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Biochemical and Biophysical Research Communications 327 (2005) 94–99
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Distinct localization of lipid rafts and externalized phosphatidylserine at the surface of apoptotic cells

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Received 17 November 2004
Available online 8 December 2004

Abstract

Externalization of phosphatidylserine (PS) takes place in apoptotic cells as well as in viable cells under certain circumstances. Recent studies showed that externalized PS is localized at the lipid raft in viable activated immune cells. We found that lipid rafts and PS existed in a mutually exclusive manner in apoptotic cells. The number of PS-exposing apoptotic cells decreased when lipid rafts were disrupted. BCθ, which binds selectively to cholesterol in a cholesterol-rich region, did not effectively recognize lipid rafts of apoptotic cells. Lipid rafts rich in GM1 were successfully prepared from apoptotic cells, but the lipid raft protein LAT was not enriched in the preparation. Furthermore, the amount of PS and phosphatidylethanolamine but not of cholesterol in lipid rafts appeared to change after induction of apoptosis. These results suggest that lipid rafts are structurally modified during apoptosis and, despite being localized differently from PS, are involved in the externalization of PS.

Keywords: Apoptosis; Cholesterol; Lipid raft; Phosphatidylserine

The composition of the plasma membrane is not identical between the two leaflets of the membrane bilayer or in the lateral dimension. Different lipid species are asymmetrically distributed at the exoplasmic and cytoplasmic sides of the plasma membrane, and this allows functional lipids to be located at appropriate positions [1,2]. The asymmetrical distribution of phospholipids seems to be disrupted in apoptotic cells [3,4] as well as in viable blood cells or tumor cells [2]. This change in phospholipid distribution, at least in apoptotic cells, is presumed to be due to alteration of the actions of phospholipid transporters [3,4]. As a result, sphingomyelin, which is predominantly present at the exoplasmic leaflet in viable cells, comes to face the cytoplasm, and the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE), which are normally confined to the cytoplasmic leaflet, are exposed on the cell surface. This makes these phospholipids exert functions that are not observed in viable cells: most notably, PS serves as a marker for apoptotic cells to be recognized and engulfed by phagocytes [5,6]. Regarding the lateral organization of the plasma membrane, sphingolipids and cholesterol are packed into particular portions (~50 nm in diameter) of the membrane that associate with specific proteins. This architecture is called the plasma membrane microdomain, and the presence of two distinct microdomains has been proposed so far, namely, lipid rafts and caveolae [7,8],

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.11.135

which are both involved in a variety of biological events under either physiological or pathological conditions [8–14]. The lipid raft is a liquid-ordered membrane microdomain with a unique composition of lipids and proteins. It is rich in cholesterol, sphingomyelin, and glycosphingolipids, and this makes it more ordered and less fluid than other parts of the plasma membrane. As a consequence, lipid rafts are resistant to solubilization with detergents at low temperature. Lipid rafts contain proteins that are associated with membranes in a variety of manners: they are attached to the exoplasmic leaflet by glycosylphosphatidylinositol anchors, are attached to the cytoplasmic leaflet by acyl tails, and penetrate the membrane using their hydrophobic transmembrane domains. Most of these proteins are involved in the inward and outward transmission of materials and information. The caveolae, the other plasma membrane microdomain, possess a lipid and protein composition quite similar to that of the lipid raft. However, caveolae show a unique configuration, a flask-shaped invagination toward the cytoplasmic side, owing to the action of the caveolae-specific protein caveolin-1 [15–17]. In contrast to the phospholipid distribution between the two layers of the plasma membrane, whether these lateral organizations change during apoptosis remains to be examined.

Externalization of aminophospholipids in some viable cells seems to take place at lipid rafts, because externalized PS and lipid raft markers are co-localized in activated immune cells [18,19]. Although the presence of lipid rafts is likely to be needed for the occurrence of PS externalization in calcium ionophore-treated cells [20], the relative localization of externalized PS and lipid rafts in those cells is not known. In the present study, we addressed these issues by histochemically locating PS and lipid rafts at the surface of apoptotic cells and biochemically characterizing lipid rafts prepared from apoptotic cells.

