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Caveolae-like domains (CLDs) have been hypothesized to mediate apoptosis, since they contain sphingomyelin and initiate the conversion of sphingomyelin to ceramide. To address whether CLDs are directly involved in apoptosis, CLDs from U937 cells were isolated, taking advantage of their detergent insolubility and low density. The CLDs contained alkaline phosphatase as well as many signaling molecules, including Fyn, protein kinase Ca, Raf-1, phospholipase Cγ1, and tyrosine phosphoproteins. Immunoblotting and immunofluorescent data showed that TNF receptor 1 colocalized with CD36 in CLDs, suggesting that TNF-α-initiated apoptosis occurs in CLDs. When cells were incubated with lipoprotein-deficient medium, the cholesterol concentration was greatly decreased in CLDs but not in other fractions, implying that the CLDs were selectively disrupted. In the CLD-disrupted cells, the surface expression of TNF receptor 1 and CD36 was significantly reduced. Analysis of cellular morphology, percent DNA fragmentation, DNA ladder, and caspase-3 activity showed that TNF-α-mediated apoptosis was blocked in CLD-disrupted cells, whereas anti-Fas-mediated apoptosis was not. Since Fas was not found in CLDs of Jurkat cells, apoptosis by Fas ligation might not require CLDs. Taken together, these data strongly imply that TNF-α-mediated apoptosis is initiated in CLDs. The Journal of Immunology, 1999, 162: 7217–7223.

Apoptosis, also termed programmed cell death, is a genetically regulated mechanism, and its machinery is conserved throughout evolution from metazoan development to mammalian homeostasis. In mammalian cells, TNF-α is known to trigger apoptosis by binding to a specific receptor, the TNF receptor (TNFR)1–3. The TNFR superfamily also includes Fas, nerve growth factor receptor, CD40, CD30, and others (4, 5). Whereas all these receptors have sequence homology in cysteine-rich pseudorepeats in their extracellular domains, the C-terminals of TNFR1 and Fas also share a sequence homology, defined as the death domain, that is required for apoptosis signaling (4). Using the yeast two-hybrid system, other death domain-containing proteins, such as TNFR-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD), have also been identified (6, 7). Upon cellular activation by TNF-α or Fas ligand (FasL), TRADD and FADD are recruited to TNFR1 and Fas, respectively (6, 7). In addition, TRADD interacts directly with FADD (8), implying that TNF and FasL signaling might converge at FADD for the purpose of cell death.

Ceramide, which is generated from sphingomyelin by sphingomyelinase (SMase) activation, is known to be a second messenger inducing apoptosis (9, 10). Although it is unclear whether the ceramide produced upon TNF stimulation is directly involved in apoptosis, the ceramide produced by neutral SMase mediates the Raf/MEK/MAP kinase pathway (MEK = MAP/extracellular signal-related kinase kinase) (11–15). The site of ceramide formation and the possible compartmentalization of signaling pools of sphingomyelin remain poorly understood. However, detergent-insoluble complexes or caveolae have been suggested to be the plasma membrane compartments producing ceramide, since they are enriched in sphingomyelin (16, 17) and mediate IL-1β- or nerve growth factor-activated conversion of sphingomyelin to ceramide (18, 19).

Caveolae are flask-shaped invaginations of plasma membrane coated by a 22-kDa structural protein called caveolin (20). Caveolae have been isolated from other membrane components on the basis of their detergent insolubility and low density (21, 22). The detergent-insoluble complexes are enriched in caveolin, glycosphingolipid (21). However, caveolin-rich domains could be separated from GPI-anchored protein-rich domains by coating endothelial cells with silica beads (23). In addition, neuroblastoma cells and lymphocytes present detergent-insoluble complexes without caveolin expression and morphologic plasma membrane invaginations, i.e., caveolae, implying that caveolin is not necessary to form a detergent-insoluble complex (24, 25). The detergent-insoluble complexes from these cells and unicellular organisms such as Saccharomyces cerevisiae (26) and Dictyostelium discoideum (27) have the same biochemical properties as caveolae and, therefore, have been called caveolae-like domains (CLDs) (28).

