Cellular apoptosis is associated with increased caveolin-1 expression in macrophages

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Abstract **Macrophage apoptosis is an important factor in determining the efficiency of the immune response, atherosclerotic lesion stability, and clearance of aged cells by phagocytosis. The involvement of caveolin-1 in the regulation of apoptosis has been previously suggested in fibroblasts and epithelial cells. Here we show that treatment of thioglycollate-elicited mouse peritoneal macrophages with various unrelated apoptotic agents, including simvastatin, camptothecin, or glucose deprivation, is associated with a specific and large increase in caveolin-1 expression. In contrast, caveolin-2 levels remain unaffected. Induction of apoptosis was measured by changes in cell morphology, annexin V-labeling, and DNA fragmentation. We demonstrate that caveolin-1 in macrophages is present in lipid rafts and colocalizes with phosphatidylserine (PS) at the cell surface of apoptotic macrophages. Our data suggest that caveolin-1 increase is an early event, closely accompanied by PS externalization and independent of caspase activation and nuclear DNA fragmentation. The increase in caveolin-1 levels does not require new protein synthesis, as cycloheximide does not prevent the apoptosis-mediated increase in caveolin-1 levels. We propose that increased levels of caveolin-1 characterize the apoptotic phenotype of macrophages. Caveolin-1 may be involved in the efficient externalization of PS at the surface of the apoptotic cells.**—Gargalovic, P., and L. Dory. **Cellular apoptosis is associated with increased caveolin-1 expression in macrophages.** ***J. Lipid Res.*** **2003.** 44: **1622–1632.**

Supplementary key words caveolae • lipid raft • membrane phospholipids • phosphatidylserine • mouse

Caveolins are membrane-anchored proteins associated with specific cholesterol- and sphingolipid-rich domains, often referred to as caveolae or lipid rafts (1, 2). The caveolin family consists of three distinct proteins of 21–24 kDa: caveolin-1 and caveolin-2 are expressed ubiquitously (3, 4), while the expression of caveolin-3 is restricted to striated muscle cells (5).

The proximity of caveolin-1 is generally associated with the inhibition of signaling pathways at the plasma membrane level. Many of these pathways belong to the proproliferative, oncogenic category (6, 7). The caveolin-1 gene is located in the putative tumor suppressor locus, which contains deletions in a variety of human tumors (8). The expression of caveolin-1 is decreased upon activation of oncogenes and in numerous tumor-derived cell lines (9). The hypercellularity of the lung parenchyma and the increased proliferative phenotype of mouse embryo fibroblasts from caveolin-1 knockout mice are also consistent with the anti-proliferative properties of caveolin-1 (10, 11). Overexpression of caveolin-1 in fibroblasts and epithelial cells, on the other hand, has been reported to sensitize these cells to apoptotic stimuli, possibly via inhibition of PI_3K and/or activation of caspase-3 (12, 13). Contrary to this pattern of caveolin expression and cellular proliferation properties, metastatic prostate cancer cells exhibit increased expression of caveolin-1 (14, 15), and caveolin-1 expression appears to suppress *c-myc*-induced apoptosis in certain prostate cancer-derived cell types (16). The involvement of caveolin-1 in the regulation of proliferation or cell death may therefore be cell specific or depend on the cellular context.

Apoptosis is a highly regulated, ATP-dependent form of cell death, characterized by caspase activation, internucleosomal cleavage of DNA, nuclear condensation, phosphatidylserine (PS) (1) exposure on the cell surface, and cellular fragmentation (17). Monocyte-derived macrophages play a critical role in the development of atherosclerosis and are intimately involved in arterial lesion formation (18). Apoptotic macrophage-foam cells represent a prominent feature of advanced atherosclerotic lesions (19–23). It has been suggested that macrophage apoptosis or lack of clearance of apoptotic cells may significantly contribute to the complications associated with lesion rupture and acute thrombosis (24). The cause of macrophage-foam cell apoptosis in advanced lesions has yet to be deter-

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Abbreviations: PS, phosphatidylserine; PVDF, polyvinylidene fluoride; tg-MPM, thioglycollate-elicited mouse peritoneal macrophage.

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mined. It may be due to the accumulation of toxic levels of intracellular unesterified cholesterol (25, 26) or exposure to oxidized lipoproteins and inflammation-associated molecules (27, 28).

Clearance of apoptotic cells is mediated via the exposure of PS on the cell surface (29, 30). Lipid raft-caveolae domains are selectively enriched in this phospholipid (31), and are directly implicated in PS externalization (32, 33).

We and others have recently demonstrated that primary mouse peritoneal macrophages express both caveolin-1 and caveolin-2 (34–36). In studies designed to examine the role of cellular cholesterol in regulating macrophage caveolin expression, we noted that treatment of cells with simvastatin results in significant cell death and in an increase in cellular caveolin-1 content. We now report that the induction of apoptosis in general, by a number of unrelated agents, leads to a large increase in caveolin-1 but not caveolin-2 expression. We also show that accumulation of caveolin-1 in apoptotic macrophages does not require protein synthesis, is independent of caspase activation and nuclear DNA fragmentation, and correlates with the appearance of PS on the cell surface.

EXPERIMENTAL PROCEDURES

Materials

Anti-caveolin-1 polyclonal (C-13630), anti-caveolin-2 monoclonal (C-57820), and anti-clathrin heavy chain (C-43820) monoclonal antibodies were purchased from Transduction Laboratories (San Diego, CA). Anti-actin (A-2066) was purchased from Sigma Chemical Co. (St. Louis, MO). Secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies were purchased from Jackson Immunochemicals (West Grove, PA). Simvastatin was a gift from Dr. Roger Newton (Parke Davis Co.). Camptothecin, cycloheximide, and aurintricarboxylic acid were purchased from Sigma Chemical Co. Caspase inhibitor Z-VAD-Fmk was purchased from Enzyme Systems Products (Livermore, CA). Alexa 488 goat anti-rabbit and Alexa 568 goat anti-rabbit antibodies, and the annexin V-based apoptosis assay kit "Vybrant # 2" were purchased from Molecular Probes (Eugene, OR). DMEM was purchased from GIBCO BRL (Grand Island, NY), and Brewer's thioglycollate was purchased from Difco Laboratories (Detroit, MI). All other chemicals were from Sigma Chemical Co.

