addition, the drug did not affect the removal of cell surface sialic acid, which was evident from a decrease in the amount of sialic acid-recognizing lectins bound to cells. Essentially the same results were obtained with three different lectins, WGA, MAM, and SAS (Fig. 3D).

No Effect of Membrane Blebbing on Susceptibility to Phagocytosis—We previously reported that doxorubicin-treated Jurkat cells are phagocytosed by thioglycollate-induced mouse peritoneal macrophages in a manner partly mediated by PS expressed on the surface of target cells during apoptosis (*33*). The removal of cell surface sialic acid, another change that occurs at the surface of apoptotic cells, has been shown to be necessary for efficient phagocytosis (*35*). We therefore examined whether or not the presence of plasma membrane blebs is involved in the phagocytosis of apoptotic cells by macrophages. To do so, the phagocytosis assay was conducted with Jurkat cells that had been induced to undergo apoptosis by doxorubicin with and without Y-27632 pre-treatment (Fig. 4). The extent of phagocytosis was determined based on the ratio of macrophages that accomplished phagocytosis or on the number of engulfed cells in each macrophage. Cells pre-treated with Y-27632 were phagocytosed with a time course and efficiency similar to those of normally apoptosing cells using either criterion (Fig. 4A). Phagocytosis was partly inhibited by the addition of PS-containing liposomes, and the extent of inhibition was almost equal in reactions with apoptotic Jurkat cells treated or not treated with Y-27632 (Fig. 4B). This indicates that plasma membrane blebbing is not involved in phagocytosis either mediated or not mediated by cell surface PS.

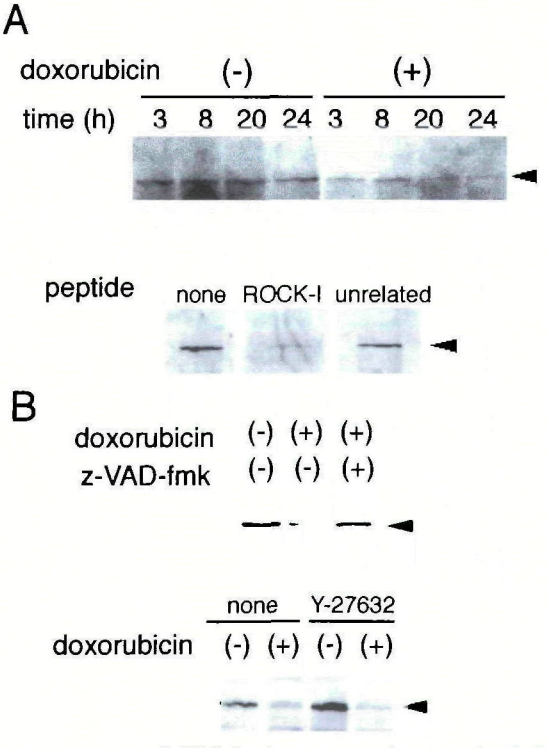

Fig. 2. **Cleavage of ROCK-I during apoptosis.** A: (top) Whole-cell lysates of Jurkat cells treated or not treated with doxorubicin for the indicated periods were analyzed by Western blotting with anti-ROCK-I antibody. The position of a 160-kDa signal is indicated by the arrowhead. (bottom) The reaction with anti-ROCK-I antibody was performed in the presence or absence of the corresponding antigen peptide (ROCK-I) or a peptide with an unrelated sequence. B: Jurkat cells were pre-treated for 1 h with z-VAD-fmk or Y-27632 (100 μM) prior to the addition of doxorubicin and analyzed after 18 h (top) or 24 h (bottom) as in (A).

