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Discrimination of Early and Late Apoptotic Cells by NBD-phosphatidylserine-labelling and Time-lapse Observation of Phagocytosis of Apoptotic Cells by Macrophages

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Since free apoptotic cells are not detected in normal tissues, it is generally believed that apoptotic cells are removed as soon as they appear *in vivo*. A fluorescent derivative of phosphatidylserine, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl]-*sn*-glycero-3-phospho-L-serine (NBD-PS) is known to be incorporated into living cells, and thereafter gradually absorbed into either fatty acid-free bovine serum albumin or fetal calf serum from the outer leaflet of the cell membrane. When thymocytes were irradiated with X-ray and cultured in the presence of NBD-PS, cells became less fluorescent as apoptosis advanced, but early apoptotic cells were still positive for NBD-PS. We then co-cultured such early apoptotic thymocytes with resident peritoneal macrophages. Upon examination under a time-lapse fluorescence microscope, it was found that the attachment of early apoptotic cells to macrophages does not cause rapid phagocytosis, as compared with late apoptotic cells, suggesting the possibility that, in contrast to the widely held view, early apoptotic cells may not be quickly removed by phagocytes *in vivo*.

Key words: apoptotic cells; macrophages, phagocytosis, phosphatidylserine, time-lapse observation.

Abbreviations: NBD-PS, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phospho-L-serine; PS, phosphatidylserine; BSA, bovine serum albumin; FCS, fetal calf serum; GST, glutathione S transferase; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; ANX buffer, annexin buffer; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; PI, propidium iodide.

When cells are infected with a virus, reach the end of their life span or cease to function properly, they undergo apoptosis (*1–3*). Because a large number of apoptotic cells are constantly generated *in vivo* and because there are few free apoptotic cells in normal tissues, phagocytes such as macrophages should remove apoptotic cells as soon as they appear. In support of this, we previously found that cells rich in early apoptotic cells are indeed phagocytosed by macrophages (*4*). It should be noted, however, that late apoptotic cells are much more significantly phagocytosed than early apoptotic cells (*4*). Since the apoptotic cell population contains varying degrees of late apoptotic cells even in the population rich in early apoptotic cells, the possibility remains that macrophages phagocytose such late apoptotic cells in the population rich in early apoptotic cells.

Macrophages recognize multiple 'eat me signals', such as modification of cell surface antigens and loss of asymmetry of the plasma membrane phospholipid bilayer, on apoptotic cells (*7, 8*). In normal cells, PS is mostly located in the inner leaflet of the cell membrane, whereas, when apoptosis occurs, cells start losing their plasma membrane asymmetry with the externalization of PS (*9*). Macrophages should recognize externalized PS on the surface of apoptotic cells if the level of it is higher than a certain threshold (*10*). Although externalized PS is often detected with fluorescent annexin V, annexin V is reported to inhibit phagocytosis (*11*), and so the alternative method is required to observe phagocytosis of early apoptotic cells under a time-lapse microscope.

A fluorescent derivative of PS (NBD-PS) is incorporated into living cells by ATP-dependent aminophospholipid translocase. NBD-PS then moves to the outer surface of a membrane by means of scramblase, and thereafter gradually absorbed into either fatty acid-free bovine serum albumin (BSA) or fetal calf serum (FCS) from the outer leaflet of the cell membrane (*12*). Since apoptotic cells express much more PS on the outer surface, the amount of NBD-PS should be lower than that in living cells. It should be noted that late apoptotic cells lose ATP-dependent aminophospholipid translocase activity (*13, 14*). However, it was not known what stage(s) of apoptotic cells retained NBD-PS.

In this study, we examined whether or not early apoptotic cells were still positive for NBD-PS. We then compared phagocytosis of early apoptotic cells with that

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of late apoptotic cells under a time-lapse fluorescence microscope.

MATERIALS AND METHODS

Animals—Male C57BL/6 mice were purchased from Sankyo Lab. Service Co. (Tokyo, Japan) and maintained in a conventional animal facility.

