Fas Signaling Induces Raft Coalescence That Is Blocked by Cholesterol Depletion in Human RPE Cells Undergoing Apoptosis

James E. Lincoln,[1] *Marie Boling,*[1] *Atul N. Parikh,*[2] *Yin Yeh,*[2] *David G. Gilchrist,*[1] *and Lawrence S. Morse*[3]

Purpose. To investigate whether the signaling events occurring in Fas-mediated apoptosis alter raft membrane formation in human RPE cells.

Methods. Formation of lipid rafts in cultured human retinal pigment epithelial cells (ARPE-19) was studied by confocal microscopy, with fluorescein-labeled cholera toxin subunit B binding protein (BODIPY)–labeled ganglioside GM1 lipid after Fas-L induction of apoptosis. Apoptosis was assessed by fluorescein-labeled annexin V detection of phosphatidylserine externalization and quadrant analysis with flow cytometry. Membrane rafts were localized into membrane vesicles by passing BODIPY-labeled GM1 RPE cells through a 2-μm-pore polycarbonate membrane using an extruder device. The labeled fractions, containing vesicles enriched in GM1, were detected by flow cytometry and then analyzed for the presence of Fas protein.

Results. Differential punctate staining of membrane rafts was demonstrated in normal and FasL-induced apoptotic human ARPE-19 cells in culture by confocal microscopy, using cholera toxin B and GM1 labeling of extruded vesicles. The lipid raft–associated vesicles were derived by plasma membrane dissociation, via a newly developed whole-cell extrusion technique that produced 2-μm vesicles with both GM1 lipid and Fas protein abundance enriched in a subpopulation of the membrane-derived vesicles. The amount of Fas protein in the vesicles containing raft domains markedly increased in FasL-treated cells. Treatment of human ARPE 19 cells with methyl β-cyclodextrin after FasL induction of apoptosis resulted in cellular cholesterol depletion and markedly reduced the incidence of Fas-receptor localization in GM1 rafts.

Conclusions. Human ARPE-19 cells in culture contain membrane rafts with apoptotic signaling effectors uniformly distributed in the native state. The cells stimulated to undergo apoptosis appear to use membrane rafts in the death-signaling process by mobilization of rafts to localized regions of the membrane that are now enriched with apoptotic signaling effectors. Fas signaling induces apoptotic raft formation that results in polar condensation, or capping, of the rafts in the late stages of apoptosis. A novel extrusion technique is described that allows localization and enrichment of rafts into membrane vesicles, which can be assayed by flow cytometry. Cholesterol depletion, after Fas ligand activation of apoptosis, reduced raft formation in cells induced to undergo apoptosis. Therapeutic implications for the treatment of retinal disorders are discussed. (*Invest Ophthalmol Vis Sci.* 2006;47:2172–2178) DOI:10.1167/iovs.05-1167

Fundamental cellular processes like proliferation, differentiation, and cell death are triggered by locally and temporally stimulated activation of protein and lipid components of signal transduction pathways initiating at the cell membrane surface. The resultant signals are transmitted internally to the cytoplasm and nucleus, where the functional activation of cell proliferation or death occurs. In response to a variety of inducers, such as oxidative stress, UV light, and ceramides, retinal pigment epithelium (RPE) cells die by a process of programmed cell death called apoptosis.[1–3] The death of the RPE cells causes degeneration of the photoreceptor, severely affecting central vision in patients with age-related macular degeneration. Recent evidence suggests that the death signals are transmitted through the RPE membrane, spanning proteins from the plasma membrane to cytoplasmic proteins, which then interact with mitochondrial proteins where the terminal stages of apoptosis are initiated. How signaling occurs across cell membranes and the interacting factors in the signal transduction pathways are the focuses of much current investigation. Many of the signaling events that take place when human RPE cells undergo apoptosis have been demonstrated both in vitro and in vivo.[1–13]

Apoptosis is a highly ordered and regulated process of programmed cell death that functions in developmental and cell-replacement processes and in disease. Characteristics of the apoptotic process include chromatin condensation, DNA fragmentation, cellular shrinkage, phosphatidylserine externalization, membrane blebbing, and the formation of apoptotic bodies. A common feature of the physiological changes involved in apoptosis is the activation of specific cysteine proteases, known as caspases, that cleave targeted substrates leading to destabilization of cellular homeostasis.[7–11] Apoptosis has been divided into two principal signaling pathways: intrinsic and extrinsic. The intrinsic pathway is activated by internal cellular stress—specifically, mitochondrial stress caused by factors such as DNA damage and heat shock. The extrinsic pathway is activated by extracellular apoptosis-inducing ligands, such as Fas ligand (FasL) which binds to the Fas receptor in the plasma membrane, initiating a signaling cascade leading to caspase activation and DNA fragmentation internally. Fas is a member of the TNFR family that triggers cell death through the presence of a death domain in its cytoplasmic portion after receptor engagement with FasL or agonistic anti-Fas antibodies. Additional evidence suggests that, under specific stresses,

From the Departments of [1]Plant Pathology, [2]Applied Science, and [3]Ophthalmology, University of California, Davis, Sacramento, California.