Cell culture

Thioglycollate-elicited mouse peritoneal macrophages (tg-MPMs) were isolated from Swiss Webster mice by peritoneal lavage using PBS 4 days following an intraperitoneal injection of 1.5 ml of sterile thioglycollate broth, as previously described (37). Cell viability/cytotoxicity was assessed by trypan blue dye exclusion and by the lactate dehydrogenase detection kit (Roche).

Internucleosomal DNA fragmentation

Assessment of internucleosomal DNA fragmentation was done by the method of Roy and Nicholson (38) with minor modifications. Following specific treatments, cells (2×10^6/dish) were scraped off on ice. Cells and floating apoptotic bodies were sedimented at 10,000 *g* for 10 min at 4°C. The pellets were resuspended in 500 μl of lysis buffer consisting of 0.6% SDS, 10 mM EDTA, and 1 M NaCl (pH 7.5) and incubated for 24 h at 4°C. Supernatants were clarified by sedimentation at 14,000 *g* for 20 min and incubated with 5 μl of RNase cocktail consisting of RNase A (500 U/ml) and RNase T1 (20,000 U/ml) (Ambion Inc., Austin, TX) for 45 min at 37°C. DNA was extracted with phenol-chloroform and precipitated with isopropanol. Equal amounts of DNA were fractionated on a 1.2% agarose gel and visualized with ethidium bromide under UV light.

Analysis of cell surface PS

The extent of PS display on the cell surface (a measure of apoptosis) was determined by the Vybrant # 2 kit according to the manufacturer's instructions. Briefly, macrophages were seeded at a density of 2×10^6 cells/glass coverslip. Following specific treatments, cells were washed twice with PBS and incubated for 15 min at room temperature with the labeling mixture consisting of 5 μl Alexa fluor 488-annexin V conjugate and 0.1 μg of propidium iodide/100 μl of binding buffer [10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ (pH 7.4)]. After incubations, cells were washed twice with the binding buffer, mounted onto slides using Aqua Poly/Mount (Polyscience), and viewed with a fluorescence microscope (Nikon Microphot FxA) equipped with imaging software "IPLab" (Scanalytics Inc., Fairfax, VA). Cells labeled with annexin V but negative for propidium iodide staining were counted and expressed as a percentage of the total cell population. At least 1,000 cells were counted per each treatment and experiment. The presence of an asterisk (*) indicates that the data are statistically significantly different from bars without the asterisk ($P < 0.05$).

Immunoblotting

Cells and floating apoptotic bodies were collected by sedimentation at 10,000 *g* for 10 min at 4°C. Protein samples were dissolved, sonicated, and boiled in Laemmli buffer (39) for 10 min, and equal protein loads were separated by SDS-PAGE using 12% gels and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) at 35 V for 16 h. After blocking with TBS containing Tween-20 [TBST, 20 mM Tris (pH 7.6), 137 mM NaCl, 0.4% Tween 20] in the presence of 5% nonfat dry milk, the membranes were incubated with the primary antibodies in TBST containing 1% nonfat dry milk at 4°C for 16 h. Secondary antibodies in TBST containing 1% nonfat dry milk were added for 2 h at room temperature. Bound secondary antibodies were detected using an ECL detection system (Amersham Pharmacia Biotech). For sequential reprobing, the antibodies were stripped off the PVDF membranes [stripping buffer consisted of 2% SDS, 100 mM β-mercaptoethanol, 62.5 mM TRIS-HCl (pH 6.8)] for 30 min at 70°C, washed with TBS, and incubated with antibodies, as described above. Fold changes in caveolin-1 or -2 levels were corrected for β-actin concentrations within each sample.

Isolation of lipid rafts

Lipid raft-caveolae membranes were isolated as described by Machleidt et al. (40). Briefly, 60×10^6 cells were solubilized in 1 ml of 1% Triton X-100 at 4°C for 20 min, followed by homogenization in a Dounce homogenizer. The homogenate was transferred to a SW41 centrifuge tube and mixed with an equal volume of 2.5 M sucrose. The sample was overlaid with a 10–30% linear sucrose gradient. After centrifugation for 21 h in a SW41 rotor, fractions (1–14) were collected from the top, precipitated with TCA and, together with the pelleted material, analyzed by SDS-PAGE and immunoblotting.

Alkaline phosphatase assay

Alkaline phosphatase assay was performed as described by Liu and Anderson (41). The PVDF membrane was first wetted in methanol, washed several times in PBS and, together with a wet filter

paper, placed in a Minifold Dot-Blot apparatus (Schleicher and Schuell, Inc.). The excess PBS was removed by suction, and 100 μl of sample was applied to each well. Vacuum was applied to transfer proteins from the samples to the PVDF membrane. The membrane was washed with 50 ml of PBS and developed using 50 ml of alkaline phosphatase substrate according to the manufacturer's instructions (Bio-Rad, cat #170-6432). The color reaction was stopped by washing the membrane with water.

Immunofluorescence

Peritoneal macrophages were seeded on coverslips at a density of 2×10^6 per well. After treatment, cells were fixed in 3% paraformaldehyde for 10 min at room temperature and incubated with 0.1% Triton X-100 on ice for 5 min. After washing with PBS, coverslips were sequentially incubated at room temperature in PBS containing 4% goat serum for 60 min, anti-caveolin-1 primary antibody, and fluorescent-tagged secondary antibody in 0.8% BSA-PBS, also for 60 min each. The coverslips were then mounted on glass slides using Aqua Poly/Mount (Polyscience) and viewed with a Nikon fluorescence microscope (Microphot FxA) equipped with imaging software "IPLab" from Scanalytics Inc. (Fairfax, VA) and deconvolution software "Power Microtome" from Vaytek Inc. (Fairfield, IA). When double-immunolabeling for PS and caveolin-1, Annexin V was added to the cells before the fixation procedure and caveolin-1 staining.