Materials and methods

Cell culture and induction of apoptosis. Jurkat cells, a human leukemic T-cell line, were maintained in RPMI 1640 medium containing 10% (v/v) fetal bovine serum at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air. For the induction of apoptosis, cells (5×10^5 cells/ml) were incubated in the same medium containing doxorubicin (Sigma–Aldrich, St. Louis, MO, USA) (1.5 μg/ml) for 24–30 h or CH11 (MBL, Nagoya, Japan), an agonistic anti-Fas monoclonal antibody (0.1 μg/ml) for 3 h. The occurrence of apoptosis was monitored either by detecting PS externalization in a flow cytometer using 5-carboxyfluorescein-labeled annexin V or by determining the extent of chromatin condensation under a fluorescence microscope after staining cells with the DNA-binding fluorochrome Hoechst 33342 as described previously [21].

Cytochemical analysis of lipid rafts. Localization of lipid rafts at the cell surface was cytochemically determined using probes that bind to the lipid raft markers GM1 and cholesterol. To detect GM1, cells were incubated first with biotin-conjugated cholera toxin B subunit (CTB) (Sigma–Aldrich) (20 μg/ml) and then with Alexa 488-labeled streptavidin, Alexa 546-labeled streptavidin or fluorescein isothiocyanate (FITC)-labeled avidin D, or first treated with the anti-GM1 monoclonal antibody clone KM638 (a gift from Kyowa Hakko Kogyo) (20 μg/ml) followed by incubation with Cy3-labeled anti-mouse IgM antibody. To detect cholesterol present in a cholesterol-rich region, cells were first treated with a protease-nicked and biotinylated derivative of perfringolysin O (BCθ) [22] (10–100 μg/ml), and then incubated with FITC-labeled avidin D. The cells were then examined for the binding of the probes by confocal laser-scanning microscopy (LSM510; Carl Zeiss, Jena, Germany) or flow cytometry (Epics-XL; Coulter, Hialeah, FL, USA). To disrupt lipid rafts, cells (5×10^6 cells/ml) were incubated with methyl-β-cyclodextrin (MCD) (Sigma–Aldrich) (10 mM) for 30 min at 37 °C in a buffer consisting of 10 mM Hepes–NaOH (pH 7.4), 0.15 M NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$.

Preparation of lipid rafts. Lipid rafts were prepared by a flotation centrifugation method [23]. Jurkat cells (1×10^8) suspended in 5 mM EDTA, 5 mM dithiothreitol, 0.15 M NaCl, and 25 mM Tris–HCl (pH 7.4) (raft buffer) containing protease inhibitors (Sigma–Aldrich) were disrupted using a glass/teflon homogenizer and centrifuged at 1000*g* for 5 min at 4 °C, and supernatants were collected. The supernatants were incubated for 30 min at 4 °C after the addition of Triton X-100 at 1% (v/v) and supplemented with OptiPrep (Axis Shield, Dundee, UK) at 35% (w/v). The samples were placed in centrifugation tubes for a swing-type rotor, overlaid by 0.65 volumes of 30% OptiPrep-containing raft buffer and then by 0.33 volumes of raft buffer, and centrifuged at 180,000*g* for 4 h at 4 °C. Six fractions (0.86 ml each) were collected from the bottom of the gradient and analyzed for the presence of the lipid raft markers GM1 and LAT as follows. For the analysis of GM1, lipids were extracted from each fraction with chloroform and methanol under acidic conditions according to the method of Bligh and Dyer [24], dried, and dissolved with a sample buffer for SDS–polyacrylamide gel electrophoresis (0.0625 M Tris–HCl (pH 6.8), 2.5% (w/v) SDS, and 2.5% (v/v) 2-mercaptoethanol). The lipid samples were electrophoretically separated on an SDS-15% (w/v) polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with phosphate-buffered saline (PBS) containing 3% (w/v) bovine serum albumin and reacted with biotin-conjugated CTB in PBS containing 1% bovine serum albumin for 1 h at 37 °C. It was washed with PBS and then incubated with PBS containing bovine serum albumin (1%) and horseradish peroxidase-conjugated streptavidin (Amersham Biosciences, Uppsala, Sweden) for 1 h at 37 °C. The membrane was washed with PBS, and signals were visualized using Western Lightning (Perkin–Elmer, Wellesley, MA, USA). For the analysis of LAT, proteins present in each fraction were concentrated using methanol and chloroform according to the method of Wessel and Flugge [25], separated by electrophoresis on an SDS-10% polyacrylamide gel, and transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with PBS containing 3% (w/v) dry skim milk and reacted with anti-LAT antibody (Upstate, Charlottesville, VA, USA) in PBS containing 3% dry skim milk overnight at 4 °C. It was washed with water and incubated with PBS containing 3% dry skim milk and horseradish peroxidase-conjugated anti-rabbit IgG for 1.5 h at room temperature. The membrane was washed with PBS containing 0.05% (v/v) Tween 20 and then with water five times, and signals were visualized using Western Lightning. The data from the analysis of GM1 and LAT were processed using Fluor-S (Bio-Rad Laboratories, Hercules, CA, USA). Fractions rich in GM1 and LAT (fraction 5 in most experiments) were collected and used in further experiments as lipid raft preparations.