Supported by The Center for Biophotonics, a National Science Foundation Science and Technology Center managed by the University of California, Davis, under Cooperative Agreement No. PHY 0120999 and by an unrestricted grant to the Department of Ophthalmology from Research to Prevent Blindness.

Submitted for publication August 31, 2005; revised December 2, 2005; accepted March 6, 2006.

Disclosure: **J.E. Lincoln**, None; **M. Boling**, None; **A.N. Parikh**, None; **Y. Yeh**, None; **D.G. Gilchrist**, None; **L.S. Morse**, None

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Corresponding author: Lawrence S. Morse, U. C. Davis Department of Ophthalmology and Vision Science, 4860 Y Street, Ste 2400, Sacramento, CA 95864; lsmorse@ucdavis.edu.

death signals may be transmitted from the outer cellular plasma membrane after the interaction of Fas/FasL proteins and sphingomyelinase (SMase) to the mitochondria by means of signaling molecules, such as ceramides, that result in a series of defined cellular changes associated with cell death.[12–18]

In cells responding through the extrinsic signaling pathway, patches of aggregated receptors migrate toward one pole of the cell and coalesce to form a cap. This morphologic change is believed to facilitate transduction of extracellular signals by local assembly of the various signaling elements.[19] Recent reports indicate that proteins capable of governing cell survival become clustered within the cellular plasma membrane and mitochondrial outer membrane within membrane structures that are glycosphingolipid-enriched microdomains called lipid rafts.[20–25] Composition of these lipid rafts within the plasma membrane includes glycosphingolipids, sphingomyelin, cholesterol, and specific membrane proteins. The functional significance of the dynamic and temporally formed lipid rafts appears to be that raft coalescence allows for control of protein–protein interactions during signaling events that mediate cell survival or death.[20–25] An emerging view is that these unique lipid microdomains provide a spatial microenvironment for the aggregation of specific sets of proteins providing for enhanced efficacy and specificity of interactions between enzymes involved in signal transduction.[19]

Consistent with these suggested roles for lipid rafts is a growing volume of evidence for a role of capping in Fas signaling of apoptosis. Ceramide elevation has been demonstrated after Fas activation in a variety of cell types, which is generated by either acid sphingomyelinase or neutral sphingomyelinase from sphingomyelin.[26–30] Furthermore, ceramide when added exogenously to homeostatic cells, induces apoptosis in almost all cell types tested.[18] Recent studies suggest a role for ceramide in the physical changes that occur in the plasma membrane during apoptosis, such as phosphatidylserine externalization, membrane blebbing, the formation of apoptotic bodies, and the enlarged raft domains seen during induction of apoptosis.[28] Additional evidence demonstrates that acid sphingomyelinase translocation to and ceramide elevation within membrane rafts occurs on Fas stimulation of hepatocytes.[19]

The role of Fas and ceramide in the apoptotic response has been corroborated in our laboratory in human ARPE-19 cells in culture by either exposure to oxidants or laser irradiation.[1–3] These cells are relevant to our present study, because of their key role in retinal degenerations including age-related macular degeneration. Clinical studies confirm a similar response in macular degenerative conditions that have been associated with aging, laser treatment, or oxidative stress in the eye.[4–6,31] In the present study, we demonstrated that Fas-induced apoptotic raft formation occurs in ARPE-19 cells, that raft formation can be blocked by cholesterol depletion, and that membrane rafts can be localized and analyzed in membrane vesicles from ARPE-19 cells undergoing apoptosis by extrusion through 2-μm pore polycarbonate membranes.

Materials and Methods

Cell Culture Conditions

All materials were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Immortal human RPE cells (ARPE-19; ATCC, Manassas, VA) were seeded from ATCC's stock and grown as described by Dunn et al.[32] The cells were grown in filter (0.1 μm)-sterilized basal growth medium DMEM/F12 (Invitrogen, CA) supplemented with 10% fetal bovine serum (Invitrogen), 1% (vol/vol) 200 mM L-glutamine (Invitrogen), 0.2% (vol/vol) 50 mg/mL gentamicin (Invitrogen), normicin (Invivogen, San Diego, CA), 1% of 10,000 units/10,000 μg penicillin/streptomycin (Invitrogen), and 3.7 g/L cell culture grade $NaHCO_3$. The complete medium was filter (0.2 μm) sterilized a second time before inoculation. Cells were grown at 37°C with 10% CO_2 atmosphere, and the medium was changed every 5 to 7 days. Cells were subcultured from near-confluent to confluent cultures after release with trypsin (0.05%)-EDTA (1 mM) in Hanks' balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} (Invitrogen). Total cell passage did not exceed 30 times.