Preparation of Recombinant Annexin V—Recombinant annexin V fused with glutathione *S* transferase (GST) was expressed in *Escherichia coli* after transfection of the expression plasmid and purified with glutathione-sepharose 4B. Alexa Fluor 568 succinimidyl ester was purchased from Invitrogen Co. (Carlsbad, CA, USA). Purified GST-annexin V was conjugated with the dye according to the standard procedure. It was noted that the binding of fluorescein isothiocyanate (FITC)-labelled annexin V (Bender MedSystems Inc., CA, USA) to apoptotic cells was inhibited by unconjugated GST annexin V in a dose-dependent manner upon flow cytometric analysis. Hereafter, GST-annexin V is called annexin V.

Induction of Apoptosis of Thymocytes—Thymocytes were obtained from C57BL/6 mice (from 6 to 8 weeks old) and suspended in RPMI 1640 medium supplemented with 7% FCS to a cell density of 5×10^6 cells/ml, followed by X-ray irradiation (Hitachi MBR-1505R2;12 Gy at 0.45 Gy/min; irradiation parameters: 150 KV, 5 mA) to induce apoptosis. NBD-PS (Avanti Polar Lipids, Inc., Alabaster, USA), diluted to 100 μg/ml with RPMI1640 without phenol red containing 0.25% fatty acid-free BSA (Sigma), was added to thymocytes to a final concentration of 10 μg/ml. After incubation for 2, 4, 10 or 24 h, labelled thymocytes were washed with cold 20 mM phosphate buffered saline (pH 7) (PBS) twice and then diluted with RPMI 1640 without phenol red supplemented with 7% FCS to a cell density of 8×10^5 cells/ml.

Binding and Phagocytic Assaying under a Fluorescence Microscope—Resident peritoneal cells were obtained from C57BL/6 mice with 4 ml of cold PBS, followed by pre-incubation in RPMI 1640 medium supplemented with 7% FCS at a cell density of 5×10^5 cells/well for 60 min at 37°C in a 24-well plate over which a cover glass (0.15–0.17 mm in thickness) had been placed. The peritoneal cells were washed with PBS three times, and then co-cultured with apoptotic thymocytes labelled with NBD-PS at a cell density of 2×10^6 cells/well in RPMI 1640 medium supplemented with 7% FCS for 30 min at 37°C. After washing with warmed PBS three times to remove unbound cells, adherent cells were immediately stained with 8 μg/mL of Alexa-588 labelled annexin V in annexin buffer (ANX buffer) containing 140 mM NaCl, 2.5 mM $CaCl_2$ and 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (Hepes), pH 7.3, in the presence of 4 mM NaN_3 for 15 min on ice. The cells were washed with ANX buffer containing NaN_3 three times. The cover glass was then removed, placed on a slide glass and examined under a fluorescence microscope (IX71, Olympus, Tokyo, Japan) with an Aqua Cosmos system (Hamamatsu Photonics, Shizuoka, Japan). The resultant glass-adherent mouse peritoneal cells include $73 \pm 10\%$ of Mac-1-positive cells and $93 \pm 3\%$ of macrophages, as judged on Diff–Quik staining (Dade Diagnostics, Maimi, FL, USA).

Time-lapse Observation under a Fluorescence Microscope—Resident peritoneal cells were added to a glass bottomed dish (35 mm in diameter) as described earlier. After washing with warmed PBS, RPMI1640 medium containing 7% FCS and 10 mM Hepes without phenol red was added to the dish. The dish was then set in the culturing unit on the stage of a time-lapse fluorescence microscope. The temperature in the dish was kept at 37°C, and the unit was filled with 5% CO_2 and 95% air. Then either a cell population rich in early apoptotic thymocytes, which were labelled with NBD-PS, or one rich in late apoptotic thymocytes was added to the dish, followed by time-lapse observation under a microscope. Whether cells are attached or phagocytosed was determined individually, based on mobility (non-attached cells can move around) and a change in shape (upon phagocytosis, macrophages embrace apoptotic cells).

RESULTS

A Change in NBD-PS Fluorescence During Apoptosis—NBD-PS is incorporated into living cells, and thereafter gradually absorbed into either fatty acid-free BSA or FCS from the outer leaflet of the cell membrane.

Thymocytes were irradiated with X-ray to induce apoptosis and then cultured in the presence or absence of NBD-PS. Flow cytometric analysis indicated that the culturing of irradiated thymocytes caused a decrease in NBD-PS and an increase in Alexa-568 labelled annexin V staining concomitantly with progression of apoptosis (Fig. 1A).