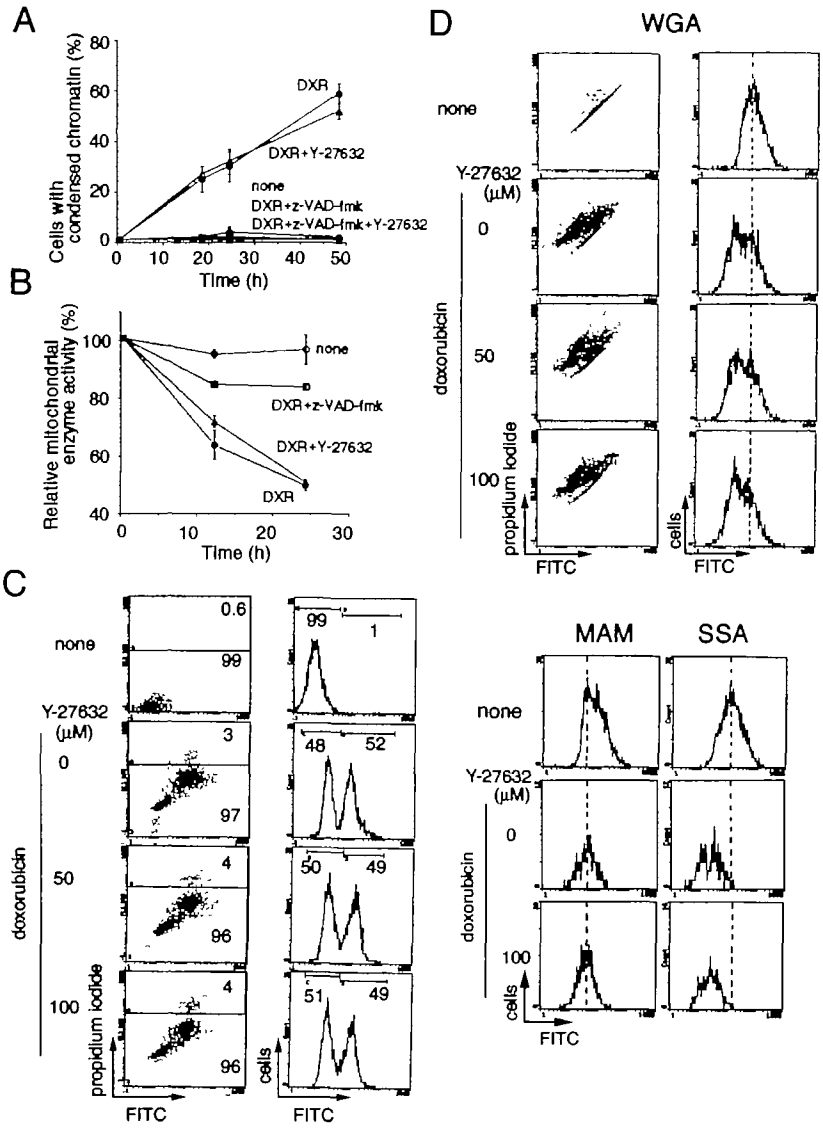

Fig. 3. **No effect of Y-27632 on other apoptotic changes.** Jurkat cells were pre-treated for 1 h with Y-27632 (at 100 μM or the indicated concentrations) and/or z-VAD-fmk, incubated in the presence or absence (indicated by the symbol 'none') of doxorubicin, and analyzed for the occurrence of apoptotic changes. A: Cells incubated in the presence or absence of doxorubicin (DXR) for the indicated periods were treated with Hoechst 33342 and examined for the presence of intensely stained nuclei. B: Cells incubated in the presence or absence of doxorubicin (DXR) were subjected to the MTT assay, and the level of mitochondrial enzyme activity is expressed relative to that of normal cells, taken as 100. C: Cells incubated for 24 h in the presence or absence of doxorubicin were examined for PS externalization by flow cytometry. Cells less intensely stained with propidium iodide (bottom area in the left panels) were analyzed for the binding of FITC-annexin V (right panels). The numbers indicate the ratios of cells (in percentage) in the corresponding areas. D: Cells were incubated for 24 h in the presence or absence of doxorubicin and examined for the binding of FITC-labeled WGA, MAM, or SAS by flow cytometry. The vertical lines indicate the mean fluorescence with cells untreated with doxorubicin.