BODIPY FL GM1 Staining to Stain for Membrane Rafts

Cells stained with BODIPY fluorescein (FL) were grown on coverslips (no. GL0140; Corning-Costar, Corning, NY) prepared for seeding by immersion in 50 mL acetone for 40 minutes, followed by 50 mL 100% ethanol for 20 minutes, then in 50 mL of 0.1 M HCl for 40 minutes, and then rinsed 10 times in 50 mL ddH_2O. Coverslips were autoclaved on a gravity cycle for at least 25 minutes. Sterile coverslips were placed into each of the wells of a rectangular eight-well plate and a 1-mL aliquot of poly-L-lysine (33 μg/mL in PBS) was placed on the coverslip. The plates were incubated at room temperature for 1 hour and rinsed two to three times with sterile ddH_2O. Treated coverslips were seeded with 1.75 mL of detached cell suspension and allowed to equilibrate for 1 day before analysis the following day.

Alexa 488 Cholera Toxin Subunit B Staining for Membrane Rafts

Coverslips were sterilized by flaming with 70% ethanol and placed into a six-well plate for seeding with 2 mL of detached ARPE-19 cell suspension. The media were changed 18 hours after seeding, and cells were imaged 40 hours after seeding.

Fas Ligand Treatment for Apoptosis Induction

ARPE-19 cells were induced with 5 ng/mL FasL (Fas ligand) protein for 16 to 20 hours, then rinsed three times with 10 mL warm HBSS, and detached with a citric saline solution (135 mM KCl, 1.4 mM sodium citrate). Warm sodium citrate (5 mL) was added to a 75-cm^2 flask and incubated at 37°C for 7 minutes or until approximately 70% of the cells (by microscopic observation) were detached, at which point a 5-mL aliquot of cold PBS was added to terminate the detachment. Cells were centrifuged immediately at 300*g* and rinsed once in 1 mL cold PBS. Cell density was measured by hemocytometer and was adjusted to 3×10^6 cells/mL with cold PBS.

Cell Labeling for Microscopy and Fluorescence-Activated Cell Sorting Analysis

For images with BODIPY FL ganglioside GM1 conjugated to BSA (Invitrogen), 100 μL of 100 nM BODIPY GM1 (in PBS) was added to a perfusion chamber (8 × 100 μL; Electron Microscopy Sciences, Fort Washington, PA) that was placed on top of a coverslip. Cells were incubated for 5 minutes at 20°C in the dark. The coverslip was rinsed three times with 2 mL cold PBS. The coverslip was then mounted upside down onto the depression (containing approximately 300 μL of PBS) of a depression slide (Fisher Scientific, Pittsburgh, PA) and the coverslip sealed with nail polish. Alexa 488 cholera toxin subunit B (CTB; Invitrogen) incubations were performed without using a perfusion chamber. Cells growing on prepared coverslips were incubated for 15 minutes in the dark in 300 μL of 1 μg/mL CTB in cold PBS. The coverslips were rinsed once with 2 mL cold PBS and mounted onto a microscope slide for imaging. For detached cells, 5 μL of 0.02 mM solution of BODIPY FL GM1 was added to cells in 100 μL PBS. The cells were incubated for 5 minutes at 20°C in the dark. PBS (300 μL) was added to the cells, and they were spun at 350*g* and resuspended in 40 μL cold PBS and transferred to a microscope slide for analysis.

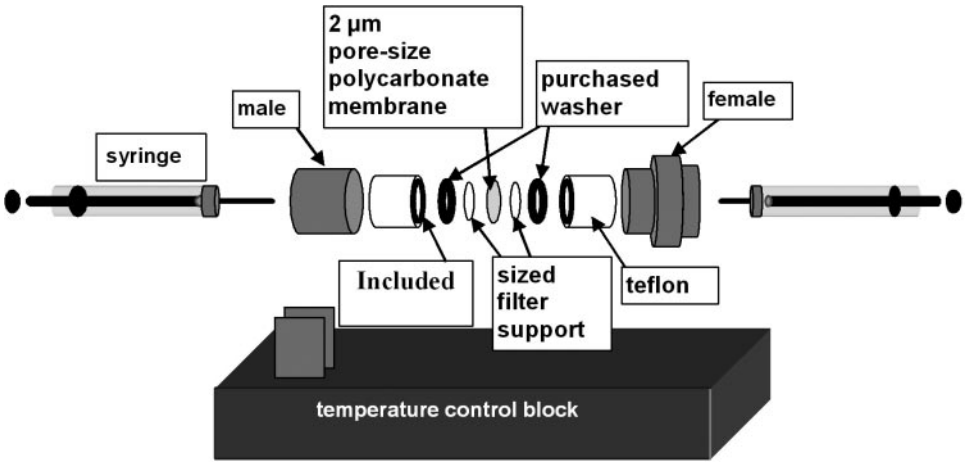


FIGURE 1. The modified extruder used to generate vesicles bounded by sections of cell membranes that express differential membrane locations of integral membrane components, including lipid rafts.

Confocal Microscopy to Image Membrane Rafts

A confocal microscope (Bio-Rad, Hercules, CA) equipped with an argon-krypton laser with 488-, 568-, and 647-nm excitation lines and fitted with filter sets gating at PMT1 (578–618 nm), PMT2 (490–522 nm), and PMT3 (664–696 nm). A 100× oil-immersion objective was used for all experiments unless otherwise noted. Unlabeled cells were used to set the baseline for the three photomultiplier tubes (PMTs).