Caveolae or CLDs have been implicated in the range of signaling events. Specific signaling events originating in caveolae or CLDs were reported as follows: epidermal growth factor-stimulated Raf-1 recruitment (29), caveolin-1 phosphorylation by Src kinase (30), the platelet-derived growth factor receptor kinase cascade (17, 31, 32), TCR signal transduction cascade (33), turnover
of phosphatidylinositol 4,5-bisphosphate (34), and conversion of cellular prion protein to scrapie prion protein (28).

To address whether CLDs are major sites mediating apoptosis signals we investigated the presence of TNFR1 in CLDs of U937 cells. We demonstrated that TNFR1 is predominantly localized in CLDs, and that apoptosis by TNF is blocked in CLD-disrupted cells, indicating that TNF-α-mediated apoptosis is initiated in the specific plasma membrane compartments, CLDs.

Materials and Methods

Material

[6-3H]Thymidine (25.0 Ci/mmol) and an enhanced chemiluminescence substrate kit were obtained from Amersham (Aylesbury, U.K.). TNF-α and anti-Fas used for apoptosis induction were purchased from Upstate Biotechnology (Lake Placid, NY), the cholesterol test kit was obtained from Boehringer Mannheim (Mannheim, Germany), and lipoprotein-deficient serum was obtained from Sigma (St. Louis, MO). The CPP32/caspase-3 colorimetric protease assay kit was obtained from Medical & Biological Laboratories Co. (Nagoya, Japan).

Antibodies for immunoblotting or immunofluorescence were obtained from the following sources. Anti-caveolin-1, anti-Fyn, anti-FKCa, anti-Raf-1, anti-Fas, and anti-tyrosine phosphoprotein were purchased from Transduction Laboratories (Lexington, KY). Anti-CD36 was purchased from SeroTech (Oxford, U.K.), and anti-TNFR1 was obtained from R & D Systems (Minneapolis, MN). Anti-PLCγ1 was obtained from Dr. P.-G. Seo (PosTech University, Pohang, Korea).

Cell culture

U937 and Jurkat cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 50 μg/ml of streptomycin and penicillin in a 5% CO₂ incubator at 37°C. NIH-3T3 cells were grown in DMEM (high glucose) supplemented with 10% heat-inactivated calf serum. For cholesterol-depleting conditions, cells grown in 10% PBS-containing medium were washed twice with serum-free medium and incubated for 24 h in medium with 10% lipoprotein-deficient serum.

Isolation of CLDs and alkaline phosphatase assay

CLDs were isolated according to the method of Pike and Casey (34) with little modification. Briefly, 8 × 10⁶ cells were rapidly chilled in liquid nitrogen and washed twice with cold PBS by centrifugation (600 × g, 5 min, 4°C). The collected cells were incubated with 1 ml of HEPES buffer (25 mM HEPES [pH 7.4], 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1 mM PMSF, aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μM leupeptin, and 1 μg/ml pepstatin A) and phosphatase inhibitors (1 mM NaF and 1 mM Na₃VO₄) for 20 min, and homogenized 20 times with a tight Dounce homogenizer (Kontes, Vinland, NJ). The extract was mixed with 1 ml of 2.5 M sucrose, transferred to an SW41 centrifuge tube, and overlaid with 6 ml of 30% sucrose solution and 4 ml of 5% sucrose solution containing 25 mM HEPES (pH 6.5) and 150 mM NaCl. The discontinuous sucrose gradients were centrifuged for 21 h at 4°C in an SW41 rotor at 39,000 rpm. The gradient was fractionated into 10 fractions from the bottom to the top. Cholesterol and protein concentrations were determined using a cholesterol test kit and the bicinchoninic acid method (Pierce, Rockford, IL), respectively, according to the manufacturer’s protocols.