Thin-layer chromatography of lipids. Cells were lysed in raft buffer containing 0.1% SDS, and lipids were extracted from the lysates with methanol and chloroform as described above. Lipids extracted from either whole cells or lipid raft preparations were dried, re-dissolved with chloroform, and analyzed by thin-layer chromatography as

described below. For the analysis of phosphatidylcholine (PC) and PE, plates (K6; Whatman, Middlesex, UK) were developed with solvent consisting of chloroform/methanol/28% (w/v) ammonium (65:35:9 in volume) [26]. The plates were incubated with 1 M NaCl saturated with amido black 10B to visualize separated phospholipids [27]. To analyze PS, lipid samples were separated together with standard PC using the same plate and solvent. Portions of the plate containing standard PC were stained with amid black, and the plate was cut off so that one portion of the plate contained lipids other than PC. This portion of the plate was developed toward the direction opposite to the previous development using solvent consisting of chloroform/acetone/acetic acid/methanol/water (50:20:15:10:5). The plate was stained as described above to visualize separated phospholipids. For the analysis of cholesterol [28], lipids were separated on the same plate using solvent consisting of diisopropyl ether/acetic acid (96:4) until the solvent crossed about two-thirds of the plate. The plate was dried and re-developed using solvent consisting of petroleum ether/diethyl ether/acetic acid (90:10:1). Separated cholesterol was detected by staining the plate with amido black as described above. The signals were digitized using a densitometer and the intensity of the signals was determined by using Molecular Analyst (Bio-Rad Laboratories). Portions of lipid samples loaded on plates were adjusted so that each lipid was quantitatively analyzed.

Results

Alteration of lipid rafts in apoptotic cells

We first histochemically detected lipid rafts at the surface of viable or apoptotic cells by confocal microscopy using probes that specifically bind to lipid raft markers. The probes used were CTB, anti-GM1 monoclonal antibody (KM638), and BCθ, the former two of which bind to GM1 and the last of which binds to cholesterol present in a cholesterol-rich region such as the lipid raft. CTB and BCθ bound to the surface of viable Jurkat cells as patches, and most signals derived from the two probes appeared to be co-localized (Fig. 1A). The patterns of signals derived from CTB and KM638 also seemed to be almost the same (top left panels in Fig. 1B), indicating that all three probes detected the same microdomain of the plasma membrane, that is, lipid rafts. We next examined the binding of those probes to Jurkat cells undergoing doxorubicin-induced apoptosis. CTB and KM638 bound to apoptotic cells as they did to viable cells, whereas signals of BCθ were almost undetectable with apoptotic cells (Fig. 1B). To confirm this by flow cytometry, doxorubicin-treated Jurkat cells were first analyzed for the externalization of PS using annexin V as a probe, and cells positive (apoptotic) and negative (viable) for the binding of annexin V were individually examined for the binding of CTB and BCθ. CTB effectively bound to cells irrespective of PS externalization, but BCθ only bound to viable cells with no PS at their surface (Fig. 1C). These results suggested that lipid rafts were somewhat altered after the induction of apoptosis, probably in the amount or distribution of cholesterol.