Cell Extrusion for Vesicle Formation

Cells were grown in 75-cm^2 flasks, as indicated earlier, subcultured 1 day before induction, and the complete growth media were exchanged at least 3 hours before induction. Cells were induced with 5 ng/mL Fas ligand (FasL) for 24 hours. Induced cells were rinsed three times with 10 mL HBSS, these rinsing solutions (containing dislodged cells) were added to a 50-mL centrifuge tube and detachment of remaining adherent cells was triggered by addition of 5 mL 0.2% EDTA. When approximately 70% of cells (by microscopic observation) were detached, the cells were transferred to the 50-mL centrifuge tube containing the rinses and were centrifuged at 300*g* for 5 minutes. Cells were rinsed in cold PBS. Cell density was measured by a hemocytometer and resuspended in 100 μL cold PBS at 5×10^6 cells/mL.

Fas-Phycoerythrin Antibody Incubations

Twenty microliters of Fas-phycoerythrin (PE) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to both 10^6 untreated cells and 10^6 Fas-ligand-induced cells in 100 μL PBS and incubated at 20°C in the dark for 15 to 30 minutes. A GM1-only control was prepared in a similar manner. The induced and control cell suspensions were centrifuged and resuspended in 400 μL PBS. A 300-μL aliquot was extruded immediately (see section on Extrusion) to minimize additional changes in the cell membrane.

Methyl-β-Cyclodextrin Treatments for Cholesterol Depletion

Both 10- and 40-minute treatments with 1 M methyl-β-cyclodextrin (MBCD) at 5°C were initiated. For the final 5 minutes of each treatment, 2.5 μL of 0.01 mM BODIPY FL GM1 complexed to BSA was added to the cells. Control cells were treated for 5 minutes with BODIPY-GM1 only. Cold PBS (1 mL) was added to both the treated and control cells, mixed by inversion, and centrifuged at 300*g* for 5 minutes. The cells were resuspended in 300 μL cold PBS and extruded immediately (described later) and then were labeled with BODIPY-GM1.

Cell Extrusion for Membrane Vesicle Preparations

All extrusions were performed with a kit (Polar Lipids Mini Extruder Kit; Avanti, Alabaster, AL), equipped with 1-mL gas-tight syringes (Hamilton, Reno, NV), according to the diagram in Figure 1. The temperature control block was precooled to 5°C and maintained at less than 15°C during the extrusion process. The extruder was modified as shown with the filter supports sized with a no. 4 brass hole punch (Humboldt Manufacturing Co., Norridge, IL). The filter supports and the polycarbonate membrane (2-μm pore size and 13 mm in diameter; Osmonics Inc., Westborough, MA) were changed between each extrusion. The purchased O-ring measured 7⁄16 in. (outer diameter) × 1⁄4 in. (inner diameter). The treated ARPE-19 cells were collected by one syringe and placed into the female port of extruder. The syringe was carefully depressed, and the opposing (vesicle collection) syringe was allowed to expand as cell suspension was forced through the 2-μm membrane. The collected suspension was placed in a precooled 5-mL polystyrene tube for analysis by flow cytometry within 4 hours of extrusion. The method of dynamic light scattering,[33] was used to measure the hydrodynamic diameters of extruded vesicles (BI-9000 AT; Brookhaven Instruments, Holtsville, NY). The extruded vesicles fell in two size categories: a small cluster at 200 ± 50 nm (this size class, possibly mitochondria, is too small for proper flow cytometry and is excluded by gating from CellQuest analysis; BD Biosciences, Bedford, MA) and a larger sized cluster centered at 1300 ± 150 nm. The larger-sized vesicles were used in the fluorescence-activated cell-sorting (FACS) analysis (BD Biosciences).

Flow Cytometry for Viability Assessment: Annexin V, Propidium Staining, and Quadrant Analysis

The presence of apoptotic cells was scored by monitoring the loss of cell membrane phospholipid asymmetry, resulting in the externalization of phosphatidylserine (PS) to the outer membrane without loss of membrane integrity, as described elsewhere.[34] For these assays ARPE-19 cells were dislodged by trypsinization, washed once in cold binding buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$) and resuspended at 2×10^6 cells/mL in 100 μL binding buffer. A 5-μL aliquot of annexin-V-Alexa Fluor 488 (Invitrogen) was added to all tubes except the binding buffer–only and propidium iodide (PI)–only tubes, and 5 μL of 50 μg/mL PI (Invitrogen) was added to all tubes except the binding buffer–only and Annexin-V–only tubes. Cells were incubated on ice in the dark for 15 minutes, then 400 μL of cold binding buffer was added to each tube, and samples were placed back on ice in the dark. Cells were analyzed by flow cytometry within 1 hour of annexin-V staining.

