<table>
<thead>
<tr>
<th>著者</th>
<th>磐戸斐子，中西義信</th>
</tr>
</thead>
<tbody>
<tr>
<td>稿名</td>
<td>Phosphatidylserine-mediated phagocytosis of anticancer drug-treated cells by macrophages</td>
</tr>
<tr>
<td>原論文誌</td>
<td>Journal of Biochemistry</td>
</tr>
<tr>
<td>巻</td>
<td>126</td>
</tr>
<tr>
<td>号</td>
<td>6</td>
</tr>
<tr>
<td>頁面</td>
<td>1101-1106</td>
</tr>
<tr>
<td>年</td>
<td>1999-01-01</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2297/14557">http://hdl.handle.net/2297/14557</a></td>
</tr>
<tr>
<td>doi</td>
<td><a href="https://doi.org/10.1093/oxfordjournals.jbchem.a022555">https://doi.org/10.1093/oxfordjournals.jbchem.a022555</a></td>
</tr>
</tbody>
</table>
Phosphatidylserine-Mediated Phagocytosis of Anticancer Drug-Treated Cells by Macrophages

Akiko Shiratsuchi and Yoshinobu Nakanishi
Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-0934

Received July 19, 1999; accepted September 29, 1999

Apoptotic cells are rapidly phagocytosed and eliminated from the organism. Although cancer cells apoptose when treated with anticancer drugs, how those cells are recognized by phagocytic cells has remained unclear. The human leukemia cell line Jurkat was cultured with doxorubicin or bufalin and induced to undergo apoptosis accompanied by phosphatidylserine externalization. When apoptotic Jurkat cells were mixed with mouse peritoneal macrophages, efficient phagocytosis was observed. Apoptosis and phagocytosis of Jurkat cells were both inhibited by Z-VAD-FMK, and phagocytosis was significantly reduced in the presence of phosphatidylserine-containing liposomes. These results suggest that anticancer drugs induce apoptosis-dependent and phosphatidylserine-mediated phagocytosis in cancer cells.

Key words: anticancer drug, apoptosis, phagocytosis, phosphatidylserine.

Apoptotic cells are engulfed by phagocytic cells, and this event has been presumed to prevent damage to surrounding tissues by the noxious contents of dead cells (reviewed in Refs. 1, 2). Another important role of phagocytosis of apoptotic cells has recently been proposed: that is, phagocytic cells present antigens, which apoptotic cells contain, on their surface and stimulate T lymphocytes (3–7). Furthermore, injection of macrophages that had phagocytosed apoptotic tumor cells led to tumor regression in rats (7). These observations strongly suggest that apoptotic cell phagocytosis occurs to eliminate unwanted cells not only directly but also indirectly by activating the immune response. It is important to elucidate the mechanism by which phagocytic cells recognize and phagocytose apoptotic cells in order to apply this phenomenon to the development of novel therapeutics against cancer.

Phagocytes should distinguish target apoptosing cells from living cells to selectively engulf the former. A variety of molecules, such as proteins, sugars, and phospholipids, have been suggested to act as a "phagocytosis marker," which is exposed on the surface of apoptotic cells and recognized by phagocytes (reviewed in Refs. 8–10). Among them, the membrane phospholipid PS has been most extensively characterized (reviewed in Ref. 11). In many apoptosing cells, including lymphocytes (12), vascular smooth muscle cells (13), vascular endothelial cells (14), spermatogenic cells (15), and Fas-overexpressing HeLa cells (16), externalized PS serves as a marker to allow phagocytes to recognize target cells. Many anticancer drugs induce apoptosis in cancer cells (reviewed in Refs. 17–19), and this is often accompanied by PS externalization (20). Apoptotic cells with externalized PS are, however, not necessarily phagocytosed in the PS-mediated manner (21, 22). We investigated here how anticancer drug-treated apoptotic cancer cells are phagocytosed by macrophages.