We then examined whether or not early apoptotic cells were positive for NBD-PS flow cytometrically. After culturing X-irradiated thymocytes for 4 or 10 h in the presence of NBD-PS, the cells were then stained with Alexa-568 labelled annexin V or propidium iodide (PI), respectively. After a 4-h culture, NBD-PS positive cells comprised annexin V-positive ones and annexin V-negative ones, and all the cells were PI-negative (Fig. 1B left), indicating that cells are at an early stage of apoptosis. It should be noted that the amount of NBD-PS in annexin V-positive cells is lower than that in annexin V-negative ones. In other words, NBD-PS dull positive cells appear to correspond to annexin V-positive ones. On the other hand, after a 10-h culture, NBD-PS dull positive cells comprised PI-negative ones and PI-positive ones, and all the cells were annexin V-positive (Fig. 1B, right), indicating that cells are at an early and a late stages of apoptosis. As apoptosis advanced, the percentage of annexin V-positive cells increased concomitantly with that of NBD-PS dull positive ones (Fig. 1C), further suggesting that NBD-PS dull positive cells correspond to annexin V-positive ones.

We then determined under a fluorescence microscope what stage(s) of apoptotic cells were positive for NBD-PS and how often a cell population rich in early apoptotic cells is phagocytosed. X-irradiated thymocytes were labelled with NBD-PS for 4 h, and then co-cultured with macrophages, followed by washing and staining with Alexa 568-labelled annexin V (red). In Fig. 2, there were thymocytes positive for NBD-PS and annexin V