Flow Cytometry

A flow cytometer equipped with 488 Argon laser (FACSort BD Biosciences) was used. Before the samples were run, the flow cytometer was calibrated (with FACSComp software and Calibrite Beads; BD Biosciences) for three-color analysis. The compensation value was

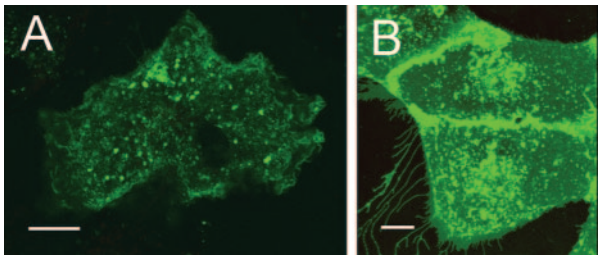


FIGURE 2. Detection of the ganglioside GM1 in RPE cells revealed the location of rafts. Confocal microscopy of untreated human RPE cells stained with (**A**) cholera toxin subunit B labeled with Alexa-488 (1 μg/mL incubated for 15 minutes) or with (**B**) GM1 labeled with BODIPY FL (100 nM for 5 minutes at 4°C, followed by two washes with PBS) showed a relatively uniform distribution of small rafts during normal cellular functions. Both staining methods gave similar patterns of small dispersed rafts. Scale bar, 10 μm.

used for the vesicle analysis, since the labels BODIPY-FL and PE had spectral properties similar to the calibration beads, FITC, and PE. The system software was used for data acquisition and analysis of events (CellQuest; BD Biosciences).

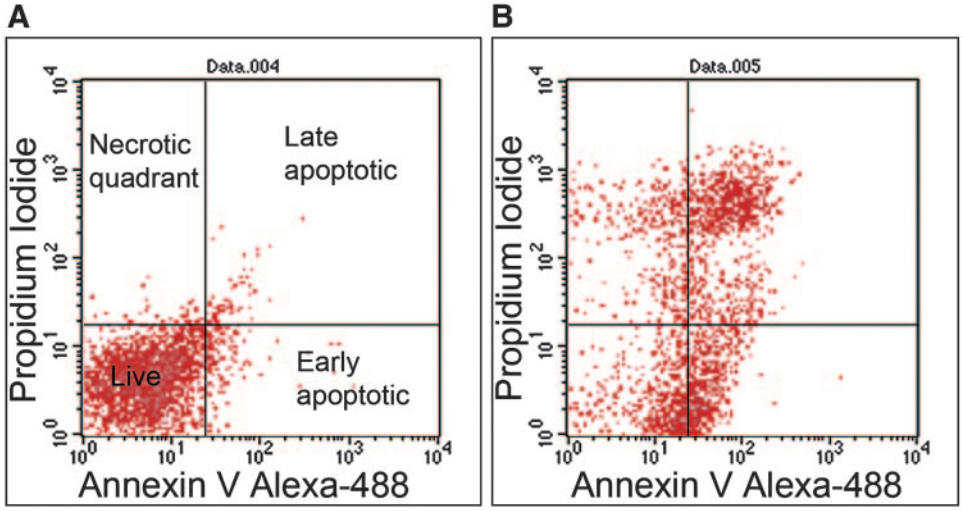
Annexin-V analysis was visualized using forward- and side-scatter detectors set to analyze whole-cell population (cells were 20–40 μm in diameter). The whole cells were gated and then plotted as log annexin-V-FITC intensity versus log PI intensity. Controls (binding buffer only, PI only, and annexin-V only) were run to set appropriate detector gains, compensation, and quadrant gates. The treated cells were evaluated by the following categories: cells negative for both PI and Annexin V staining are live cells; PI-negative, annexin V–positive staining cells were early apoptotic cells; and PI-positive annexin-V–positive staining cells are primarily cells in late stages of apoptosis where membrane integrity is lost. Quadrant analysis of the sorted cells was performed on computer (CellQuest software; BD Bioscience). Twenty thousand cells were analyzed for each sample. An analysis region was set to exclude cell aggregates and debris, and the green channel (fluorescein-annexin V) was set to score <1% of the signals from untreated control cells.

Vesicle analysis was performed after forward- and side-scatter detectors were adjusted to resolve the 1-μm vesicle population. These events were gated and analyzed on the basis of BODIPY FL (conjugated to GM1) log intensity and PE (Fas-PE antibody) log intensity. Controls of unlabeled, BODIPY FL GM1-only–labeled, and PE-only–labeled vesicles were used to set appropriate gates and detector gains.

RESULTS

Our objective was to examine the connection between Fas induced cellular apoptosis and the coalescence of lipid rafts in ARPE-19 cells and to develop methods for the isolation and characterization of these rafts. Cholera toxin subunit B (CTB), which binds to ganglioside lipid GM1[35] an integral raft component, was used to localize lipid raft domains[36,37] in the apoptotic ARPE-19 cells. To enable additional analysis of the induced cell changes associated with induction of apoptosis, we tested whether fluorescently labeled lipids known to reside in rafts could be used to visualize raft domains, and therefore changes in rafts, in induced ARPE-19 cells. We compared the localization of CTB staining, which could confound the analysis by autonomously inducing detrimental pore formation, to that of a trace amount of exogenously added fluorescein labeled GM1 for staining and found the raft domains, revealed by patterns of fluorescent CTB or fluorescent GM1, to be comparable with respect to size and distribution (Fig. 2) of the markers. We did not observe any significant detrimental effect of GM1 staining on cell viability, and interpret these results to indicate that a fluorescent GM1 probe was suitable for identifying raft domains in all subsequent experiments.