RESULTS

The effect of simvastatin on caveolin-1 expression in macrophages

We previously characterized caveolin-1 and caveolin-2 expression in tg-MPMs and showed that increased cellular cholesterol content does not affect the level of caveolins in these cells (34). Previous studies by others have shown, however, that a 72 h treatment of MDCK cells with the HMG-CoA reductase inhibitor simvastatin leads to a decrease in cellular caveolin-1 levels (42). To examine the effect of HMG-CoA reductase inhibition on caveolin-1 and -2 levels, tg-MPMs were incubated with increasing concentrations of simvastatin for 72 h. As shown in **Fig. 1**, simvastatin treatment at concentrations of 1 μM and higher leads to a specific and dose-dependent increase in cellular caveolin-1 content. In contrast, the levels of caveolin-2 change little. Quantitative analysis indicates that 10 μM simvastatin increases caveolin-1, on average, 20-fold (data not shown).

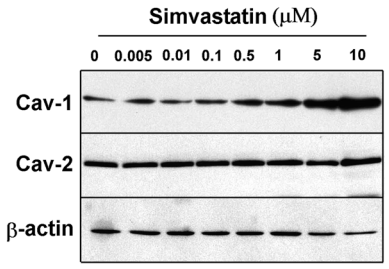

Fig. 1. Caveolin expression in simvastatin-treated mouse macrophages. Thioglycollate-elicited mouse peritoneal macrophages (tg-MPMs) were plated at a density of 2×10^6 cells per 35 mm well and treated with the indicated concentrations of simvastatin for 72 h in serum-free medium. After treatment, equal amounts of protein from whole-cell lysates were resolved by SDS-PAGE and immunoblotted for caveolin-1. After detection, polyvinylidene fluoride (PVDF) membranes were stripped-off and reblotted for cav-2 and β-actin, as described in the Experimental Procedures.

Simvastatin treatment and induction of apoptosis in macrophages

Light microscopy of simvastatin-treated cells (10 μM) reveals visible changes in their morphology when compared with control, untreated cells. Simvastatin-treated cells exhibit cell rounding, loss of attachment sites, and philopodia, as well as increased cellular fragmentation; phenomena suggestive of apoptosis. Statins have been previously shown to induce apoptosis in other cell types (43, 44). PS exposure on the cell surface is an early indicator of apoptosis (45) and it mediates the selective removal of apoptotic cells by phagocytosis (46). The extent of apoptosis in simvastatin-treated tg-MPMs was determined by double-labeling of tg-MPMs with annexin V and propidium iodide to separate apoptotic cells (annexin V-positive) from necrotic cells (annexin V- and propidium iodide-positive).

In the typical experiment shown in **Fig. 2**, an incubation of tg-MPMs with 10 μM simvastatin for 72 h leads to a large and specific increase in caveolin-1 expression (Fig. 2A), detectable DNA fragmentation (Fig. 2B), and a 7-fold increase in apoptosis as measured by PS externalization (Fig. 2C). As also shown, all of these effects can be reversed by 100 μM mevalonate in the incubation medium, which is consistent with studies conducted in vascular smooth muscle cells (43). Examination of caveolin-1 expression shows that the effect of 10 μM simvastatin is clearly discernible at 24 h and is nearly maximal at 48 h of treatment (**Fig. 3A**). The increase in caveolin-1 expression is accompanied by a time-dependent increase in DNA fragmentation and PS externalization (Fig. 3B, C).

Effect of other apoptotic agents

Because of the apparent correlation between caveolin-1 expression and the induction of apoptosis, we also examined the expression of caveolin-1 in cells induced to undergo apoptosis by agents unrelated to simvastatin. Incubation of tg-MPMs in glucose-free medium has been previously shown to induce apoptosis (47). We therefore measured the extent of PS externalization, DNA fragmentation, and caveolin expression under these conditions. The results of a typical experiment are shown in **Fig. 4**. As shown, glucose deprivation for 72 h results in extensive nuclear DNA fragmentation (Fig. 4B) and an 8-fold increase in PS-positive cells (Fig. 4C) with characteristic apoptotic changes in cell morphology (not shown). Importantly, these changes are associated with a specific and massive ~70-fold increase in caveolin-1 but not caveolin-2 content (Fig. 4A).

Caveolin-1 expression was also examined in tg-MPMs treated with camptothecin, a topoisomerase I inhibitor and a known apoptotic agent (48). As shown in **Fig. 5B**, treatment of cells with camptothecin at concentrations ≥5 μM leads to a high degree of DNA fragmentation. After 48 h of treatment at concentrations of ≥5 μM, cells were at the late stages of apoptosis with extensive (>50%) detachment from the culture dish, as determined by light

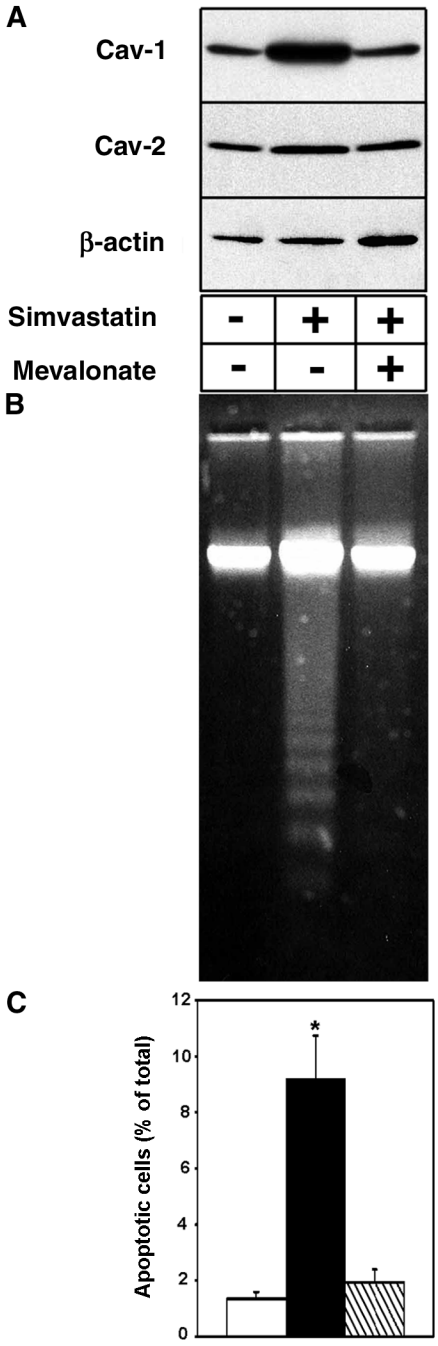

Fig. 2. Simvastatin-induced apoptosis in tg-MPM: effect of mevalonate. tg-MPMs were treated with 10 μM simvastatin in the absence or presence of mevalonate (100 μM) for 72 h. Isolated whole-cell lysates were immunoblotted for caveolins and β-actin (A). Internucleosomal fragmentation of isolated DNA was examined by agarose gel electrophoresis (B), as described in Experimental Procedures. Apoptotic cells [phosphatidylserine (PS)-positive] were expressed as a percentage of the total cell population (C). The data shown represent means ± SEM of a typical experiment. Five such experiments were carried out.

