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Cholesterol Enrichment of Human Monocyte/Macrophages Induces Surface Exposure of Phosphatidylserine and the Release of Biologically-Active Tissue Factor–Positive Microvesicles

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Abstract

Objective—Biologically significant amounts of two procoagulant molecules, phosphatidylserine (PS) and tissue factor (TF), are transported by monocyte/macrophage-derived microvesicles (MVs). Because cellular cholesterol accumulation is an important feature of atherosclerotic vascular disease, we now examined effects of cholesterol enrichment on MV release from human monocytes and macrophages.

Methods and Results—Cholesterol enrichment of human THP-1 monocytes, alone or in combination with lipopolysaccharide (LPS), tripled their total MV generation, as quantified by flow cytometry based on particle size and PS exposure. The subset of these MVs that were also TF-positive was likewise increased by cellular cholesterol enrichment, and these TF-positive MVs exhibited a striking 10-fold increase in procoagulant activity. Moreover, cholesterol enrichment of primary human monocyte-derived macrophages also increased their total as well as TF-positive MV release, and these TF-positive MVs exhibited a similar 10-fold increase in procoagulant activity. To explore the mechanisms of enhanced MV release, we found that cholesterol enrichment of monocytes caused PS exposure on the cell surface by as early as 2 hours and genomic DNA fragmentation in a minority of cells by 20 hours. Addition of a caspase inhibitor at the beginning of these incubations blunted both cholesterol-induced apoptosis and MV release.

Conclusions—Cholesterol enrichment of human monocyte/macrophages induces the generation of highly biologically active, PS-positive MVs, at least in part through induction of apoptosis. Cholesterol-induced monocyte/macrophage MVs, both TF-positive and TF-negative, may be novel contributors to atherothrombosis.

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Disclosures

None.

Keywords

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Microvesicles (MV) are small membranous structures that are released from cells on activation or during apoptosis.[1,2] Monocyte/macrophage-derived MVs transport biologically significant amounts of phosphatidylserine (PS), a membrane lipid that enhances clot propagation,[3] and tissue factor (TF), a potent initiator of coagulation in vivo.[1,4,5] Based on several observations, we hypothesized a link between cellular cholesterol enrichment and monocyte/macrophage MV generation. First, clinical studies have shown an association between hyperlipidemia and elevated numbers of circulating monocyte/macrophage-derived PS-positive MVs,[6–9] and monocyte/macrophage-derived MVs have been found in human atherosclerotic lesions as well.[4,10] Second, monocyte/macrophages accumulate unesterified cholesterol (UC) in vivo during atherogenesis,[11] presumably owing to their uptake of lipoproteins that have been retained and modified within the arterial wall.[12–14] Third, cholesterol loading of primary human monocyte-derived macrophages (MDM) in vitro was reported to stimulate the expression of TF,[15] and increased TF expression was found in atherosclerotic vessels from cholesterol-fed rabbits,[16,17] in human lipid-rich plaque macrophages,[18] and in monocytes from hypercholesterolemic patients.[19] Moreover, our group recently reported that hyperlipidemia induces a prothrombotic state in vivo in animals,[20] although none of these aforementioned studies examined TF-positive MV release. Fourth, plasma lipid lowering by medication,[17] diet,[21,22] or extracorporeal low-density lipoprotein apheresis[23] reduced TF levels in vivo, and extensive cholesterol depletion of monocytes in vitro for the purpose of disrupting normal plasma membrane structure was recently shown to inhibit their calcium ionophore-stimulated release of total MVs.[24] Nonetheless, direct effects of cholesterol enrichment on monocyte/macrophage MV release have not been reported. In the current study, we directly loaded human THP-1 monocytic cells and primary human MDMs with exogenous cholesterol and assessed their vesiculation, as well as the biologic activity of the released MVs. LPS, a well-known stimulus for MV generation by THP-1 monocytes,[25] served as a positive control.

Methods

Cell Culture and Cholesterol Treatment

THP-1 cells (ATCC, Manassas, Va) were maintained in suspension culture in RPMI-1640 supplemented with 7% FBS, 1% penicillin/streptomycin, and 0.05 mmol 2-mercaptoethanol/L, as recommended by the ATCC. Before each experiment, cells were transferred to serum-free RPMI-1640 containing 0.2% BSA (RPMI/BSA), followed by a 1-hour preincubation at 37°C. Next, the cells were incubated at 37°C without (control) or with 10 μg unesterified cholesterol (UC)/mL, with or without 10 μg LPS/mL, for 0 to 20 hours in serum-free RPMI/BSA. The UC was delivered as a water-soluble complex with methyl-β-cyclodextrin (UC/mcd; 1:6 molar ratio of UC:mcd; Sigma), which has been widely used to modify the cholesterol content of cultured cells without potentially confounding effects from receptor engagement.[26–28] We verified that all UC/mcd preparations contained no detectable endotoxin by the Limulus amoebocyte lysate assay (Associates of Cape Cod, Inc).