ARPE-19 cells induced to undergo apoptosis by treatment with FasL and analyzed by FACS using annexin V and PI to detect both early and late stages of apoptosis in both untreated and treated populations of ARPE-9 cells (Fig. 3). FACS analysis revealed very few apoptotic cells in the native untreated population of RPE cells (Figs 3A). In contrast, ARPE-19 cells treated with FasL for 24 hours revealed a quadrant consisting of approximately 75% of the cells in either late or early stages of apoptosis (Fig. 3B).



Cell state	% of gated
Live	92.54
Early Apoptotic	3.99
Necrotic/Late Apoptotic	3.46

Cell state	% of gated
Live	24.60
Early Apoptotic	22.74
Necrotic/Late Apoptotic	52.66

FIGURE 3. Human RPE cells died by apoptosis in response to the presence of Fas ligand. RPE cells were either (**A**) untreated or (**B**) treated with 5 ng/mL Fas ligand for 24 hours. Flow cytometry and quadrant analysis were performed. Stimulation of RPE cells with Fas ligand resulted in approximately 70% of the cells dying by apoptosis after 24 hours.

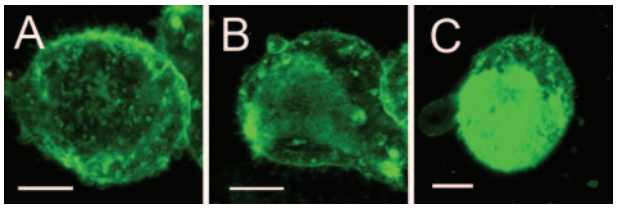


FIGURE 4. Raft coalescence occurs during apoptosis of RPE cells. Apoptosis initiated in RPE cells with FasL treatment (5 ng/mL) caused raft coalescence, resulting finally in capping of the cells with a single large raft or platform. Lipid rafts were visualized after 19 hours by staining with BODIPY-FL-labeled GM1 as in Figure 1. (**A**) A healthy cell; (**B**) early stage of raft coalescence; (**C**) late stage "capping. " Images were visualized by confocal microscopy. Scale bar, 10 μm.

Cells also were evaluated by confocal microscopy with either CTB or GM1 fluorescent labels to detect the presence of raft microdomains. (Fig. 4) similar to those described elsewhere.[20] The analysis revealed an initial distribution of small separated raftlike microdomains in ARPE-19 cells that was replaced temporally in apoptotic cells by large raftlike platforms, formed from raft coalescence, or capping (Figs. 4B, 4C). The occurrence of capping is quite dramatic when viewed through the confocal microscope, but FACS analysis did not show any increase in CTB-fluorescence per cell, indicating that the cap appearance was due to a redistribution of GM1 (coalescence) and not new synthesis.

Although isolation of raft domains is generally done by isolating membrane components that are resistant to detergents and has been used for many cell types,[38] the method described herein provides for the isolation and characterization of both raft and non–raft-containing membrane domains. The critical feature of this method relies on extrusion of whole cells through a small pore membrane resulting in formation of small lipid-containing vesicles composed of plasma membrane segments, thereby providing for the creation of vesicles potentially containing raft domains, which can be visualized by flow cytometry. The resultant vesicles were sorted and analyzed by FACS based on GM1-fluorescence, as a measure of raft content. FACS analysis of vesicles from healthy ARPE-19 cells and FasL-treated RPE cells undergoing apoptosis revealed a dramatic increase in high GM1 raft-containing vesicles in dying cells (Fig. 5). Figure 5 shows an increase in high GM1-containing rafts in

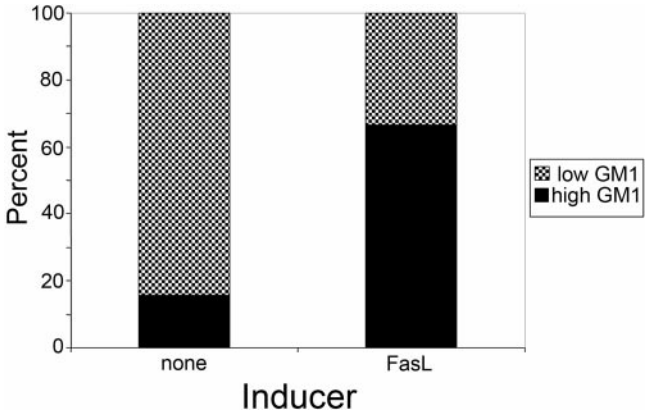


FIGURE 5. Extruded vesicles of high GM1 content increased during apoptosis. FACS analysis of extruded vesicles, from untreated RPE cells and RPE cells treated with Fas ligand (5 ng/mL) to induce apoptosis, resulted in an increase in high-GM1–containing vesicles in the dying cells. Analysis showed an increase in high-GM1 containing vesicles from 10% in untreated cells to almost 60% in FasL-treated RPE cells after 24 hours.