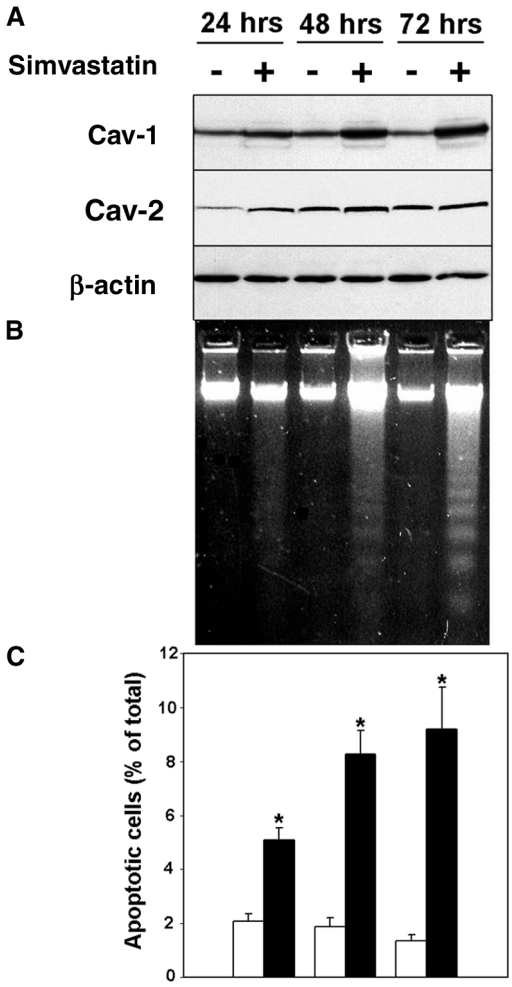

Fig. 3. Caveolin expression and apoptosis induction by simvastatin. tg-MPMs were treated with 10 μM simvastatin for up to 72 h. At indicated time points, the expression of caveolins and β-actin (A), internucleosomal fragmentation of isolated DNA (B), and the extent of apoptosis (PS-positive cells) (C) was determined, as described in Experimental Procedures.

microscopy. Consistent with our previous observations, caveolin-1 levels in these cells increase in a dose-dependent manner (Fig. 5A), while levels of caveolin-2 remained unaffected. It should be noted that caveolin-1 increase is already detectable in cells treated with 1 μM camptothecin in the absence of detectable DNA fragmentation and cell detachment, but a significant 5-fold increase in PS externalization (Fig. 5C). These observations suggest that the increase in caveolin-1 levels is associated with cellular apoptosis, precedes DNA fragmentation, and parallels the onset of PS externalization. Extensive cellular detachment and fragmentation of cells treated with camptothecin at concentrations ⩾5 μM prevented us from determining the extent of PS-positive/propidium iodide-negative apoptotic cells, as this requires attached cells. Attached, floating, and fragmented cells were, however, harvested for the assessment of caveolin-1 levels and DNA fragmentation.

Treatment of tg-MPMs with another apoptotic agent (5% ethanol) for 24 h also produces a significant increase in caveolin-1 expression along with extensive DNA fragmentation (data not shown).

Caspases and caveolin levels

Activation of caspases plays a central role in the apoptotic process, leading to cleavage of multiple cellular proteins and ultimately to cell death (49). We were interested

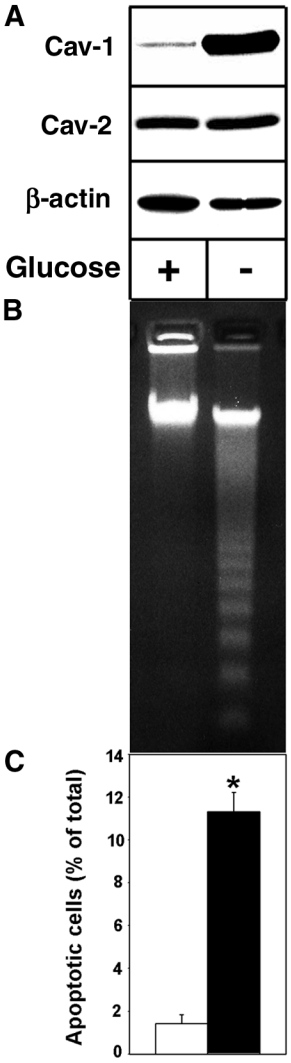

Fig. 4. Glucose deprivation of tg-MPMs induces apoptosis and increases caveolin expression. Cells were incubated in serum-free medium in the presence or absence of glucose (4,500 mg/l) for 72 h. The extent of caveolin-1 and -2 and β-actin expression was examined by immunoblotting (A). Internucleosomal DNA fragmentation (B) and the extent of apoptosis (C) are also shown.