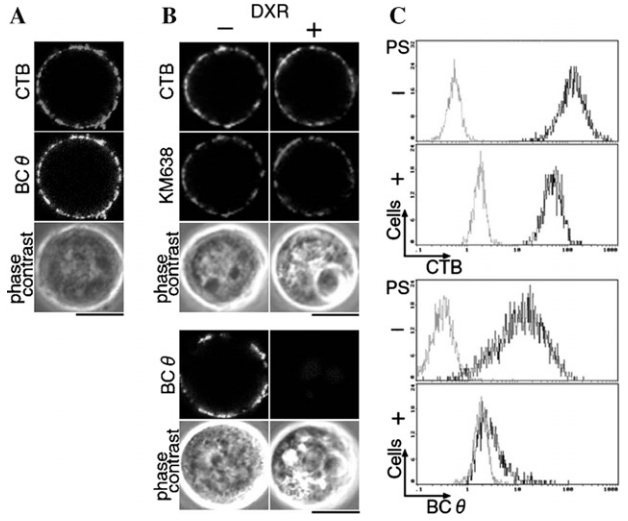

Fig. 1. Detection of lipid rafts at the surface of viable and apoptotic cells. (A) Viable Jurkat cells were histochemically analyzed for the localization of lipid raft markers by confocal microscopy using the indicated probes as described in Materials and methods. Cells were simultaneously examined for the binding of CTB and BCθ. Fluorescence and phase contrast views of the same microscopic fields are shown in each row. Bar, 10 μm. Data from one experiment of three with similar results are presented. (B) Jurkat cells left untreated (−) or treated (+) with doxorubicin (DXR) were analyzed for the binding of the indicated probes of lipid raft markers by confocal microscopy as described in Materials and methods. Either viable or apoptotic cells were examined for the binding of CTB and KM638 (top 6 panels) or of BCθ (bottom 4 panels). Bound CTB was visualized using Alexa 488-labeled streptavidin. Fluorescence and phase contrast views of the same microscopic fields are shown for each set of experiments. Bars, 10 μm. Data from one experiment of three with similar results are presented. (C) Jurkat cells treated with doxorubicin were incubated with a mixture of 5-carboxyfluorescein-labeled annexin V, propidium iodide (PI), CTB, and FITC-labeled streptavidin, or with a mixture of 5-carboxyfluorescein-labeled annexin V, PI, BCθ, and FITC-labeled streptavidin, analyzed by flow cytometry. Cell populations that were annexin V negative/PI negative (PS−) and annexin V positive/PI negative (PS+) were gated and analyzed for staining with FITC to determine the level of the binding of CTB (top) or BCθ (bottom). Thin and thick lines represent results from experiments without and with CTB or BCθ, respectively. Data from one experiment of three with similar results are presented.

Distinct localization of lipid rafts and externalized PS at the surface of apoptotic cells

The localization of lipid rafts and externalized PS was then compared in apoptotic cells. For this purpose, Jurkat cells, either viable or apoptotic, were simultaneously treated with CTB and annexin V for locating lipid rafts and PS, respectively, and examined by confocal microscopy. As expected, viable cells only bound to CTB, while cells undergoing doxorubicin-induced apoptosis gave signals derived from both probes (top and middle panels in Fig. 2). The localization of the two probes at the surface of apoptotic Jurkat cells appeared to be complementary (middle panels in Fig. 2). Essentially the same results were obtained in experiments using KM638 as

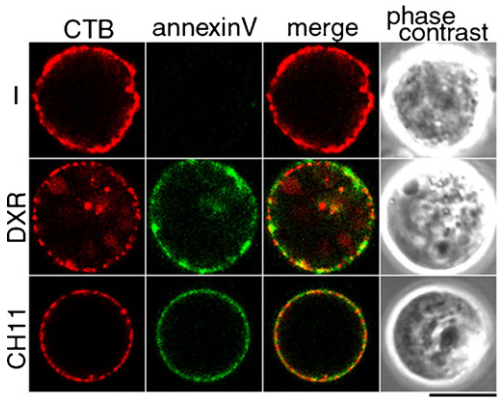

Fig. 2. Localization of lipid rafts and externalized PS at the surface of apoptotic cells. Jurkat cells left untreated (−), treated with doxorubicin (DXR), or treated with CH11 were analyzed for the binding of CTB and annexin V by confocal microscopy as described in Materials and methods. Bound CTB was visualized using Alexa 546-labeled streptavidin. Fluorescence and phase contrast views of the same microscopic fields are shown in each row. Bar, 10 μm. Data from one experiment of three with similar results are presented.