Alkaline phosphatase activity was measured according to the method of Liu and Anderson (18). PBS-washed filter paper was placed in a Bio-Rad Bio-Rad filter apparatus (Hercules, CA) and overlaid with nitrocellulose membrane that was washed with PBS. The excess PBS was removed by suction, and 50 μl of sample was loaded in each well. A vacuum was applied to the apparatus to transfer proteins in the sample on a nitrocellulose membrane. The membrane was then washed with 50 ml of PBS and developed using 50 ml of substrate from Bio-Rad alkaline phosphatase substrate kit. The reaction was stopped by membrane washing with water.

SDS-PAGE and Western blotting

Proteins were concentrated by TCA/aceton precipitation and resuspended in 300 μl of 2× SDS sample buffer. After 35 μl of proteins were run in SDS-polyacrylamide gels and transferred to a nitrocellulose membrane, Ags were visualized by sequential treatment with specific Abs, HRP-conjugated secondary antibodies, and an enhanced chemiluminescence substrate kit.

Immunofluorescence

Cells were fixed in 10% formaldehyde in PBS for 20 min and permeabilized in 0.5% Triton X-100 in PBS for 5 min at room temperature. The cells were incubated with blocking buffer (5% BSA in PBS), and primary Abs (goat anti-TNFRI and mouse anti-CD36 or goat anti-TNFRI and mouse anti-caveolin-1). The primary antibodies were detected with fluorescein-conjugated anti-goat IgG and rhodamine-conjugated anti-mouse IgG. Cells were observed with a Bio-Rad confocal microscope (MRC 1024).

Aptosis assay

Cells (2 × 10⁵ cells) were lysed in 500 μl of lysis buffer (100 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.2 M NaCl, 0.2% SDS, and 0.1 mg/ml proteinase K). After overnight incubation at 37°C, DNA was precipitated by adding an equal volume of isopropanol, dissolved in 20 μl of Triton-HCl, pH 8.0, containing 1 mM EDTA and 0.1 mg/ml RNase A, and incubated at 37°C for 30 min. The DNA was analyzed by 2% agarose gel electrophoresis with 0.5 μg/ml ethidium bromide.

The quantitative apoptosis assay was followed according to the method of Duke and Cohen (35). Briefly, 1 × 10⁶/ml cells were incubated with [3H]thymidine (1 μCi/ml) for 2 h to label DNA, then washed with serum-free medium. After treatment with TNF-α or anti-Fas, cells were transferred to a microcentrifuge tube labeled B and microcentrifuged at 4000 rpm for 10 min at 4°C, and the supernatant was transferred to a tube labeled T. The cell pellet was lysed with TTE solution (10 mM Tris (pH 7.4), 1 mM EDTA, and 0.2% Triton X-100) for 10 min and microcentrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was transferred to a tube labeled T. The pellet after microcentrifugation was resuspended with 0.5 ml of STE solution (1% SDS, 10 mM Tris (pH 7.4), and 1 mM EDTA). The radioactivity from each tube was determined by liquid scintillation counting, and the percent DNA fragmentation was calculated as following: % DNA fragmentation = 100 × (S + T)/(S + T + B).

Measurement of caspase-3 activity

Caspase-3 activity was measured according to the manufacturer’s protocol. Cells (7 × 10⁵) were lysed with 300 μl of chilled cell lysis buffer. After microcentrifugation (10,000 × g, 1 min, 4°C), the clear supernatant was used for caspase-3 colorimetric protease assay. The protein (170 μg) was mixed with 50 μl of 2 × reaction buffer (containing 10 mM DTT) and 5 μl of 4 mM Asp-Glu-Val-Asp-p-nitroanilide (200 μM final concentration). After incubation at 37°C for 2 h, samples were read at 405 nm in a microtiter plate reader.