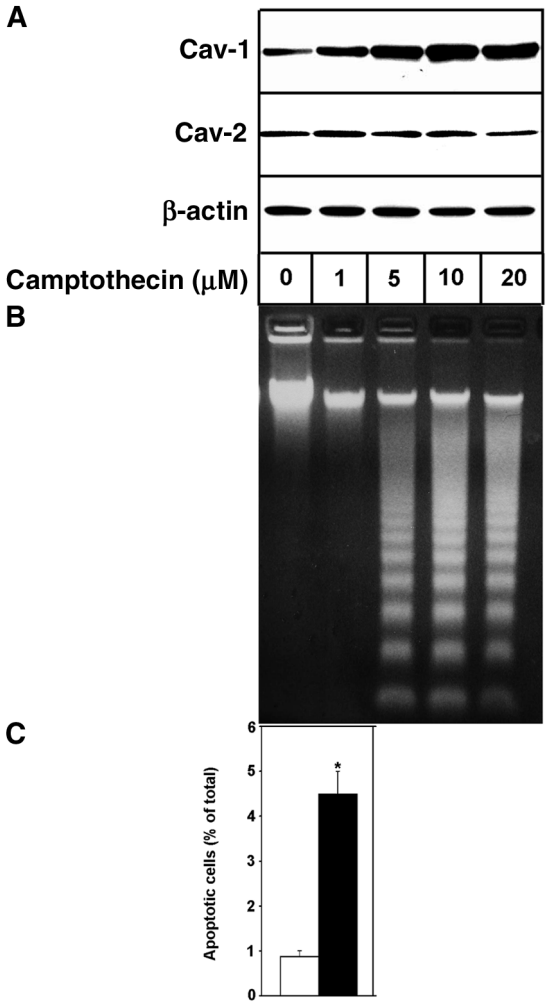

Fig. 5. Camptothecin-induced apoptosis and caveolin expression. Cells were treated with the indicated concentrations of camptothecin for 48 h in serum-free medium. After treatment, caveolin expression in whole-cell lysates was measured by immunoblotting (A), and DNA fragmentation was assessed by agarose-gel electrophoresis (B), as described in Experimental Procedures. The extent of apoptosis in cells treated with 1 μM camptothecin (no DNA fragmentation) as determined by annexin V assay is also shown (C).

in examining the potential role of caspases in the induction of caveolin-1 expression. tg-MPMs were therefore treated with 10 μM simvastatin for 48 h in the presence or absence of a general caspase inhibitor, Z-VAD-Fmk. The addition of simvastatin induces apoptosis with observable DNA fragmentation (**Fig. 6B**) and PS externalization (Fig. 6C). The pan-caspase inhibitor Z-VAD-Fmk prevents the simvastatin-induced DNA fragmentation (Fig. 6B) but fails to significantly prevent PS externalization (Fig. 6C) or the induction of caveolin-1 accumulation (Fig. 6A). The morphology of cells treated with Z-VAD-Fmk in the presence of simvastatin is identical to that of cells treated with simvastatin alone. In both cases, cells display shrinking and rounding, with visible blebbing and disintegration into apoptotic bodies (not shown). These observations suggest that caspase inhibition and inhibition of DNA fragmentation do not alter the overall fate of death in simvastatin-treated cells. Both the observed increase in caveolin-1 levels and PS externalization are independent of caspase activation and caspase-induced DNA fragmentation.

Localization of caveolin-1 in apoptotic macrophages

To examine the subcellular distribution of caveolin-1 in apoptotic macrophages, we immunolabeled control and simvastatin-treated cells with specific caveolin-1 antibody and analyzed the distribution by immunofluorescence microscopy. In control cells, caveolin-1 expression is relatively low (**Fig. 7A**), as was expected on the basis of our earlier observations (34, 50). Induction of apoptosis with simvastatin is associated with a significant increase in staining intensity (Fig. 7B), and is observed primarily in cells that display early morphological changes characteristic of apoptosis, such as cell rounding, shrinking, and loss of cellular pseudopodia.

Caveolin-1 and PS are both present at the cell surface.

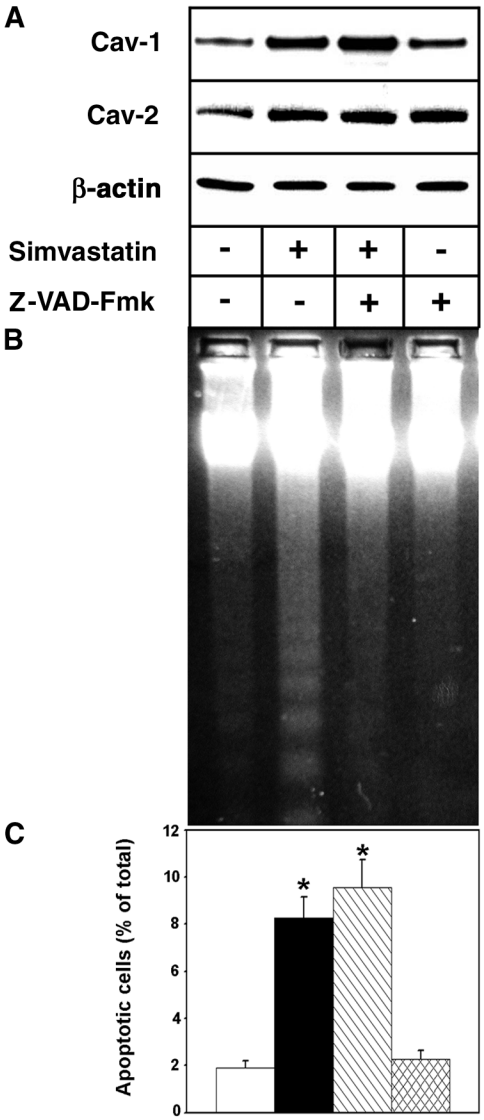

Fig. 6. Caspases and caveolin expression in apoptotic tg-MPMs. Cells were treated with serum-free medium alone (control) or in combinations of simvastatin (10 μM) and the general caspase inhibitor Z-VAD-Fmk (50 μM) for 48 h. Z-VAD-Fmk was added to the culture medium an hour before the addition of simvastatin and again after 24 h. The expression of caveolins and β-actin was assessed by immunoblotting (A), DNA fragmentation by agarose gel electrophoresis (B), and apoptosis by PS externalization (C).

The early and coordinate expression of caveolin-1 and PS externalization suggests a potential relationship between these two events. To examine whether caveolin-1 distribution in apoptotic cells resembles that of PS on the cell surface, we double-immunolabeled simvastatin-treated macrophages for caveolin-1 and PS (Fig. 7C, D, E). As shown at higher magnification (100×), caveolin-1 (Fig. 7C) and PS (Fig. 7D) are both localized at the periphery of the apoptotic cell and exhibit extensive colocalization (Fig. 7E).