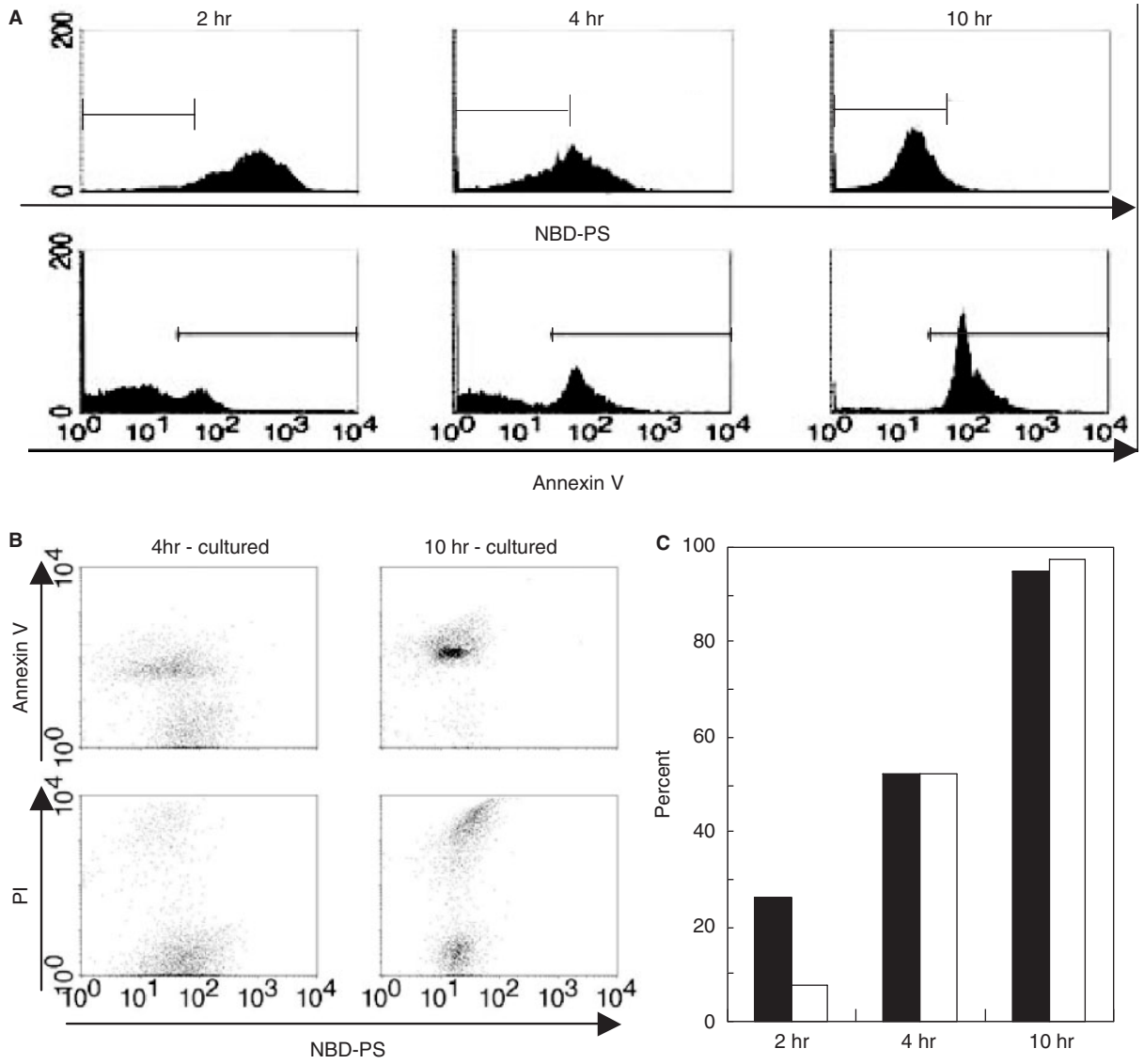

Fig. 1. **A change in NBD-PS fluorescence during apoptosis.** (A) After X-irradiation, thymocytes were cultured in the presence or absence of NBD-PS for various times. The cells not labelled with NBD-PS were then stained with Alexa-568 labelled annexin V. These cells were analysed by a flow cytometer (FACScan, Becton Dickinson). The bars indicate NBD-PS dull and annexin V-positive cells. (B) After X-irradiation, thymocytes were cultured in the presence of NBD-PS for 4 or 10 h, followed by staining with Alexa-568 labelled annexin V or PI, respectively. These cells were analysed by a flow cytometer (FACScan, Becton Dickinson). (C) The percentages of annexin V-positive cells (black) and those of NBD-PS dull positive cells (white) were shown. All the experiments were carried out several times, and the representative results were shown.

(indicated by arrows), being in good agreement with the result in Fig. 1. Some of these thymocytes were stained with annexin V and NBD-PS at the same sites where apoptotic thymocytes were attached to macrophages (Fig. 2, indicated by bold arrows), whereas others were stained with annexin V and NBD-PS at the different sites (Fig. 2, indicated by a thin arrow). There was a thymocyte positive for NBD-PS but not annexin V (Fig. 2, indicated by an arrowhead), which was presumed to be a very early apoptotic cell. On the other hand, there were thymocytes positive for annexin V but not NBD-PS, which were presumed to be an early but somewhat advanced apoptotic cell (Fig. 2, indicated by asterisks). In Fig. 2, almost no thymocytes were phagocytosed by macrophages.

Time-lapse Observation—We then examined the attachment and phagocytosis of a population rich in early apoptotic cells under a time-lapse fluorescence microscope. Thymocytes were cultured for 4 h in the presence of NBD-PS after X-ray irradiation to obtain a population rich in early apoptotic cells, which was then co-cultured with macrophages for 30 min in total. During the co-culture, we made a time-lapse observation under a fluorescence microscope. As shown in Fig. 3A (a right column, fluorescent field), under a time-lapse fluorescence microscope, only two NBD-PS-labelled apoptotic

thymocytes were phagocytosed within 10 min, as indicated by an arrow, an arrowhead and a change in colour from white to grey, the others only adhering, i.e., not being phagocytosed. Moreover, when macrophages showed a change in shape or location, thymocytes adhered to the macrophages remained attached suggesting that apoptotic thymocytes are firmly attached to macrophages. As shown in Fig. 3A (a left column, bright field), three cells were phagocytosed within 20 or 30 min, as indicated arrows, an arrowhead and a change in colour from black to grey. As shown in Fig. 3B (white), although 76% of thymocytes attached to macrophages were NBD-PS positive, only $8 \pm 7\%$ of NBD-PS positive thymocytes attached to macrophages were phagocytosed after incubation for 30 min. On the other hand, $45 \pm 14\%$ of NBD-PS negative thymocytes attached to macrophages were phagocytosed (Fig. 3B, grey).