Adherent primary human MDMs were prepared from fresh Buffy coats by low-speed Ficoll density gradient centrifugation to purify the mononuclear cell fraction, followed by positive selection of CD14+ monocytes using magnetic beads coated with a monoclonal anti-CD14 antibody (Miltenyi Biotec Inc.). The CD14+ monocytes were differentiated into macrophages by a 7-day incubation at 37°C in DMEM supplemented with 10% FBS, 10% horse serum, 0.5 ng granulocyte-macrophage colony stimulating factor (CSF)/mL, and 0.5 ng macrophage CSF/mL.[29,30] Because concentrations of UC/mcd up to 380 μg UC/mL of medium, based on the mass of cholesterol in the UC/mcd complexes, have been used to cholesterol-enrich primary macrophages,[26] we chose an intermediate concentration, 100 μg UC/mL, for our studies. Adherent MDMs were incubated at 37°C for 20 hours without (control) or with UC/mcd in RPMI/BSA.

Characterization of MVs and Cells

For flow cytometry, we left the THP-1 cells, which grow in suspension, in their conditioned media. At each time point, samples of cells and media were fixed by addition of paraformaldehyde (PFA) to a final concentration of 2%, followed by storage at −80°C until analysis, as described.[31,32] For adherent primary human MDMs, supernatants without cells were fixed in 2% PFA, whereas the cell monolayers were scraped and fixed in suspension in 1% PFA and then 70% ethanol. Consistent with prior literature,[31,32] our initial studies indicated that PFA fixation of MVs and cells protects them from changes induced by a single freezing and thawing. Moreover, we specifically verified that the cholesterol-induced increase in MV generation measured after fixation and freezing was the same as that seen using unfixed, unfrozen material (data not shown). The number of MVs in each sample was quantified by flow cytometry, using gating criteria based on particle size, as detected by forward scatter, and surface exposure of PS, as detected by staining with phycoerythrin (PE)-labeled annexin-V (BD Pharmingen).[25] The portion of these PS-positive MVs that were also TF-positive was quantified by simultaneous staining with a fluorescein isothiocyanate (FITC)-labeled anti-human TF monoclonal antibody (Cat# 4508CJ, American Diagnostica). For THP-1 cell suspensions, results are reported as MVs per 1000 cells. Because MDMs are adherent, we added 25 000 15-μm latex beads (Molecular Probes) to each 100 μL of their conditioned medium as a reference, and our results are reported as absolute numbers of MVs per ml of conditioned culture medium. Cellular cholesterol content was quantified in isopropanol extracts of unfixed THP-1 cells by the cholesterol oxidase method (Wako Inc).[33] In some samples, late-stage apoptosis of THP-1 monocytes or primary human MDMs was assessed by terminal deoxynucleotidyl transferase FITC-dUTP nick end labeling of the cells (TUNEL; APO-DIRECT kit, BD Pharmingen), followed by flow cytometric quantification. For causal studies of the role of apoptosis, a subset of incubations included a caspase-3 inhibitor, Z-DEVD-FMK, at the manufacturer's highest recommended concentration (100 μmol/L; R&D Systems).

Assessments of Tissue Factor Antigen and Tissue Factor Procoagulant Activity

Cellular release of TF antigen was quantified by ELISA. Culture suspensions (THP-1 monocytes) or conditioned media (primary human MDMs) were centrifuged at low speed (200*g* for 10 minutes) to remove cells or large debris while leaving MVs in the supernatant.

Tissue factor concentrations were then determined (IMUBIND Tissue Factor ELISA kit, American Diagnostica).