Caveolin-1 and caveolin-2 expression in lipid rafts during apoptosis

We have shown previously that, in contrast to fibroblasts and other cell types with abundant caveolae, caveolin-1 and -2 do not colocalize in primary peritoneal macrophages (34). Our data demonstrated that in macrophages, caveolin-1 is localized at the cell surface, while caveolin-2 is present in the Golgi compartment. Recently, we also provided evidence for the presence of caveolin-1, but not caveolin-2, in lipid rafts isolated from mouse primary macrophages (50). Lipid raft membranes have been recently identified as potentially important sites for the efficient externalization of PS to the cell surface (32). To address the presence of caveolins in lipid rafts in apoptotic macrophages, we isolated Triton X-100 insoluble raft membranes and assessed the distribution of caveolins in these cells (**Fig. 8**). As expected, in control macrophages, caveolin-1 partitions almost exclusively into the insoluble lipid raft membrane fractions (Fig. 8A, fractions 5–7) identified by the marker alkaline phosphatase (41). In contrast to caveolin-1, Golgi-associated caveolin-2 partitions into the nonraft (soluble) fraction at the bottom of the gradient with the bulk of cellular proteins (e.g., β-actin and clathrin). Consistent with our earlier observations, the induction of apoptosis is associated with a specific increase in caveolin-1 (Fig. 8B), while other proteins, including caveolin-2, remain unchanged. A significant portion of the observed increase in caveolin-1 is associated with the lipid raft fractions (fractions 5–8), but an increase in the nonraft membranes is also seen.

Up-regulation of caveolin-1 is independent of de novo protein synthesis

On the presented results, the onset of caveolin-1 increase appears to be an early event in macrophage apoptosis, as is the appearance of the PS at the cell surface. To gain more insight into the mechanism of caveolin-1 up-regulation, camptothecin-treated macrophages were incubated in the presence of cycloheximide (30 μM and 100 μM), an inhibitor of protein synthesis, and the results are shown in **Fig. 9**. The induction of apoptosis by camptothecin is accompanied by increased cellular expression of caveolin-1, even in the presence of cycloheximide. Cycloheximide treatment has no effect on caveolin-2 or β-actin levels, but profoundly affects the levels of cellular apolipoprotein E, a rapidly turning-over protein (51). Cycloheximide treatment alone induces changes in cellular morphology and caveolin-1 expression that are consistent with and characteristic of apoptosis. Indeed, suppression of protein synthesis for an extended period (24 h) is the most likely reason for cycloheximide-induced apoptosis in macrophages (52). The increase in caveolin-1 levels under these conditions is therefore unequivocal evidence that protein synthesis is not required; caveolin-1 levels are only increased relative to those of other cellular proteins.

DISCUSSION

Macrophages play an important role in immunity, atherosclerotic lesion formation, and tissue remodeling. The extent of macrophage apoptosis and apoptotic cell clearance may therefore have a significant effect on these pro-

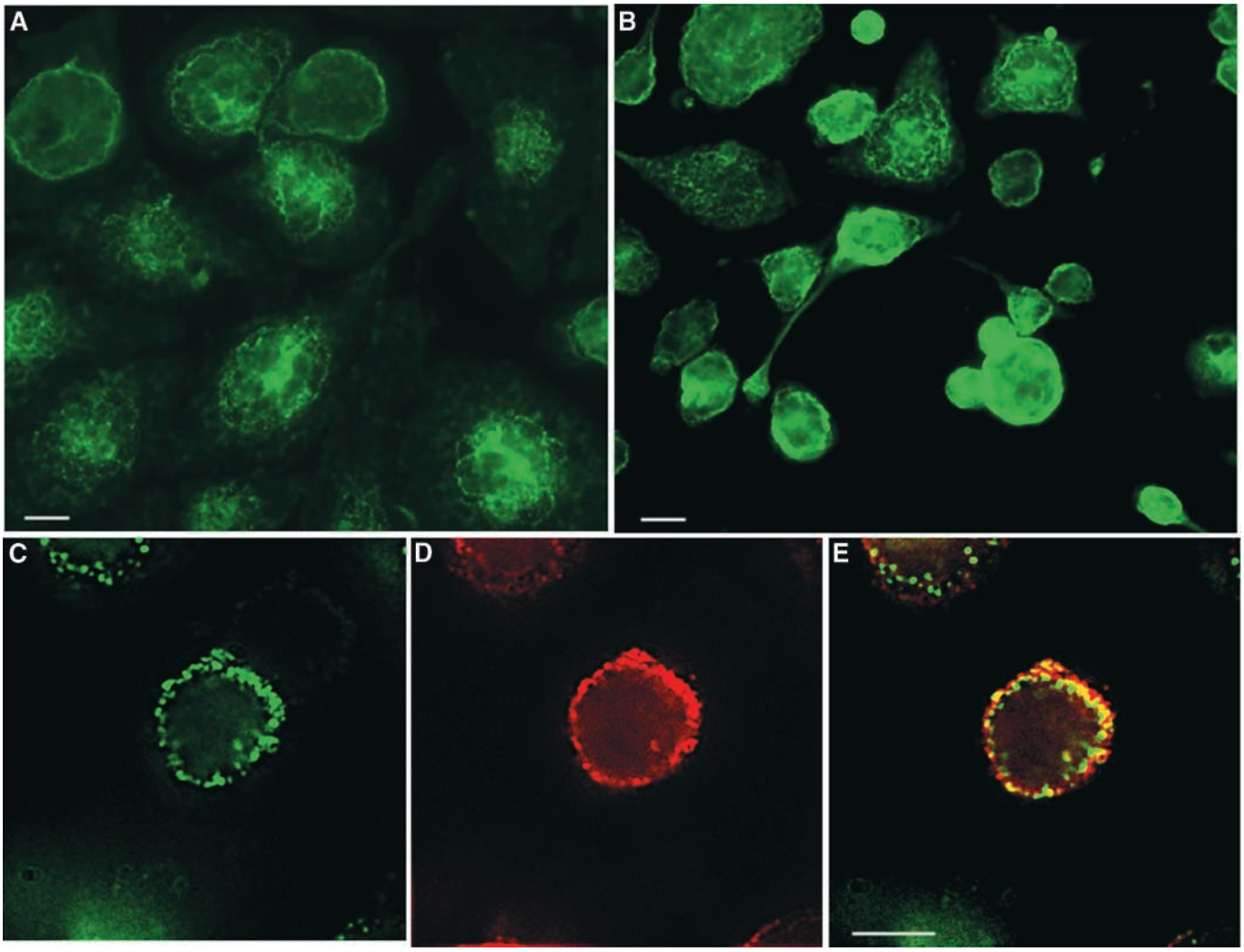

Fig. 7. Subcellular distribution of caveolin-1 in apoptotic cells. Control and simvastatin-treated (10 μM for 72 h) macrophages were immunolabeled with pAb directed against caveolin-1. The bound caveolin-1 primary antibody was visualized by incubation with fluorescent-tagged secondary antibody (see Experimental Procedures). The increase in fluorescence intensity of caveolin-1 in simvastatin-treated cells (B) versus control cells (A) is observed primarily in cells showing early signs of apoptosis. Double immunolabeling of simvastatin-treated cells with caveolin-1 pAb (C) and with Annexin V (D) shows a significant degree of colocalization on the cell surface when the images are superimposed (E). Images in A and B were taken at 40× magnification, while the images in C–E were taken at 100× magnification. Bars represent 10 μm.