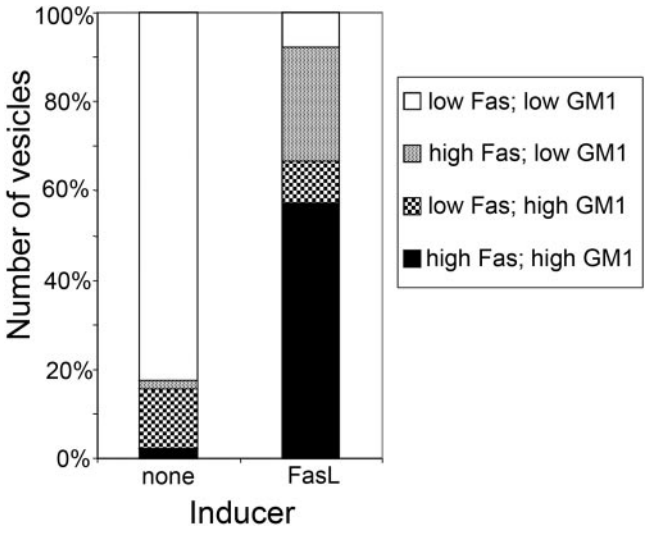


FIGURE 6. Fas receptor proteins redistributed into raft vesicles after induction of apoptosis. Extruded vesicles isolated from untreated or FasL-stimulated (5 ng/mL for 24 hours) RPE cells were stained with both labeled Fas antibody and labeled GM1. The vesicles were then subjected to FACS analysis to determine the subpopulations containing a high content of Fas and/or GM1.

untreated cells (10%) to almost 60% in FasL-treated ARPE-19 cells. In addressing the biological objectives of this project, it was necessary to develop a technique to obtain subfractions of the cell membrane, which could be used to both analyze and characterize the location and distribution of rafts in cells induced to undergo programmed cell death. To that end, the extrusion and enrichment techniques used herein should be applicable as a novel strategy for molecular characterization of the raft domains concentrated in vesicles enriched for raft components or to obtain subpopulations of membrane components suitable for FACS or chemical analysis.

In addition to localization of lipids in the rafts, we used FACS analysis to investigate the distribution of the Fas proteins within rafts using antibodies against protein components of the membrane. Cells were immediately extruded after treatment to prevent redistribution of plasma membrane raft domains. A major redistribution of Fas receptor was observed in FasL-treated ARPE-19 cells by the increased percentage of vesicles with colocalization of GM1 and Fas receptor protein (Fig. 6).

To test the hypothesis that signaling involves cholesterol containing rafts, we analyzed the amount of Fas receptor in raft versus non–raft-containing vesicles after MBCD treatment. MBCD is used to deplete cholesterol from membranes. The data reported in Figure 7 indicate that that treatment with MBCD decreased the ability of Fas receptor to localize to GM1-containing rafts.

DISCUSSION

Identification and Isolation of Membrane Rafts on ARPE-19 Cells

Lipid rafts are dynamic, temporally formed microdomains proposed to be involved in the signaling events that control protein–protein and protein–lipid interactions that mediate cell survival or death.[20,38] Our results demonstrate that both cholera toxin subunit B (CTB) and GM1-ganglioside lipid colocalize on the surface of the ARPE-19 cell in both homeostatic and apoptotic conditions. These results with ARPE-19 cells are consistent with those reported by others indicating that both CTB and GM1 are unique markers of the raft microdomain

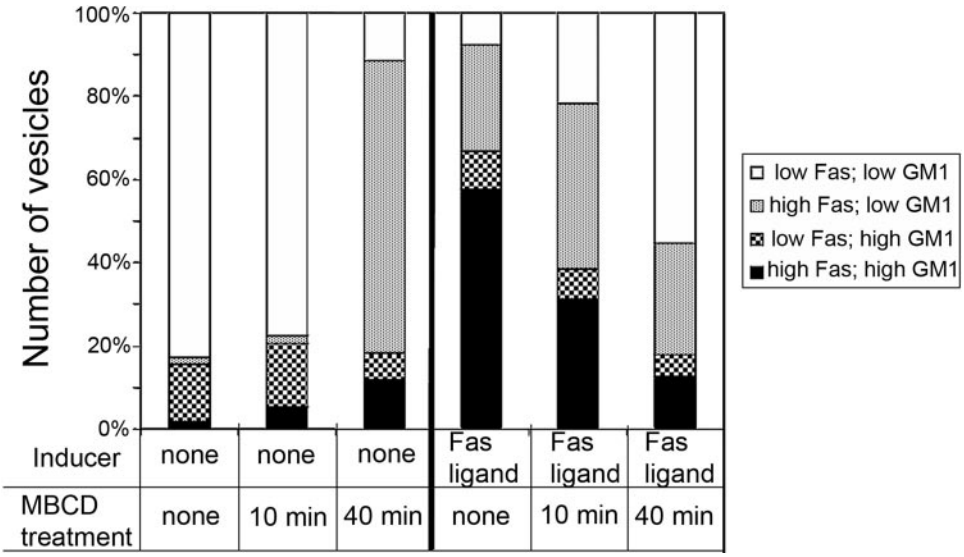


FIGURE 7. Raft formation is inhibited by depletion of cholesterol from RPE cell membranes. RPE cells were either untreated or treated with FasL (5 ng/mL) for 24 hours, and then MBCD was added. The cells extruded immediately before addition or 10 or 40 minutes after the addition of MBCD. FACS analysis of extruded vesicles was performed as in Figure 5. The percentage of high Fas receptor–containing rafts decreased during depletion of cholesterol from the membrane.

found in the lipid bilayers of the plasma membrane of both plant and animal cells. The demonstration that ARPE-19 cells contain membrane rafts in both healthy and dying cells allowed us to focus on two main investigative thrusts: (1) Do membrane rafts change when ARPE-19 cells undergo apoptosis? and (2) can these rafts be visualized in membrane fractions enriched for domain components?