We then examined the attachment and phagocytosis of a population rich in late apoptotic thymocytes under a time-lapse microscope. Thymocytes were cultured for 24 h in the presence of NBD-PS after X-ray irradiation to obtain the population. However, late apoptotic cells did not retain NBD-PS (*10,12*), and so only bright field images are shown. After late apoptotic thymocytes had become attached to macrophages (indicated by arrows in Fig. 4A), they were phagocytosed by macrophages within 10 min (indicated by a change in colour from black to grey). As shown in Fig. 4B, $77 \pm 8\%$ of thymocytes attached to macrophages were phagocytosed after incubation for 30 min. Latex microbeads were phagocytosed by macrophages as quickly as late apoptotic thymocytes. These results demonstrated that late apoptotic thymocytes were more rapidly phagocytosed by macrophages than early apoptotic thymocytes.

DISCUSSION

Early apoptotic cells are usually defined as annexin V-positive and PI-negative, while late apoptotic cells as annexin V-positive and PI-positive. Although NBD-PS dull positive cells appeared to be largely identical to annexin V-positive PI-negative ones upon flow cytometric analysis (Fig. 1B), there were NBD-PS-positive and annexin V-negative cells (very early apoptotic cells) and NBD-PS-negative and annexin V-positive cells (an early but somewhat advanced apoptotic cell) upon fluorescence microscopic analysis (Fig. 2). These findings indicated that NBD-PS is relatively well retained in very early and early apoptotic cells, but not late apoptotic cells. Consequently, NBD-PS-labelling allowed us to discriminate early apoptotic cells from late apoptotic cells without further staining.

Time-lapse observation demonstrated that, among the population rich in early apoptotic cells, NBD-PS-negative cells were more readily phagocytosed than NBD-PS-positive cells. Since the population was almost PI-negative, it is likely that NBD-PS-negative cells in the population were also PI-negative but somewhat advanced ones, as compared with NBD-PS-positive ones. Time-lapse observation also demonstrated that the population rich in late apoptotic cells was readily phagocytosed. Our study thus tended to support the previous work showing that only late apoptotic neuronal cells are phagocytosed by microglia (*15*), and suggested that early apoptotic cells can become attached to macrophages but are not immediately phagocytosed.

NBD-PS dull positive cells appeared to be largely identical to annexin V-positive PI-negative ones, and so PS is presumably involved in the attachment of early apoptotic cells to macrophages. Alternatively, it is possible that carbohydrate is involved in the attachment (*16, 17*), because the authors claimed that such recognition occurs when apoptotic cells begin to express PS (*17*). Under their conditions, however, approximately 20–25% of such cells attached to macrophages were phagocytosed during the 2-h assay (*16*), suggesting that, in contrast with our results, even very early apoptotic cells are phagocytosed by macrophages under certain conditions. It should be pointed out here that there are several differences in experimental conditions between these two studies (this study, *16*), including incubation time (30 min *vs*. 2 h), assay methods (observation at a single cell level *vs*. observation at a population level), apoptotic cells (X-irradiated mouse thymocytes *vs*. etoposide-treated human leukaemic cells) and macrophages (mouse peritoneal resident macrophages *vs*. PMA-treated THP-1 cells).

Apoptotic cells are rarely found in normal tissues because phagocytes dispose of apoptotic cells as soon as they capture them. It should be noted, however, that apoptosis is often detected with the TUNEL method and/or anti-ssDNA Abs staining (*18, 19*) in such studies. Since DNA fragmentation follows PS externalization in the apoptotic process, it is possible that early apoptotic cells should be observed adjacently to macrophages *in vivo*.