Tissue factor procoagulant activity was measured with fresh (unfixed, unfrozen) samples using a chromogenic assay for the activation of clotting factor X (active factor Xa) by MVs captured by platelets, as previously described.[24] In brief, MV-containing culture supernatants were obtained by low-speed centrifugation of conditioned medium from THP-1 cells or MDMs after treatment without or with UC. Washed human platelets were activated with 5 μg collagen/mL and then incubated with these MV-containing supernatants for 1 hour at 37°C with continuous rocking. Unbound MVs were then removed by sedimenting the platelets and captured MVs at 1600*g* for 10 minutes at RT and discarding the supernatants. The pelleted platelets with bound MVs were then resuspended in buffer containing $CaCl_2$, exogenous factor VIIa, and inactive factor X (American Diagnostica) and incubated at 37°C for 30 minutes. To stop the reaction, platelets with bound MVs were removed by filtration (0.2 μm). The filtrate, which contained any activated factor Xa, was portioned into aliquots in a 96-well plate and mixed with a chromogenic Xa substrate, Spectrozyme-Xa (0.5 mmol/L, American Diagnostica). After 60 minutes at 37°C, absorbances at 405 nm were recorded in a microplate reader. As negative controls, TF activity was measured in samples in which factor VIIa, factor X, or Spectrozyme-Xa had each been omitted from the reaction. Lipidated TF (American Diagnostica) was used as standards (0, 10, 20, and 40 pmol/L). We also measured TF procoagulant activity of washed MVs, which were isolated from 1 mL of THP-1 culture supernatant by high-speed centrifugation (100 000*g* at 4°C for 1 hour), then washed and resuspended in 1 mL RPMI/BSA for analysis of activity, as above.

Statistical Analyses

Values are shown as means±SEM, n=4 to 5. Comparisons among several groups were performed using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test, with $P<0.05$ considered significant. Comparisons between two groups used Student unpaired *t* test.

Results and Discussion

We found that cholesterol enrichment of human THP-1 monocytes provoked a significant increase in the release of PS-positive MVs (Figure 1A and 1B). The effect was evident as early as 2 hours and persisted for the entire 20-hour period we examined (Figure 1A and 1B). By 20 hours, the total MV count from cholesterol-enriched THP-1 cells was nearly 3 times the value from untreated control cells. As a positive control,[25] LPS also stimulated THP-1 monocyte MV release, but we now found that cholesterol given simultaneously with LPS increased microvesiculation at each time point as compared with either agent alone (Figure 1B). To establish that cholesterol was responsible for these effects, we verified that incubation with UC/mcd caused significant cholesterol enrichment of THP-1 monocytes throughout the 20-hour time-course (51.1±4.0 at 0 hours, 86.3±12.7 at 2 hours, and 123.0±13.2 μg cholesterol/mg cell protein at 20 hours, n=4, $P<0.01$ for 2 hours and for 20 hours versus 0 hours). Similar cholesterol enrichment was seen in cells incubated with the combination of UC and LPS, whereas no changes in cellular cholesterol content were

observed in the control or LPS-only groups (data not shown). Furthermore, media supplemented with the same low concentration of methyl-β-cyclodextrin (0.15 mmol/L), but not complexed to cholesterol, failed to measurably affect THP-1 MV generation over time (eg, the MV count after 20 hours in the presence of methyl-β-cyclodextrin without UC was 93%±3% of the control value in unsupplemented medium, NS).

In addition to increasing total cellular output of MVs, cholesterol loading of THP-1 cells in the absence or presence of LPS caused large increases in the cellular release of TF-positive MVs, as measured either by flow cytometry of the cell culture suspension (Figure 2A), or by TF ELISA of cell-culture supernatants (supplemental Figure I, available online at http://atvb.ahajournals.org). Importantly, cellular cholesterol loading of THP-1 cells caused a remarkable 10-fold increase in the TF procoagulant activity of MV-containing cell culture supernatants (Figure 2B) and of washed THP-1 MVs (not shown), consistent with the increase in TF mass (supplemental Figure I) and possibly TF de-encryption (see references[34–36]). To examine these effects in a primary cell, we found that cholesterol-enrichment of human MDMs caused large, statistically significant increases in total (Figure 3A) and TF-positive (supplemental Figure II) MV release compared with control. Moreover, MV-containing cell culture supernatants from cholesterol-enriched primary human MDMs also showed a striking 10-fold increase in TF procoagulant activity over control (Figure 3B). These results suggest that enhanced release of procoagulant MVs from cholesterol-enriched monocyte/macrophages could contribute to the general prothrombotic state in hypercholesterolemia,[19,20,23,37] and to the prothrombotic interior of lipid-rich, vulnerable plaques.[4,5,10,14]