Results

Signaling molecules in CLDs of U937 cells

Using their detergent insolubility and low density, CLDs were isolated from U937 cells. After ultracentrifugation, there was an opaque band at a density of the boundary between 30 and 5% sucrose, which contained the CLDs. The sucrose gradient was fractionated into 10 fractions for further analysis. To certify whether the Triton X-100-insoluble fraction contained the signaling molecules reported to be present in CLDs, we analyzed each fraction for these species by immunoblotting. Caveolin-1, a caveolar marker protein, was not present in any fraction (data not shown), suggesting that U937 cells do not have morphologic caveolar structures. Fig. 1 shows that alkaline phosphatase, a GPI-anchored protein, as well as Fyn and phosphotyrosine proteins were predominantly present in the CLDs. In addition, small quantities of PKCa, Raf-1, and PLCγ1 were also found in the CLDs. Especially, PLCγ1 found in the CLDs could activate the turnover of phosphatidylinositol 4,5-bisphosphate, which is mediated by the Triton X-100-insoluble complex (34). The presence of signaling molecules in the CLDs suggests that the CLDs of U937 cells might be involved in various signaling events.

Localization of TNFR1 in CLDs

To address the possibility that CLDs mediate the apoptotic pathway, we analyzed TNFR1 and CD36 in CLDs by immunoblotting and immunofluorescence with anti-TNFRI; since CD36 is enriched in the Triton X-100-insoluble complex (21, 36), CD36 was used as a marker of CLDs. Immunoblotting data (Fig. 2A) showed
that TNFR1 exists predominantly in CLDs, strongly suggesting that TNF-α-mediated apoptosis could be initiated in CLDs. Exactly the same pattern was seen when the samples were immunoblotted with anti-CD36.

Both CD36 and TNFR1 were detected on the surface of intact cells using the confocal microscope (Fig. 2B). In optical sections through the middle of the cell, staining of CD36 and TNFR1 appeared in small, bright patches around the cell periphery (Fig. 2B). This pattern was quite distinct from the faint, nonspecific fluorescence seen in cells stained with secondary antibody alone. Also, the presence of CD36 and TNFR1 was visualized by double immunofluorescence to demonstrate that these molecules are colocalized. Fig. 2B indicates that these two proteins are indeed colocalized, showing the same patched and punctate staining pattern.

We used NIH-3T3 cells to verify the location of TNFR1. Anti-TNFR1 IgG fluorescence staining was concentrated in patches on the cell surface (Fig. 2C). These patches were appeared as stretches along the margin of the cell or irregularly shaped regions over the cell body. The same cell processed using an anti-caveolin-1 IgG had nearly an identical staining pattern. A comparison of both images showed extensive colocalization of the two Ags. This suggests that TNFR1 is concentrated in caveolin-rich domains. Since molecules in caveolae or caveolae-like domains
appeared colocalized in indirect immunofluorescence, we conclude that TNFR1 is indeed localized in caveolae or caveolae-like domains.

The disruption of CLDs reduces the surface expression of TNFR1 and CD36

Cholesterol is a building block of caveolae or CLDs and appears to be required for the maintenance of their structure (22). Caveolae disappeared in cells that were depleted of cholesterol or exposed to sterol-binding agents, such as filipin and nystatin (22). Disassembly of caveolae means disruption of signal transduction via caveolae (37). MDCK cells incubated in low density lipoprotein-deficient medium showed the reduced surface expression of gD1-DAF (38), a GPI-anchored protein that is predominantly localized in detergent-insoluble complexes. In addition, cells treated with lovastatin, a cholesterol synthesis inhibitor, lost their ability to convert cellular prions to scrapie prions (39), both forms of which are enriched in CLDs (28). These results indicate that cholesterol depletion inhibits the cell surface expression of molecules present in caveolae or CLDs and blocks signaling events through caveolae or CLDs.