The finding that the binding of early apoptotic cells to macrophages does not cause immediate phagocytosis may be related to a balance between 'eat me' signals such as PS and 'leave me away' signals such as CD31 (*20, 21*). According to a recent report, vital cells become attached to macrophages *via* a homophilic interaction through CD31 on both macrophages and vital cells, but CD31 on vital cells can deliver signals to macrophages telling them to leave the vital cells (*22*). In contrast, apoptotic cells deliver 'eat me' signals but not 'leave me away' signals to macrophages to ensure phagocytosis. In this context, the 'early apoptotic' cells in our study appear to be at an intermittent stage between vital cells and 'apoptotic' cells described in the above article in the sense of a balance between 'leave me away' signals and 'eat me' ones. Our findings may also be related to regulation of cytokine production upon interaction of macrophages with apoptotic cells. Macrophages produce anti-inflammatory cytokines upon interaction with early apoptotic cells (*23, 24*), whereas macrophages produce inflammatory cytokines upon interaction with late apoptotic cells (*25*). The underlying mechanism for switching from anti-inflammatory to inflammatory cytokines may also be related to a balance of 'leave me away' signals and 'eat me' ones. Indeed, much work is needed to examine this possibility.

In conclusion, it is likely that, when early apoptotic cells become attached to macrophages, they are not

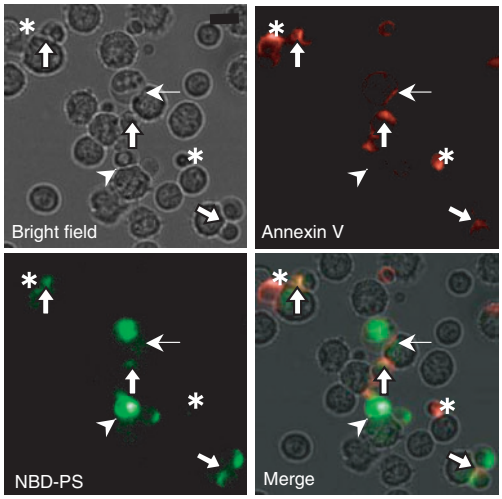

Fig. 2. **Observation of apoptotic cells labelled with NBD-PS and annexin V under a fluorescence microscope.** Thymocytes were cultured for 4 h in the presence of NBD-PS after X-irradiation as described under Materials and Methods. They were then co-cultured with macrophages for 30 min, followed by staining with Alexa-568 labelled annexin V and observation under a fluorescence microscope. The cells indicated by bold arrows and a thin arrow are labelled with NBD-PS and annexin V. The cell indicated by an arrowhead is labelled with NBD-PS but not annexin V. The cells indicated by asterisks are labelled with annexin V but not NBD-PS.