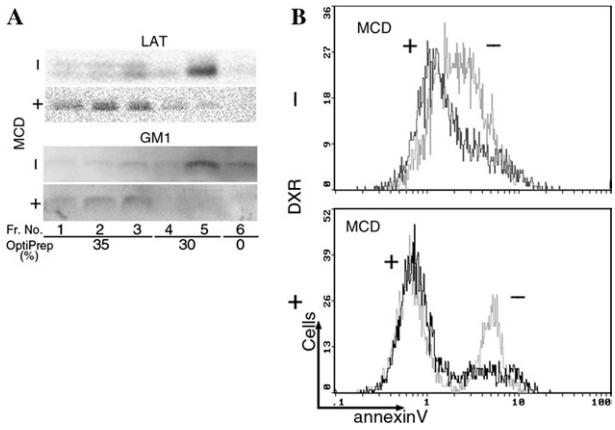

Fig. 3. Effect of MCD on the integrity of lipid rafts and PS externalization in apoptotic cells. (A) Viable Jurkat cells were left untreated (−) or incubated (+) with MCD, and their lysates were subjected to OptiPrep gradient centrifugation. Each fraction of the gradient was examined for the presence of LAT or GM1 as described in Materials and methods. Data from one experiment of three with similar results are presented. (B) Jurkat cells that had been left untreated (−) or treated (+) with doxorubicin (DXR) were incubated with (+) or without (−) MCD. The cells were then treated with 5-carboxyfluorescein-labeled annexin V and propidium iodide, and analyzed by flow cytometry. Cells less intensely stained with propidium iodide were gated and examined for the level of bound annexin V. Data from one experiment of three with similar results are presented.

a probe to detect GM1 (data not shown) or with cells undergoing Fas-mediated apoptosis (bottom panels in Fig. 2). These results indicated that PS externalization in apoptotic cells occurs at portions of the plasma membrane where lipid rafts are absent.

Requirement for lipid rafts for externalization of PS in apoptotic cells

We next examined whether lipid rafts are involved in PS externalization. For this purpose, cells were treated with MCD, which depletes cholesterol from membranes and therefore disrupts lipid rafts. We first included MCD during incubation of cells with doxorubicin but found that the presence of MCD by itself induced apoptosis in Jurkat cells (data not shown). We thus examined the effect of MCD on cells that had already exposed PS by treatment only for a short period. To examine if this treatment disrupts lipid rafts without inducing apoptosis, we treated viable Jurkat cells with MCD for 30 min and then examined the presence of lipid rafts as well as the occurrence of apoptosis. For this purpose, lipid rafts were prepared from cells by a flotation centrifugation method. Lysates of cells either treated or not treated with MCD were solubilized with a detergent and fractionated on a discontinuous OptiPrep gradient, and the fractions obtained were examined for the presence of LAT, a protein marker for lipid rafts, and GM1 (Fig. 3A). We found that both LAT and GM1 were concentrated in a fraction with 30% OptiPrep with lysates prepared from cells not treated with MCD, indicating that detergent-insoluble lipid rafts were successfully prepared. However, this was not the case when lysates of cells treated with MCD were similarly processed: neither LAT nor GM1 accumulated in the corresponding fraction. The treatment with MCD did not seem to affect cell viability, because the level of the binding of annexin V to the cell surface continued to be low (top panel in Fig. 3B). These results indicated that a 30-min treatment with MCD disrupted most lipid rafts in Jurkat cells without inducing apoptosis. We then determined the effect of MCD treatment on PS externalization in apoptotic Jurkat cells, about 40% of which were positive for PS externalization. We found that MCD treatment caused a dramatic reduction of PS-exposing cells: less than 20% of cells were positive for the binding of annexin V after treatment with MCD (bottom panel in Fig. 3B). Such cells seemed to remain apoptotic, because the extent of chromatin condensation (about 50%) did not change after incubation with MCD (data not shown). These results suggested that the presence of lipid rafts is required for the maintenance of PS externalization in apoptotic cells.