MATERIALS AND METHODS

Cell Culture and Apoptosis Analysis—Jurkat cells, a human leukemia T-cell line, were grown in RPMI 1640 (Nissui Pharmaceutical, Tokyo) containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂ in air. The cells were treated with either doxorubicin (Sigma, MO) (0.3 µg/ml) or bufalin (provided by K. Nakaya) (10⁻⁷ M). Cell viability and chromatin condensation were analyzed under a microscope after staining cells with trypan blue or Hoechst 33342, respectively. PS externalization was determined by flow cytometry (EPICS-XL; Coulter, FL) as described previously (23, 24). Briefly, test cells were treated with FITC-labeled annexin V (Bender MedSystems, Vienna, Austria) and propidium iodide, and cells that were less intensely stained with propidium iodide were gated and analyzed for the binding of FITC-annexin V. For confirmation of cell surface binding of FITC-annexin V, cells mixed with the fluorescent dyes were further treated with trypan blue (2 mg/ml) at pH 4.5 prior to flow cytometry. Trypan blue, a plasma membrane nonpermeable dye, binds to FITC and diminishes its fluorescence because of energy transfer (quenching) (25). To inhibit apoptosis, the caspase inhibitor Z-VAD-FMK...
Macrophage Preparation and Phagocytosis Assay—Macrophages were prepared from peritoneal fluids of thioglycollate-inoculated BDF1 mice (female, 8 weeks old) as described previously (16) and cultivated on cover slips (15 mm diameter) in RPMI 1640 containing 10% fetal bovine serum at 37°C until use. The phagocytosis assay was performed essentially as described (15). Briefly, target cells were labeled with biotin (NHS-LS-Biotin; Pierce, Rockford, IL), mixed with macrophages (at a ratio of 10 target cells to one macrophage), and incubated at 37°C for 2-3 h. The mixture was treated with trypsin (0.5 mg/ml) to remove Jurkat cells free from or lightly attaching to macrophages. The remaining cells were further treated with PBS containing paraformaldehyde (2%), glutaraldehyde (0.5%), and Triton X-100 (0.05%), then supplemented with FITC-conjugated avidin (fluorescein-avidin D; Vector Laborstories, Burlingame, CA). To analyze chromatin condensation and nuclear morphology of phagocytosed Jurkat cells, the fixed cells were further treated with propidium iodide, which binds to DNA. The number of macrophages containing engulfed cells was determined under a fluorescence/phase-contrast microscope and expressed relative to that of total macrophages, i.e., as the phagocytic index. The mean and SD of a typical example from at least three independent experiments are presented. For liposome preparations, phospholipids (Avanti Polar Lipids, Alabaster, AL) were dried as films, suspended in PBS, and sonicated. The liposomes were composed of either PC only (PC-liposomes) or a combination of PC and PS at a molar ratio of 7:3 (PS-liposomes).

RESULTS

Apoptosis of Anticancer Drug-Treated Jurkat Cells—We chose two apoptosis-inducing anticancer drugs, doxorubicin and bufalin. The drugs possess distinct modes of action: doxorubicin intercalates DNA and mainly affects nucleic acid synthesis (27), while bufalin inhibits Na+,K+-ATPase in the plasma membrane (28). Human leukemic Jurkat cells were cultured in the presence of these drugs, and cell viability and chromatin condensation were examined (Fig. 1A). Both drugs showed similar effects: cell viability decreased (left panel) and the number of cells with condensed chromatin increased (right panel) as the time of drug treatment was prolonged. Chromatin condensation caused by either drug was significantly inhibited in the presence of a caspase inhibitor, Z-VAD-FMK (Fig. 1B). These results indicated that doxorubicin and bufalin induce apoptosis in Jurkat cells.

We next examined whether this apoptosis is accompanied by PS externalization. To do so, drug-treated Jurkat cells were stained with FITC-annexin V and propidium iodide, and analyzed by flow cytometry. When the cells less intensely stained with propidium iodide were examined for the binding of FITC-annexin V, an extra peak with more fluorescence appeared after treatment with either drug, although the size of the peaks was different (left panels in Fig. 2A). Such peaks completely disappeared when the cells...
were treated with propidium iodide (zone A in the left-most panel) were analyzed for the binding of annexin V (histograms at the right). The numbers indicate the percentages of cells in the corresponding areas. (B) The same assay was conducted with cells that were treated with Z-VAD-FMK (20 or 40 μM).}

were treated with trypan blue to quench fluorescence from the cell surface (right panels), indicating that cells corresponding to the extra peaks contained surface-bound FITC-annexin V. Moreover, the addition of Z-VAD-FMK inhibited the appearance of such peaks (Fig. 2B). From these results, we concluded that PS, which is normally restricted to the cytoplasmic side of living cells, was externalized and exposed on the surface of apoptotic Jurkat cells. PS externalization was evident in anticancer drug-treated Jurkat cells before their chromatin condensed and nuclei fragmented. Quantification of the phagocytosis reaction revealed that Jurkat cells treated with either drug became susceptible to phagocytosis by macrophages (Fig. 3D). Phagocytosis was significantly reduced when Jurkat cells were treated with the anticancer drugs in the presence of Z-VAD-FMK (Fig. 3E), indicating that phagocytosis was dependent on apoptosis. The above results all showed that Jurkat cells treated with either doxorubicin or bufalin were phagocytosed by peritoneal macrophages at early stages of apoptosis.