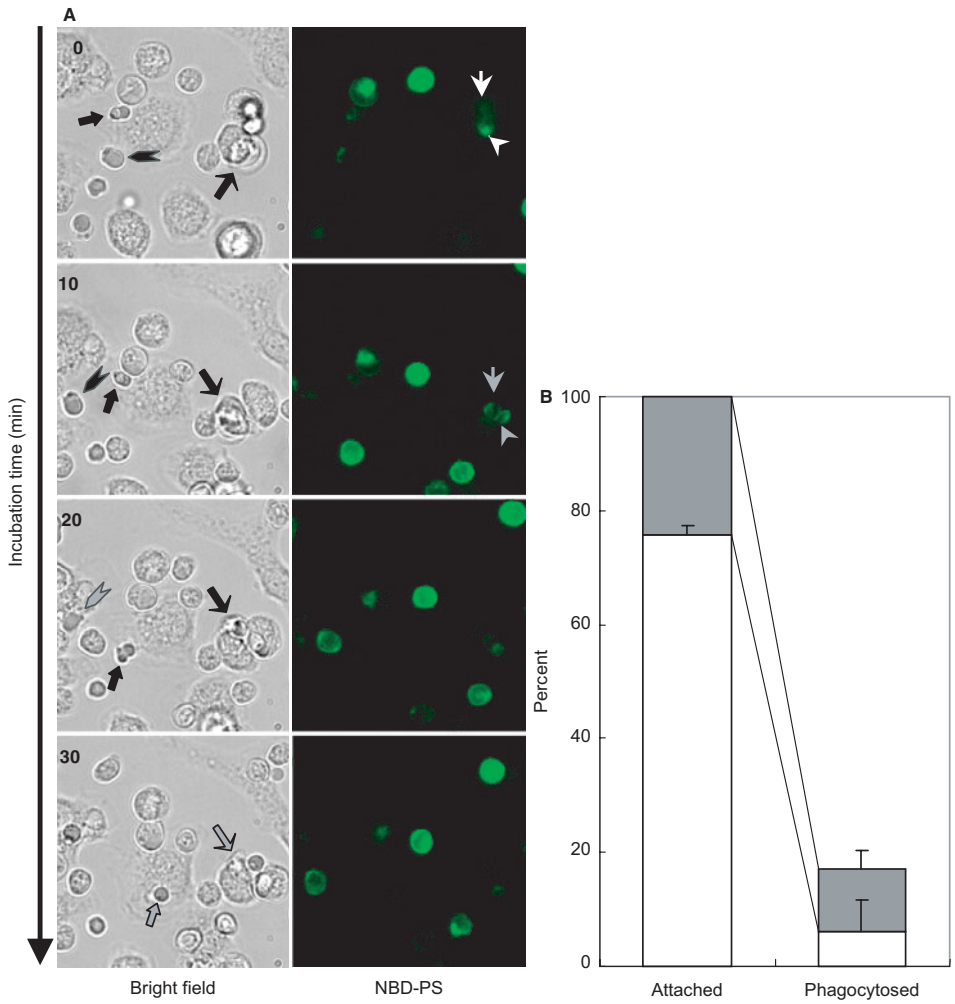

Fig. 3. **Time-lapse observation of the attachment and phagocytosis of a population rich in early apoptotic thymocytes.** Thymocytes were cultured for 4 h in the presence of NBD-PS after X-irradiation. Co-culturing with macrophages was started under a time-lapse microscope. Time-lapse observation was made for 30 min. (A) The cells indicated by arrows were phagocytosed, as indicated by the change in colour from black or white to grey. (B) The percentages of NBD-PS positive (white) or negative (grey) thymocytes attached to macrophages and those of phagocytosed ones during a co-culture for 30 min were determined in several experiments, when the total percentage of attached cells was taken as 100. The data were expressed as the means ± SD.

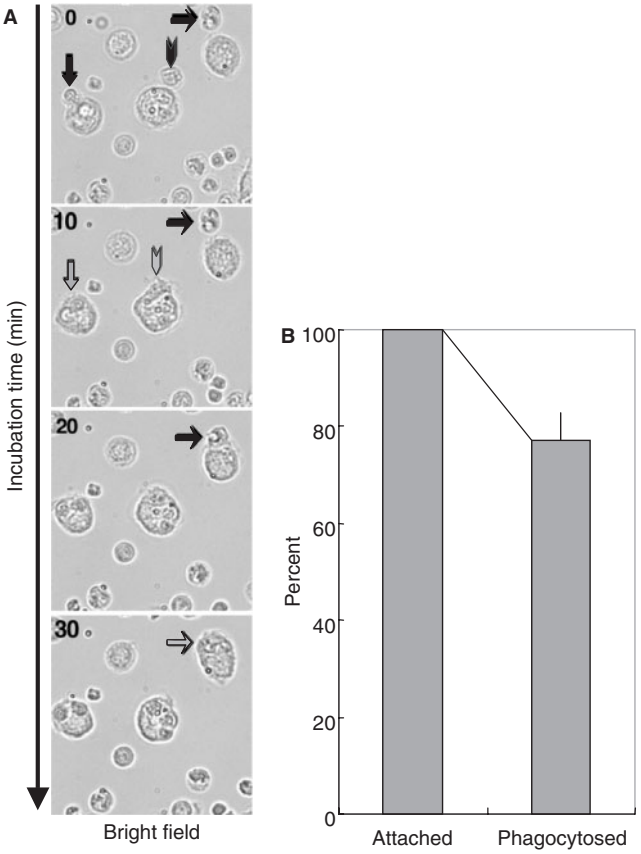

Fig. 4. **Time-lapse observation of the attachment and phagocytosis of a population rich in late apoptotic thymocytes.** Thymocytes were cultured for 24 h after X-irradiation, followed by co-culturing with macrophages and observation under a time-lapse microscope for 30 min. (A) The cells indicated by arrows were phagocytosed within 10 min. The change in colour from black to grey indicated phagocytosis. (B) The percentages of thymocytes attached to macrophages and phagocytosed ones during a co-culture for 30 min were determined in several experiments, when the percentage of total attached cells was taken as 100. The data were expressed as the means ± SD.

rapidly phagocytosed, but that late apoptotic cells are phagocytosed quickly after binding to macrophages.

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