cesses. The involvement of caveolin-1 in the regulation of signal transduction, cholesterol trafficking, and formation of caveolae has been well documented. Its potential involvement in apoptosis has been suggested by overexpression studies rendering fibroblasts and epithelial cells more sensitive to apoptotic stimuli (12, 13). Numerous reports identify caveolin-1 as a putative tumor suppressor gene down-regulated in various tumors and oncogenically transformed cell lines (53–56). The mechanism of the proposed proapoptotic effect of caveolin-1 is not known, but it appears to be cell specific because it may suppress apoptosis in certain prostate cancer-derived cell types (15), and its expression in prostate cancer cells is associated with increased cell survival and metastasis (57). It may be of interest that this anti-apoptotic effect in metastatic prostate cancer cells is associated with caveolin-1 secretion (57).

We have previously characterized caveolin expression in macrophages (34). The present study suggests that increased caveolin-1 levels represent an indicator of macrophage apoptosis. Our data show that caveolin-1 is increased in macrophages undergoing apoptosis induced by several unrelated stimuli in the absence or presence of serum (up to 10%, data not shown). We also show that the increase in caveolin-1 is independent of protein synthesis, caspase activation, and oligonucleosomal DNA fragmentation, while it correlates with the onset of PS externalization. Importantly, caveolin-1 is present at the cell surface in normal as well as apoptotic cells, and colocalizes with outer-surface PS.

Our initial observation of simvastatin-mediated apoptosis and concomitant increase in caveolin-1 levels was surprising in light of the observations made with MDCK cells. Caveolin-1 expression in MDCK cells treated with 25 μM simvastatin for 72 h is decreased, and apoptosis is not observed (42). In agreement with our observations, simvasta-

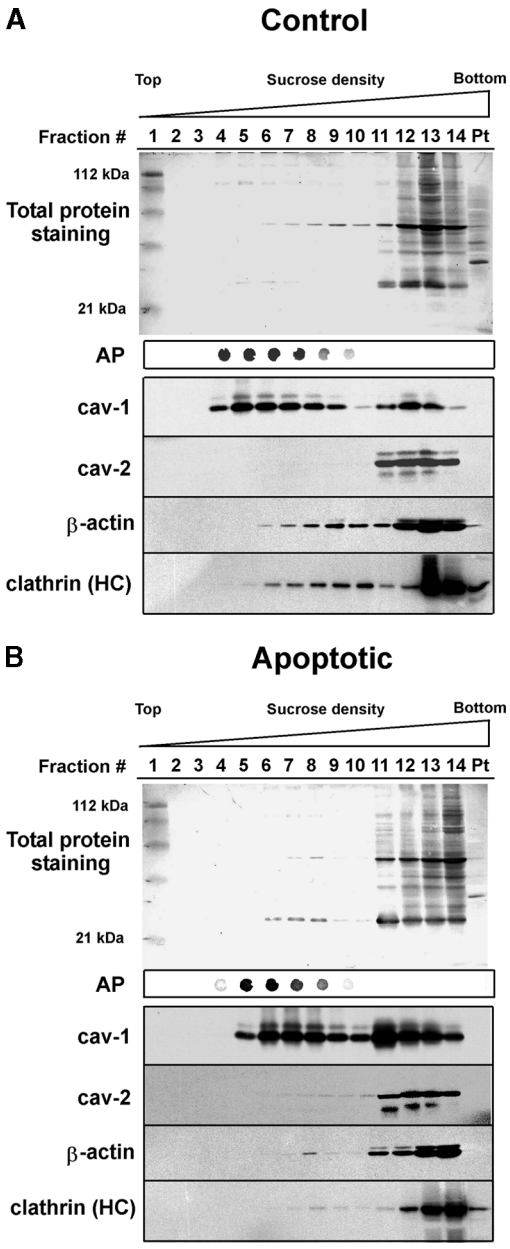

Fig. 8. Lipid raft distribution of caveolins in apoptosis. Lipid raft membranes were isolated using the 1% Triton X-100 method as described in Experimental Procedures. Control (A) and 10 μM simvastatin-treated (B) macrophages were homogenized in the lysis buffer containing 1% Triton X-100 and subjected to sucrose gradient centrifugation, which separates lipid raft membranes from the bulk of the cellular constituents. Fractions were collected from the top (1–14), and together with the pelleted material (Pt) analyzed by SDS-PAGE. Total protein loading after transfer to PVDF membrane was visualized by Ponceau S staining. Caveolin-1, caveolin-2, β-actin, and clathrin heavy-chain (HC) expression was analyzed by immunoblotting. A portion of each fraction was also analyzed for alkaline phosphatase activity (AP) (a lipid raft/caveolae marker), as described in Experimental Procedures.