Biochemical characterization of lipid rafts in apoptotic cells

We next directly examined possible changes in the composition of lipid rafts after the induction of apoptosis. When lipid rafts were prepared from doxorubicin-treated Jurkat cells by a flotation centrifugation method, GM1 but not LAT accumulated in a fraction containing 30% OptiPrep (Fig. 4). This result indicated that lipid

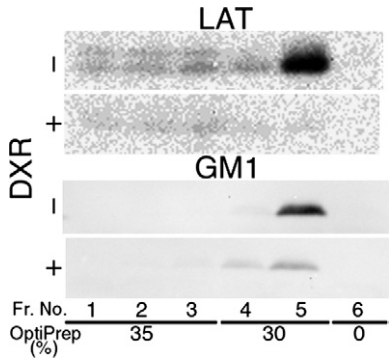

Fig. 4. Preparation of lipid rafts from apoptotic cells. Jurkat cells were left untreated (−) or treated (+) with doxorubicin (DXR), and their lysates were subjected to OptiPrep gradient centrifugation. Each fraction of the gradient was examined for the presence of LAT or GM1 as described in Materials and methods. Data from one experiment of three with similar results are presented.

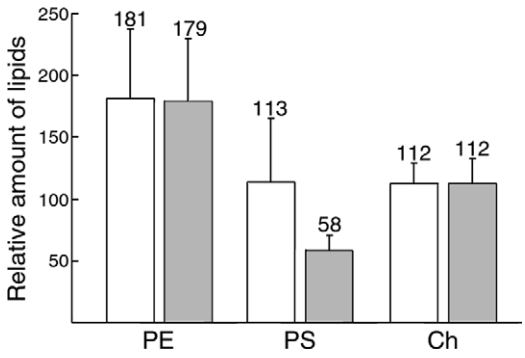

Fig. 5. Lipid content in lipid rafts. Lipids were extracted from lipid raft preparations or total lysates of doxorubicin-treated or untreated Jurkat cells, and analyzed by thin-layer chromatography. The intensity of the signals derived from each lipid obtained with lipid samples from apoptotic cells was expressed relative (in percentage) to that from viable cells. Open and shaded columns represent results with lipid samples prepared from whole cells and lipid rafts, respectively. Ch, cholesterol. Data from three independent experiments are shown as means ± SD.

rafts did exist in apoptotic cells, but contained less LAT than lipid rafts of viable cells do. Taking this and the results shown in Fig. 1 into consideration, it seems likely that lipid rafts are structurally altered in terms of the content of both lipids and proteins upon induction of apoptosis.

We next analyzed lipids present in lipid rafts prepared from viable and apoptotic Jurkat cells. Lipids were extracted from lipid raft preparations as well as from whole cells and analyzed for the amounts of phospholipids and cholesterol by thin-layer chromatography. We determined the amount of each lipid relative to that of PC, because the presence of multiple steps in the preparation of lipid rafts made determination of the absolute amount of lipids difficult. The extracted lipids were first analyzed to standardize the amount of PC between the preparations from viable and apoptotic cells, and then portions of the samples containing the same amount of PC were examined for the amounts of other lipids. The content of two phospholipids, PS and PE, and cholesterol was determined in this way by thin-layer chromatography with different solvents and development procedures (see the Materials and methods) (data not shown). The intensities of the signals on chromatograms were determined using a densitometer, and the relative content of each lipid was compared between viable and apoptotic cells (Fig. 5). We found that the content of PE increased in both lipid rafts and whole cells, and that of PS in lipid rafts decreased during apoptosis. In contrast, the content of cholesterol in either lipid rafts or whole cells did not seem to differ before and after the induction of apoptosis. These results indicated that the content of certain kinds of phospholipids in lipid rafts changes during apoptosis, and suggested that the failure of BCθ to bind to the surface of apoptotic cells is not due to a decrease in the amount of cholesterol in lipid rafts but probably due to a change of its distribution.