DISCUSSION

Plasma membrane blebbing is one of the hallmarks of apoptosis, but its mechanism and physiological consequences remain to be elucidated. In the present study, we examined whether there is a causative connection between membrane blebbing and other intracellular as well as surface changes evident during apoptosis. For this purpose, chromatin condensation, inactivation of mitochondrial enzymes, PS externalization, and cell surface desialylation were determined in apoptotic Jurkat cells treated with a drug that completely inhibits plasma membrane blebbing. We found that all of these biochemical changes occurred in apoptotic cells with no blebs on the plasma membrane. This clearly indicates that plasma membrane blebbing does not occur upstream of these changes in the apoptotic pathway. Both caspase-dependent and -independent mechanisms seem to be responsible for blebbing of the plasma membrane (*29, 30, 40–44*). Our results show that the appearance of membrane blebs in doxorubicin-treated Jurkat cells depends on caspases and is completely inhibited by the ROCK-I inhibitor Y-27632. However, the cleavage of ROCK-I, which reportedly leads to the activation of this protein kinase, was evident even in the presence of Y-27632. This was also the case when another apoptosis inducer, an agonistic anti-Fas antibody, was used to kill Jurkat cells (data not shown). Given that Y-27632 specifically targets ROCK-I, the drug might abrogate the function of ROCK-I not by inhibiting the partial cleavage in the inactive precursor, but by inhibiting the action of the cleaved and thus activated ROCK-I. However, this is only speculative, and further studies are needed to resolve the issue.

Plasma membrane blebbing is a change that occurs at the surface of apoptotic cells in addition to alterations in the composition of phospholipids and the structure of sugar moieties. Among the surface changes reported so far, PS externalization and desialylation make apoptotic cells more susceptible to phagocytosis, an important biological consequence of apoptosis. Inhibition of plasma membrane blebbing did not influence the extent of either PS externalization or desialylation, indicating that the latter two

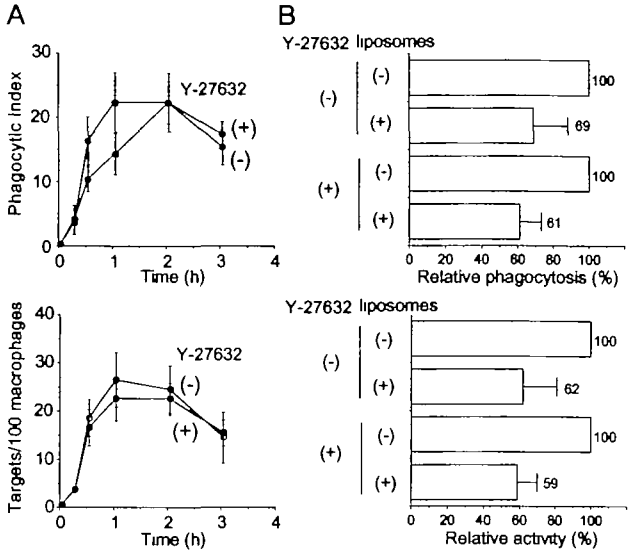

Fig. 4. **Efficient phagocytosis by macrophages of apoptotic cells with no membrane blebs.** Jurkat cells induced to undergo apoptosis by doxorubicin for 24 h with or without 1-h pre-treatment with Y-27632 were subjected to the phagocytosis assay with mouse peritoneal macrophages. A: Time course of phagocytosis reaction. The phagocytic index (top) and number of engulfed cells per 100 macrophages (bottom) were determined at the indicated time points of the phagocytosis reaction. B: Effect of PS-containing liposomes. Phagocytosis reactions were conducted for 2 h in the presence or absence of PS-containing liposomes (1 mM). The phagocytic index (top) and the number of engulfed cells (bottom) are shown relative to reactions with no added liposomes, taken as 100. The means of the phagocytic index and the number of engulfed cells in control reactions were 34 (without Y-27632) and 27 (with Y-27632), and 26 (without Y-27632) and 23 (with Y-27632), respectively.

changes occurring at the surface of apoptotic cells are unrelated to membrane blebs. Furthermore, apoptotic Jurkat cells with and without membrane blebs were equally phagocytosed by macrophages, and the addition of PS-containing liposomes inhibited phagocytosis of apoptotic Jurkat cells treated or not treated with Y-27632 to similar extents. These results indicate that plasma membrane blebs are not involved in phagocytosis either mediated or not mediated by PS. The PS-independent reactions in our assay do not seem to require factors present in serum, because the efficiency of phagocytosis was the same for reactions with and without serum (data not shown). Therefore, it remains possible that membrane blebs play a role in complement- or Fc-mediated recognition of apoptotic cells by phagocytes. In fact, C1q preferentially binds to apoptotic cells at plasma membrane blebs (*45*). Apart from phagocytosis, membrane blebs could be involved in the transmission of signals from apoptotic cells to their neighbors, as shown with apoptotic epithelial cells extruded from the epithelium (*46*). Further studies from various points of view will be necessary to clarify the physiological consequences of plasma membrane blebbing.

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