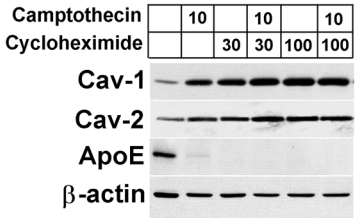

Fig. 9. Effect of cycloheximide on caveolin-1 levels in apoptotic macrophages. tg-MPMs were treated for 24 h with 10 μM camptothecin and/or indicated concentrations (μM) of cycloheximide, an inhibitor of protein synthesis. Equal amounts of protein from whole-cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting for the expression of caveolins, apolipoprotein E, and β-actin.

tin has been previously shown to induce apoptosis in other cell types (43, 44), and is noted to be toxic to human monocyte-derived macrophages at concentrations >1 μM (58). These concentrations are well above plasma concentrations in patients taking this drug, which peaks around 50 nM (59). In our studies, concentrations of simvastatin below 1 μM were not toxic to tg-MPMs, and were not associated with changes in caveolin-1 levels. On the other hand, the treatment of macrophages with simvastatin at a concentration of 1 μM and higher leads to a time- and dose-dependent increase in caveolin-1 expression, accompanied by signs of apoptotic phenotype, including DNA fragmentation and PS externalization. It has been suggested that statins cause apoptosis by inhibiting protein prenylation (60), an effect that can be prevented by the addition of 100 μM mevalonate. Both apoptosis and caveolin-1 increase in simvastatin-treated cells can be prevented by the addition of mevalonate but not squalene (not shown). These data suggest that apoptosis in this case is not caused by lack of cholesterol, but likely by the loss of the cells' ability to prenylate or farnesylate certain proteins. Caveolin-1 accumulation, on the other hand, is unlikely to be a direct result of this, since it is not modified by prenylation or farnesylation. Further and unequivocal support for the notion that an increase in caveolin-1 levels is associated with the induction of apoptosis comes from the observation that a number of other, unrelated agents that cause apoptosis through different mechanisms also leads to caveolin-1 increase. These include glucose deprivation, camptothecin, or ethanol. Macrophages and other cells expressing NO synthase exhibit high sensitivity to glucose deprivation (47). On the other hand, camptothecin is a potent inhibitor of topoisomerase I (61), while ethanol has been shown to induce apoptosis in macrophages due to an increase in TGF-β expression (62). Our studies demonstrate that all of these agents induce apoptosis in tg-MPMs and a specific increase in caveolin-1 levels. The increase in caveolin-1 levels is thus a general feature of macrophages undergoing apoptosis, regardless of the mechanism of action of the various apoptotic stimuli used.

Signals that mediate the execution of apoptosis by various stimuli converge on the activation of caspases (49). Apoptotic macrophages display the characteristic changes associated with apoptosis, such as DNA fragmentation, PS externalization, cell shrinkage, and disintegration into apoptotic bodies. DNA fragmentation in apoptotic cells is a result of activation of a specific caspase-3-activated DNase (63). The inhibition of caspases by the pan-caspase inhibitor Z-VAD-Fmk inhibits, as expected, the internucleosomal

cleavage of DNA but, perhaps surprisingly, fails to prevent cell death, PS externalization, and caveolin-1 increase. Apoptotic cell death has been previously demonstrated to be, at least in some cases, independent of caspase activation, as evidenced by failure of the caspase inhibitor Z-VAD-Fmk to prevent cell death (64–66). In many cases, inhibition of caspases prevents nuclear changes associated with apoptosis, but fails to block cell death (67–69). The failure of Z-VAD-Fmk to inhibit apoptosis in simvastatin-treated tg-MPMs suggests that other pathways must be involved. Based on our observations, the inhibition of caspases is also unable to prevent PS externalization, which is consistent with reports that caspase activation can be dissociated from PS externalization (64, 69, 70). This is also consistent with our observations that the incubation of simvastatin-treated tg-MPMs with the endonuclease inhibitor aurintricarboxylic acid (71, 72) (10 μM and 50 μM) inhibits DNA fragmentation in simvastatin-treated tg-MPMs, but fails to prevent the increase in caveolin-1, PS externalization, and cell death (not shown). Importantly, the expression of caveolin-1 is unchanged by caspase or endonuclease inhibition, but correlates closely with PS externalization.

Examination of caveolin-1 expression in apoptotic macrophages at various time points suggests its early onset, similar to that of PS externalization and preceding the later changes, such as DNA fragmentation. As predicted, the examination of cells by immunofluorescence microscopy shows that the increase in caveolin-1 expression is restricted to the population of cells showing early signs of apoptosis, such as cell rounding, loss of attachment sites, and cell shrinking. We have previously shown that caveolin-1 is localized primarily at the cell surface of macrophages, while caveolin-2 is present in the Golgi compartment (34). Closer examination of the caveolin subcellular distribution shows that caveolin-1 is found primarily at the surface of apoptotic cells in lipid rafts. Importantly, caveolin-1 exhibits extensive colocalization with PS at the surface of apoptotic macrophages. These data are consistent with the hypothesis that changes in plasma membrane phospholipid distribution, characteristic of apoptosis, such as PS externalization, are related to increased caveolin-1 levels.

Surface localization of PS plays an essential role in the recognition and removal of apoptotic cells by phagocytes (29, 73). PS exposure on the cell surface is an early event and results from activation of the scramblase and inhibition of aminophospholipid translocase (30, 74, 75).

Although potential candidates for scramblase and flippase have been cloned, the extent of their participation in PS externalization is controversial (76, 77). While we demonstrate that increased caveolin-1 levels and its colocalization with PS at the plasma membrane is associated with macrophage apoptosis, it remains to be seen if caveolin-1 is involved in the externalization of PS. Our studies using cycloheximide show that the increase in caveolin-1 levels is independent of protein synthesis. Instead, changes in caveolin-1 trafficking or inhibition of protein degradation are more likely mechanisms. The data are consistent with the notion that total caveolin-1 levels within the cells do not change during apoptosis; instead, global protein degradation leads to a profound enrichment in caveolin-1 relative to other cellular proteins, because it is rescued/protected from degradation by the virtue of its localization. It remains to be shown whether caveolin-1 remains a passive captive within the membranes of the apoptotic cells or plays an active role in mediating some of the underlying changes in phospholipid distribution.

Caveolin-1 is present in lipid rafts and colocalizes with PS on the surface of apoptotic cells. While these observations are consistent with previous studies demonstrating enrichment of PS in the lipid rafts (31) and the involvement of lipid rafts in PS externalization (32), more in-depth studies are needed to demonstrate that caveolin-1 and PS are physically present in the same lipid raft compartment. There are currently no available experimental approaches to selectively blocking the externalization of PS. Future studies using cells overexpressing caveolin-1 or macrophages from caveolin-1 knockout mice will be instrumental in demonstrating a potential connection between these two events.

In conclusion, we identify increased caveolin-1 expression as a specific marker of macrophage apoptosis. Caveolin-1 increase is an early event, independent of caspase activation and DNA fragmentation, but closely associated with the externalization of PS to the cell surface.

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