Discussion

In contrast to previous findings obtained with viable B cells [18] and neutrophils [19], we found that the localization of PS at the surface of apoptotic Jurkat cells was distinct from that of lipid rafts irrespective of the way of inducing apoptosis. This suggests that the mechanisms for PS externalization differ between viable cells and apoptotic cells. It is presumed that changes in the activity of lipid transporters, namely phospholipid scramblase and aminophospholipid translocase, are responsible for alteration of the phospholipid distribution between the two leaflets of the plasma membrane in apoptotic cells [21,29]. We therefore think that it will be necessary to determine changes in the activity of lipid transporters in viable cells upon activation that leads to PS externalization.

In spite of the distinct localization of lipid rafts from PS, the integrity of lipid rafts appeared to be necessary for the maintenance of PS externalization in apoptotic Jurkat cells. Kunzelmann-Marche et al. [20] previously reported that treatment with MCD reduced the level of PS externalization in calcium ionophore-treated human erythroleukemia cells. They treated cells with MCD prior to and during activation by calcium ionophore. In our study, Jurkat cells were treated with the drug after they had been induced to undergo apoptosis by incubation with doxorubicin, and thus already exposed PS at their surfaces. We thus concluded that lipid rafts are necessary for the maintenance of PS externalization. It still remains to be solved whether the translocation of PS to the outer leaflet that occurs at early stages of apoptosis requires the presence of lipid rafts. We also found that lipid rafts are subjected to structural modification during apoptosis. It is likely that the distribution of cholesterol within lipid rafts changes in apoptotic cells, because BCθ, which

only binds to cholesterol in a cholesterol-rich region, did not effectively recognize lipid rafts of apoptotic cells, and the content of cholesterol in lipid rafts did not change after the induction of apoptosis. In addition, the amounts of PS and PE relative to PC in lipid rafts appeared to change during apoptosis. The consequences of such changes in lipid composition occurring upon apoptosis are unclear at present, but may indicate the importance of lipid rafts for PS externalization and also to a presumed difference in the mode of PS externalization between viable cells and apoptotic cells. Surface exposure of PS is required for the phagocytic elimination of apoptotic cells [5,6]. If changes in the structure of lipid rafts are needed for PS externalization in apoptotic cells, such changes may be considered part of the self-defense mechanism by which harmful or spent cells are promptly and selectively removed from organisms to maintain tissue homeostasis.

Acknowledgments

We thank Kyowa Hakko Kogyo Co., Ltd., for a gift of KM638. We are also grateful to K. Hanada and K. Kasahara for advice. This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