Involvement of PS in Phagocytosis of Anticancer Drug-Treated Cells—Since externalized PS often serves as a marker in phagocytosis of apoptotic cells (11), we next asked if this is the case with anticancer drug-treated Jurkat cells, by conducting the phagocytosis assay in the presence of liposomes. Liposomes containing PS and PC significantly inhibited phagocytosis of Jurkat cells treated with either drug, whereas those composed of only PC showed a minimal effect (Fig. 4). These results suggested that phagocytosis of the drug-treated Jurkat cells by macrophages was mediated, at least partially, by PS exposed on the cell surface.

Significant levels of phagocytosis, however, always occurred in the presence of a maximal amount of PS-liposomes, suggesting the existence of PS-independent phagocytosis. We thus investigated the involvement of other possible phagocytosis markers (Fig. 5). A synthetic peptide containing the integrin-binding sequence RGDS; fucoidan and dextran sulfate, specific inhibitors of class A scavenger receptor types I and II; and N-acetyl-D-glucosamine, a ligand for lectin-like receptors, all showed some inhibition, but were much less effective than PS-liposomes. Furthermore, simultaneous addition of PS-liposomes and fucoidan or dextran sulfate gave no additive effect. Similar results were obtained in the phagocytosis assays of Jurkat cells treated with either doxorubicin or bufalin. These results suggest that as-yet unidentified molecules, in addition to PS, are involved in the recognition by macrophages of apoptotic Jurkat cells.

**DISCUSSION**

Although anticancer drugs induce apoptosis that is accompanied by externalization of PS in many cancer cell lines (20), it has not yet been shown whether those cells undergo PS-mediated phagocytosis. The present study showed that macrophages phagocytosed Jurkat cells, which were undergoing apoptosis induced by treatment with either doxorubicin or bufalin, in a manner inhibitable by PS-containing mixture was incubated for 2-3 h, washed, permeabilized, and supplemented with FITC-avidin. When the reacted cells were examined under a fluorescence/phase-contrast microscope, many fluorescent particles were detectable in the cytoplasm of macrophages (Fig. 3A). Jurkat cells left untreated with the drugs were not significantly engulfed by macrophages (Fig. 3B). There was no significant difference between the two drugs. When the macrophages that reacted with doxorubicin-treated Jurkat cells were further stained with propidium iodide, many phagocytosed Jurkat cells whose nuclei were not intensely stained or fragmented were observed (Fig. 3C). A similar result was obtained with bufalin-treated Jurkat cells (data not shown). These results indicated that macrophages phagocytosed drug-treated Jurkat cells before their chromatin condensed and nuclei were fragmented. Quantification of the phagocytosis reaction revealed that Jurkat cells treated with either drug became susceptible to phagocytosis by macrophages (Fig. 3D). Phagocytosis was significantly reduced when Jurkat cells were treated with the anticancer drugs in the presence of Z-VAD-FMK (Fig. 3E), indicating that phagocytosis was dependent on apoptosis. The above results all showed that Jurkat cells treated with either doxorubicin or bufalin were phagocytosed by peritoneal macrophages at early stages of apoptosis.

**Phagocytosis of Anticancer Drug-Treated Jurkat Cells by Mouse Peritoneal Macrophages**—Jurkat cells that had been cultured with the drugs for 24 h were labeled with biotin and mixed with mouse peritoneal macrophages. The
liposomes. Since these two drugs inhibit the growth of cancer cells in different ways, it is likely that anticancer drugs induce PS-mediated phagocytosis of cancer cells irrespective of their mode of action in cell growth inhibition.

PS exists predominantly in the inner leaflet of the plasma membrane bilayer of living cells, and translocates to the outer leaflet upon induction of apoptosis (reviewed in Refs. 29, 30). Our results showed that PS externalization and phagocytosis were both inhibited in the presence of an apoptosis inhibitor. Induction of apoptosis and phagocytosis are thus attributed to the same actions of anticancer drugs, at least for the drugs used in this study. Cell growth inhibition and apoptosis induction by anticancer drugs
Two enzymes called aminophospholipid translocase and scramblase (32) and (33) are believed to control the localization of the phospholipid lead to its externalization upon phagocytosis. It is thus important to elucidate the mechanism by which PS is exposed to the cell surface upon apoptotic changes, is sufficient for drug-treated cells to be recognized and phagocytosed by phagocytic cells, and such studies should provide us with new approaches for the development of novel therapeutic agents against cancer.

We thank K. Nakaya for a kind gift of bufalin, H. Yoshida for her contribution to the initial stage of this study, C. Fujii for macrophage preparation, and V. Fadok for critically reading the manuscript.

REFERENCES


22. Fadok, V.A., Henson, P., Campbell, P.A., and Henson, P.M. (1992) Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J. Immunol. 149, 4029–4035