Cell Membrane Extrusion Provides Raft-Localized Vesicles

Historically, the visualization of rafts was based on their unique property to be resistant to detergent solubilization; hence, the name detergent-resistant membranes, found in the literature.[20,38] However, we found this methodology tedious to perform and difficult to reproduce. The methodology reported in this article permits the isolation of both the raft component of the membrane with the non–raft component to permit future studies of the raft isolates in synthetic platform systems. The extrusion of the RPE cells through a polycarbonate membrane pore of 2-μm size followed by flow cytometric enrichment of GM1 lipids though quadrant gating techniques produced a reliable method capable of detecting and separating membrane vesicles enriched in raft domains from those membrane fractions that are raft free.

Fas Signaling and Capping during the Induction of Apoptosis

The ability to localize and visualize the membrane rafts allowed us to investigate whether size and distribution of membrane rafts changed when cells were undergoing apoptosis and provides an opportunity to assess raft-driven functions. Histopathologic analysis and cell culture studies of ARPE-19 cells have confirmed that cell death is mediated through Fas signaling via the Fas receptor, which is localized on the outer plasma membrane bilayer.[8,9,11] Apoptosis also can be mediated through directed clustering of Fas receptor in membrane rafts leading to Fas receptor autoactivation independent of the presence of FasL. Regardless of the exact mechanism, Fas association with membrane rafts appears to be important in amplifying Fas signaling, because lipid rafts can serve as foci for recruitment of signaling molecules at the plasma membrane.[39–41]

In our study, human ARPE-19 cells were observed to elaborate increased density of punctate staining with GM1 and Fas receptor when stimulated with FasL. Correspondingly, the proportion of apoptotic cells, determined by annexin V staining in FACS quadrant analysis, also increased (Fig. 3). Confocal microscopy confirmed that capping of the raft domains occurred. Capping is believed to be a prerequisite for signaling by many receptors, including Fas.[19,23,40] Additional studies have supported the notion that ceramide platforms form through sphingomyelinase activation which causes self-assembly of rafts and therefore play an important role in capping and activation of the receptors that regulate the biological processes of apoptosis, cellular proliferation, and immune signaling.[26,29,31,40,42]

Effect of Cholesterol Depletion on the Number of Rafts Containing Fas Receptor

Previous studies from our laboratory have shown that oxidative stress and or laser photocoagulation of RPE cells induces apoptosis that is mediated through ceramide signaling mechanisms.[2,3] Furthermore, the apparent connection between cell death and capping is supported in published reports revealing that destabilization of coalesced raft domains on selective cholesterol depletion results in increased cell survival.[19,40] In this study, we observed a decrease in Fas-associated rafts in cells treated with MBCD, which suggests that raft stability may be a pharmacologic target for possible therapeutic intervention for conditions such as age-related macular degeneration or other retinal disorders that involve apoptosis, either from the disease process itself or resulting from therapeutic intervention.[43,44] Raft formation is reduced by MBCD treatment, and the corresponding observed decrease in apoptotic activity is consistent with the hypothesis that membrane rafts drive the function of apoptosis.[19,40] Together, these findings suggest that modification of lipid, ceramide, and/or cholesterol metabolism may influence RPE membrane raft stability and modify apoptotic pathways.

Last, the ability to recover vesicles derived from membrane rafts enriched for apoptotic signaling molecules will allow us to investigate further the biological properties and mechanisms whereby rafts mediate signaling across cell membranes. Accordingly, we envision isolating and embedding components from raft-enriched vesicles into stable synthetic lipid bilayers that mimic the plasma membrane. Such methodology may lead to pharmacological strategies that allow targeting of cholesterol, lipoprotein, or sphingolipid metabolism and thereby alter

signaling mechanisms through altered raft composition or membrane stability. One distinct advantage of having a reliable technique to generate vesicles via extrusion from apoptotic cells that are enriched in rafts and ones that are depleted of rafts is to enable direct evaluation of the effect of rafts in signal exchange in synthetic bilayers. Such direct comparison is not possible with detergent-resistant membrane fractions. Furthermore the biophysical properties that promote signaling through rafts could be investigated by single molecule and single cell–vesicle spectroscopy[45,46] and may facilitate further understanding of protein–protein, protein–lipid, and lipid–lipid interactions.

Acknowledgments

The authors thank Huawen Wu for assistance with the analysis of dynamic light scattering experiments and Chanel K. Yee for technical assistance.

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