[1] A. Zachowski, Review Article. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement, Biochem. J. 294 (1993) 1–14.
[2] R.F.A. Zwaal, A.J. Schroit, Review Article. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells, Blood 89 (1997) 1121–1132.
[3] P. Williamson, R.A. Schlegel, Review. Transbilayer phospholipid movement and the clearance of apoptotic cells, Biochim. Biophys. Acta 1585 (2002) 53–63.
[4] K. Balasubramanian, A.J. Schroit, Aminophospholipid asymmetry: a matter of life and death, Annu. Rev. Physiol. 65 (2003) 701–734.
[5] V.A. Fadok, D.L. Bratton, S.C. Frasch, M.L. Warner, P.M. Henson, Review. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes, Cell Death Differ. 5 (1998) 551–562.
[6] R.A. Schlegel, P. Williamson, Review. Phosphatidylserine, a death knell, Cell Death Differ. 8 (2001) 551–563.
[7] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[8] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–39.
[9] T.V. Kurzchalia, R.G. Parton, Membrane microdomains and caveolae, Curr. Opin. Cell Biol. 11 (1999) 424–431.
[10] E.J. Smart, G.A. Graf, M.A. McNiven, W.C. Sessa, J.A. Engelman, P.E. Scherer, T. Okamoto, M.P. Lisanti, Caveolins, liquid-ordered domains, and signal transduction, Mol. Cell. Biol. 19 (1999) 7289–7304.
[11] T. Kobayashi, A. Yamaji-Hasegawa, E. Kiyokawa, Lipid domains in the endocytic pathway, Semin. Cell Dev. Biol. 12 (2001) 173–182.
[12] L.D. Zajchowski, S.M. Robbins, Review Article. Lipid rafts and little caves: compartmentalized signalling in membrane microdomains, Eur. J. Biochem. 269 (2002) 737–752.
[13] K. Simons, R. Ehehalt, Cholesterol, lipid rafts, and disease, J. Clin. Invest. 110 (2002) 597–603.
[14] I.R. Nabi, P.U. Le, Caveolae/raft-dependent endocytosis, J. Cell Biol. 161 (2003) 673–677.
[15] M. Drab, P. Verkade, M. Elger, M. Kasper, M. Lohn, B. Lauterbach, J. Menne, C. Lindschau, F. Mende, F.C. Luft, A. Schedl, H. Haller, T.V. Kurzchalia, Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice, Science 293 (2001) 2449–2452.
[16] B. Razani, J.A. Engelman, X.B. Wang, W. Schubert, X.L. Zhang, C.B. Marks, F. Macaluso, R.G. Russell, M. Li, R.G. Pestell, D. Di Vizio, H. Hou Jr., B. Kneitz, G. Lagaud, G.J. Christ, W. Edelmann, M.P. Lisanti, Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities, J. Biol. Chem. 276 (2001) 38121–38138.
[17] B. Razani, X.B. Wang, J.A. Engelman, M. Battista, G. Lagaud, X.L. Zhang, B. Kneitz, H. Hou Jr., G.J. Christ, W. Edelmann, M.P. Lisanti, Caveolin-2-deficient mice show evidence of severe pulmonary dysfunction without disruption of caveolae, Mol. Cell. Biol. 22 (2002) 2329–2344.
[18] S.R. Dillon, M. Mancini, A. Rosen, M.S. Schlissel, Annexin V binds to viable B cells and colocalizes with a marker of lipid rafts upon B cell receptor activation, J. Immunol. 164 (2000) 1322–1332.
[19] S.C. Frasch, P.M. Henson, K. Nagaosa, M.B. Fessler, N. Borregaard, D.L. Bratton, Phospholipid flip–flop and phospholipid scramblase 1 (PLSCR1) co-localize to uropod rafts in formylated Met-Leu-Phe-stimulated neutrophils, J. Biol. Chem. 279 (2004) 17625–17633.
[20] C. Kunzelmann-Marche, J.-M. Freyssinet, M.C. Martínez, Loss of plasma membrane phospholipid asymmetry requires raft integrity, J. Biol. Chem. 277 (2002) 19876–19881.
[21] A. Shiratsuchi, S. Osada, S. Kanazawa, Y. Nakanishi, Essential role of phosphatidylserine externalization in apoptosing cell phagocytosis by macrophages, Biochem. Biophys. Res. Commun. 246 (1998) 549–555.
[22] A.A. Waheed, Y. Shimada, H.F.G. Heijnen, M. Nakamura, M. Inomata, M. Hayashi, S. Iwashita, J.W. Slot, Y. Ohno-Iwashita, Selective binding of perfringolysin O derivative to cholesterol-rich membrane microdomains (rafts), Proc. Natl. Acad. Sci. USA 98 (2001) 4926–4931.
[23] F. Lafont, P. Verkade, T. Galli, C. Wimmer, D. Louvard, K. Simons, Raft association of SNAP receptors acting in apical trafficking in Madin–Darby canine kidney cells, Proc. Natl. Acad. Sci. USA 96 (1999) 3734–3738.
[24] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[25] D. Wessel, U.I. Flügge, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids, Anal. Biochem. 138 (1984) 141–143.
[26] D.A. Kennerly, Diacylglycerol metabolism in mast cells. Analysis of lipid metabolic pathways using molecular species analysis of intermediates, J. Biol. Chem. 262 (1987) 16305–16313.
[27] A.Y. Plekhanov, Rapid staining of lipids on thin-layer chromatograms with amido black 10B and other water-soluble stains, Anal. Biochem. 271 (1999) 186–187.
[28] V.P. Skipski, A.F. Smolowe, R.C. Sullivan, M. Barclay, Separation of lipid classes by thin-layer chromatography, Biochim. Biophys. Acta 106 (1965) 386–396.
[29] B. Verhoven, R.A. Schlegel, P. Williamson, Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes, J. Exp. Med. 182 (1995) 1597–1601.