The flow cytometry protocol for THP-1 monocytes allowed us to simultaneously examine MVs and the cells that generated them. Our data showed no significant changes in either cell-surface exposure of PS or cellular size when THP-1 cells were incubated in unsupplemented serum-free medium (Figure 1A and supplemental Figure III) or treated with LPS (supplemental Figure III; size data not shown). Under these conditions, a low level of cellular PS exposure was seen in the current (Figure 1A) and previous studies[25] which could play a role in generation of PS-positive MVs by these cells.[38,39] In contrast, cholesterol enrichment caused a majority of the THP-1 monocytes to promptly display strong staining for PS on their surface by 2 hours (Figure 1A and supplemental Figure III), and to gradually shrink over time, as shown by a downward shift in forward scatter (Figure 1A). Because cell-surface exposure of PS not only induces procoagulant responses[5] but may also indicate early-stage apoptosis,[5,40] we examined a definitive marker of late-stage apoptosis, namely DNA fragmentation by TUNEL. At the 20-hour time point, the proportions of TUNEL-positive cells were only 1% to 2% in serum-free medium, and only 2% to 3% with LPS stimulation, but rose to approximately 30% with cholesterol enrichment of THP-1 cells (Figure 4). Likewise, the proportions of primary human MDMs that were TUNEL-positive were only 1% in serum-free medium but rose to approximately 11% after 20 hours of cholesterol enrichment. Thus, similar to prior literature,[26] we saw significant cholesterol-induced apoptosis, which raises the possibility that at least some of the cholesterol-induced MV generation in our system might be from apoptotic blebbing. Interestingly, our human monocyte/macrophages underwent UC-induced apoptosis even in the absence of other manipulations (see reference[26]), indicating an unexpectedly robust response in these cells.

To assess a causal role for apoptosis, we used the caspase-3 inhibitor Z-DEVD-FMK. Addition of this inhibitor during cholesterol enrichment of THP-1 monocytes delayed cell-surface exposure of PS by 2 to 4 hours, but did not affect the final percentage of PS-positive cells at 20 hours (Supplemental Figure IVA). The inhibitor significantly decreased the percentage of TUNEL-positive cells at 20 hours from 28±2.8% to 12.5±2.2% (supplemental Figure IVB, $P<0.001$). Importantly, the caspase-3 inhibitor significantly decreased the number of PS-positive MVs released during UC-enrichment over time, although not entirely to the level of unenriched control cells at 6 hours and 20 hours (Figure 5 and supplemental Figure IVA). In contrast, we did not see any effect of the caspase-3 inhibitor on the number of TF-positive MVs released after UC-enrichment (data not shown), suggesting that TF synthesis and TF-positive MV release may come from a subset of these cells that are not in late-stage apoptosis (see reference[41]).