When cells were stained with trypan blue after incubation with lipoprotein-deficient medium for 24 h to disrupt CLDs, they still appeared morphologically healthy and viable, although the total cellular cholesterol concentration was 62.8% that found in normal cells. The cholesterol concentrations were 13.6 and 8.1 μg/g protein, respectively, in normal and lipoprotein-deficient conditions. Fig. 3A shows the concentrations of cholesterol and protein, respectively, in isolated CLDs. Approximately 53% of the cholesterol in normal cells was recovered in CLDs. There was a significant decrease in cholesterol in the lipoprotein-deficient condition (30% of normal condition) considering the cholesterol amount only in CLDs (fractions 4 and 5). However, the amount of cholesterol in the bottom fractions (fractions 9 and 10) did not change. The protein concentration in CLDs, accounting for <1% of the total proteins, remained constant in both conditions (Fig. 3A).

**FIGURE 3.** Cholesterol depletion reduces the cholesterol level in CLDs and the surface expression of CD36 and TNFR1. Cells incubated in normal or lipoprotein-deficient medium for 24 h were lysed with 1% Triton X-100. The same amount of protein was used for CLDs isolation. A, The concentrations of cholesterol and protein in each fraction of the sucrose gradient were determined. B, Indirect immunofluorescence of CD36 and TNFR1 in normal and lipoprotein-deficient conditions. The numbers represent the mean relative signal intensity of CD36 and TNFR1 obtained from 10 cells. The intensity was measured by confocal microscopy.
From these results, it was concluded that cells grown in lipoprotein-deficient medium have selectively disrupted CLDs. Immunofluorescence data (Fig. 3B) showed that the surface expression of TNFR1 and CD36 was greatly reduced in cells grown under lipoprotein-deficient conditions. When the relative signal densities of TNFR1 and CD36 were measured by confocal microscopy, cells grown in lipoprotein-deficient medium lost 64.7 and 64.6% of their signal density, respectively. When the amount of immunofluorescence of TNFR1 was measured by FACS scanning, cells grown in lipoprotein-deficient medium also showed a great decrease in TNFR1 fluorescence compared with cells grown under normal conditions (data not shown). With confocal microscopic and FACS scanning data, it is concluded that the surface expression of TNFR1 is greatly reduced in cells grown in lipoprotein-deficient medium.

Disruption of CLDs blocks TNF-α-mediated apoptosis, but not Fas-mediated apoptosis

To address whether CLDs are required for TNF-α signaling, apoptosis by TNF-α was measured in cells grown in normal or lipoprotein-deficient medium. The cells were preincubated with serum-free medium for 2 h and treated with 5 nM TNF-α or 0.5 μg/ml anti-Fas for 3 h, and cell morphology was observed under the inverted microscope. Following treatment with TNF-α, blebbing morphology appeared in cells grown under normal conditions, but not under lipoprotein-deficient conditions (data not shown). However, there was no difference in the blebbing morphology under both conditions after treatment with anti-Fas (data not shown). The same results were obtained with respect to intranucleosomal DNA laddering and quantitative DNA fragmentation (Fig. 4, A and B). Following treatment with TNF-α, DNA fragmentation appeared in cells grown under normal conditions, but only a small amount was observed under lipoprotein-deficient conditions. However, there was no difference in the DNA fragmentation under both conditions after treatment with anti-Fas, suggesting that Fas-mediated apoptosis is not affected in CLD-disrupted cells.

To make these data more convincing, caspase-3 protease activity was determined after treating cells with TNF-α (5 nM) or anti-Fas (1 μg/ml) for 3 h under normal and lipoprotein-deficient conditions (Fig. 4C). Following treatment of TNF-α, caspase-3 activity was highly increased (4.3-fold increase over untreated cells) in cells grown under normal conditions, but its activity under lipoprotein-deficient conditions was 39% of normal. Meanwhile, there was little difference in caspase-3 activity under both conditions after treatment with anti-Fas. Its activity under normal conditions was increased 3.4-fold over that in untreated cells, whereas under lipoprotein-deficient conditions it was 94% of normal. These data suggest that the membrane compartments for TNF and Fas signaling could be spatially separated. Since Fas was not detected in U937 cells by immunoblotting, we selected Jurkat cells expressing high level of Fas to investigate whether Fas is localized on CLDs. Fig. 5 shows that CLDs of Jurkat cells harbored exclusively
alkaline phosphatase. However, Fas was found in only the bottom fraction, not in the CLD fraction, explaining why apoptosis by Fas ligation was not influenced in cells grown in lipoprotein-deficient medium, which disrupted CLDs. Taken together, we conclude that the disruption of CLDs blocked TNF-α-mediated apoptosis, but not anti-Fas-mediated apoptosis.

Discussion
Since caveolae were first proposed to be signal transduction centers in 1993 (40), the number of signaling events delineated to occur through caveolae or CLDs has increased. Here, we demonstrate the predominant localization of TNFR1 in CLDs and the involvement of CLDs in apoptosis. This finding strongly suggests that CLDs are the plasma membrane compartments transducing signals from TNF to other TNF interacting molecules, such as FAN (factor associated with neutral SMase activation), TRADD, TNFR-associated factors, and receptor-interacting proteins (5). The number of TNF-interacting molecules has also steadily increased, as indicated by immunoprecipitation and by the yeast two-hybrid system. Since molecules such as FAN and TRADD could be recruited to TNFR1 after TNF stimulation (6, 41), CLD analysis for these molecules may open new doors for understanding the pathways of apoptosis and may facilitate the identification of new molecules interacting with TNFRs.

It has been reported that Raf-1 is recruited to the plasma membrane, especially to caveolae, after epidermal growth factor stimulation (29). Raf-1 in caveolae is able to phosphorylate MAP kinase, suggesting that the MAP kinase pathway (from receptor to MAP kinase) might be mediated via caveolae (32). If that is true, TNF-α- or ceramide-mediated MAP kinase pathway might also be mediated in caveolae or CLDs, since Raf-1 is also phosphorylated and activated by TNF or ceramide (11, 12). In addition, we cannot exclude the possibility that CLDs are major membrane compartments for recruiting FAN and neutral SMase, which are known to be localized in the plasma membrane (10, 12), and for converting sphingomyelin to ceramide after TNF-α stimulation.

The fate of any T cell, whether it is developing in the thymus or functioning in the peripheral immune system, is dependent on TCR specificity for Ags presented by MHC molecules and on the consequences of TCR-generated intracellular signaling pathways that lead to activation, anergy, or apoptosis (42, 43). Upon TCR stimulation, TCRs, phospho-ZAP70, and phosphotyrosine proteins are recruited to the detergent-insoluble microdomain (31). Apo2L/TRAIL (TNF-related apoptosis-inducing ligand) is one TNF family member and induces apoptosis by its ligation (2). Apo2L/TRAIL-mediated apoptosis is blocked by transfecting decoy receptors (44), which are GPI-anchored proteins, and might be localized in CLDs. CD20 is a nonglycosylated 33- to 37-kDa phosphoprotein involved in B cell signaling that subserves important functions in the regulation of B cell proliferation and differentiation. Extensive cross-linking of CD20 with murine anti-CD20 mAbs in the presence of either goat anti-mouse IgG or Fc receptor-expressing cells directly inhibits B cell proliferation, induces nuclear DNA fragmentation, and leads to cell death by apoptosis (45). Binding of CD20 Abs to B cells induces the rapid redistribution of up to 95% of CD20 molecules to low density, detergent-insoluble microdomains and induces the appearance of an approximately 50-kDa phosphorylated tyrosine protein in the same compartment (46, 47). With these findings for TCR, Apo2L, and CD20 and our data for TNF-α, we strongly suggest that CLDs might be a specific plasma membrane compartment regulating apoptosis.

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