Taken together, our results directly link cellular cholesterol enrichment, a known consequence of hyperlipidemia, arterial retention of lipoproteins, and atherogenesis,[12,14] with the enhanced generation of biologically active monocyte/macrophage-derived MVs, at least in part through induction of apoptosis. Related effects may occur with other cell types, though independent of apoptosis.[42] The effects of TF-positive monocyte/macrophage-derived MVs and their potential contributions to atherothrombosis have been investigated in the present as well as in previous studies.[4,43] Nevertheless, more than 80% of the MVs in our study (Figures 1B and 2A; Figure 3A and supplemental Figure II) and in prior literature[44] were PS-positive but TF-negative. We speculate that these particles may be biologically active as well, for example, by enhancing clot propagation,[3,5] causing apoptosis of nearby cells,[45,46] blocking PS receptors that could otherwise promote phagocytosis of apoptotic bodies,[47] and altering gene expression via their display of PS[48] and perhaps other biologically active molecules.[49,50] Of interest, we recently found that injection of cholesterol-induced monocyte MVs into rats provoked substantial leukocyte rolling and adherence to post-capillary venules in vivo, indicative of endothelial activation (Liu M-L, Scalia R, Williams KJ, unpublished data, 2006). Thus, cholesterol-induced PS-positive monocyte/macrophage MVs, both TF-positive and TF-negative, may be novel contributors to atherothrombosis. Because cholesterol is an exchangeable molecule that can be removed by acceptor particles, such as high-density lipoprotein, cellular production of these vesicles could be amenable to therapeutic interventions.[14,51]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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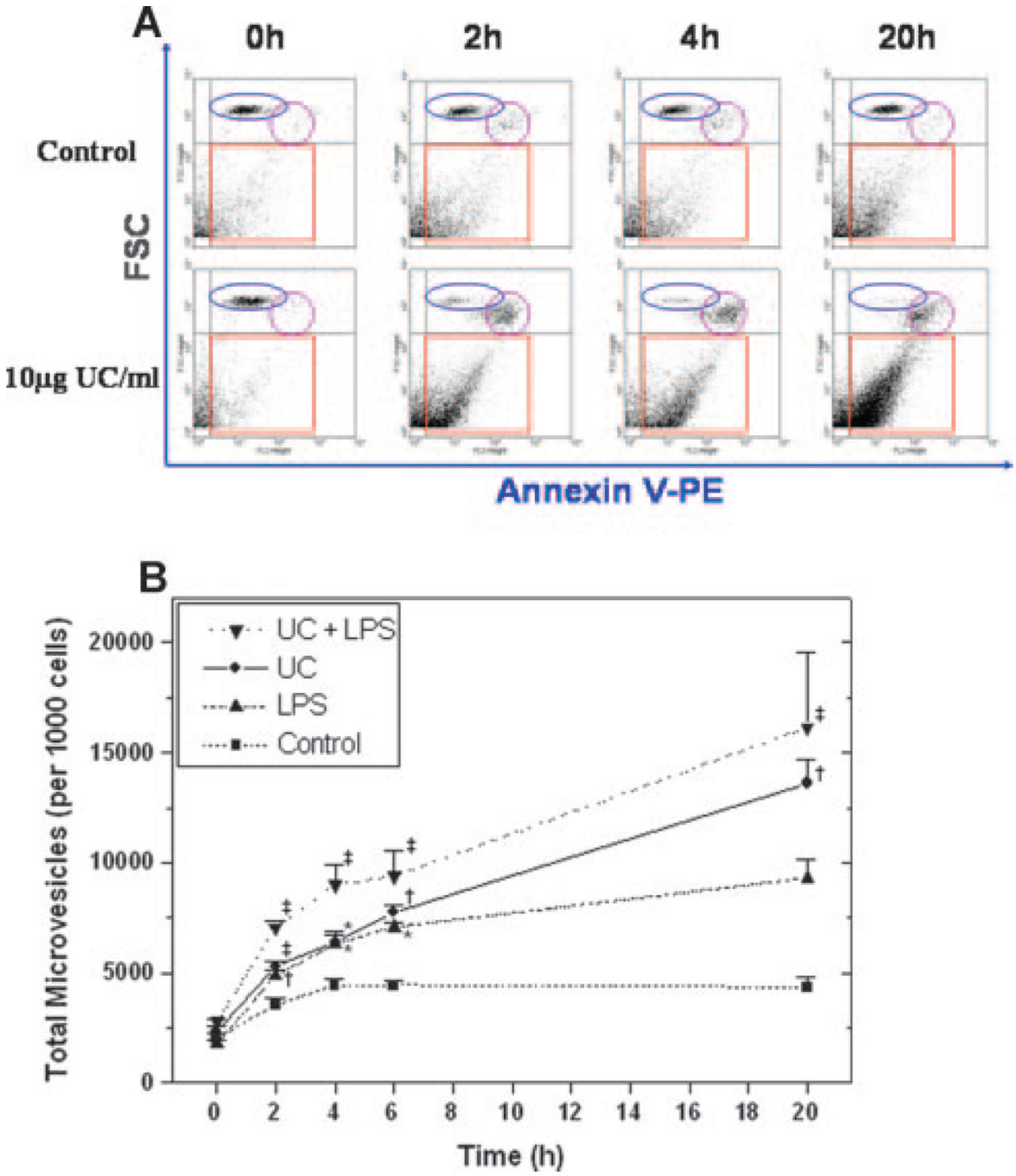

Figure 1.

Cholesterol enrichment of monocytic cells increases total MV generation. Suspensions of THP-1 monocytes were treated for 0 to 20 hours at 37°C with UC, LPS, both, or neither (Control), as indicated, and then samples were analyzed by flow cytometry. A, Representative dot-plots of forward scatter (FSC, indicating size) vs annexin V-PE staining (indicating PS exposure) for suspensions of control and UC-enriched cells. The squares enclose the population of PS-positive MVs. The ovals indicate cellular populations with the same size range and PE intensity as control THP-1 cells at t=0 hours, whereas the circles

identify a population of cells that appeared over time, particularly with cholesterol enrichment. B, Quantitative counts of PS-positive MVs generated over time under the four experimental conditions. $P<0.01$ by ANOVA. *$P<0.05$, †$P<0.01$, and ‡$P<0.001$ vs the control group by the Student-Newman-Keuls test.

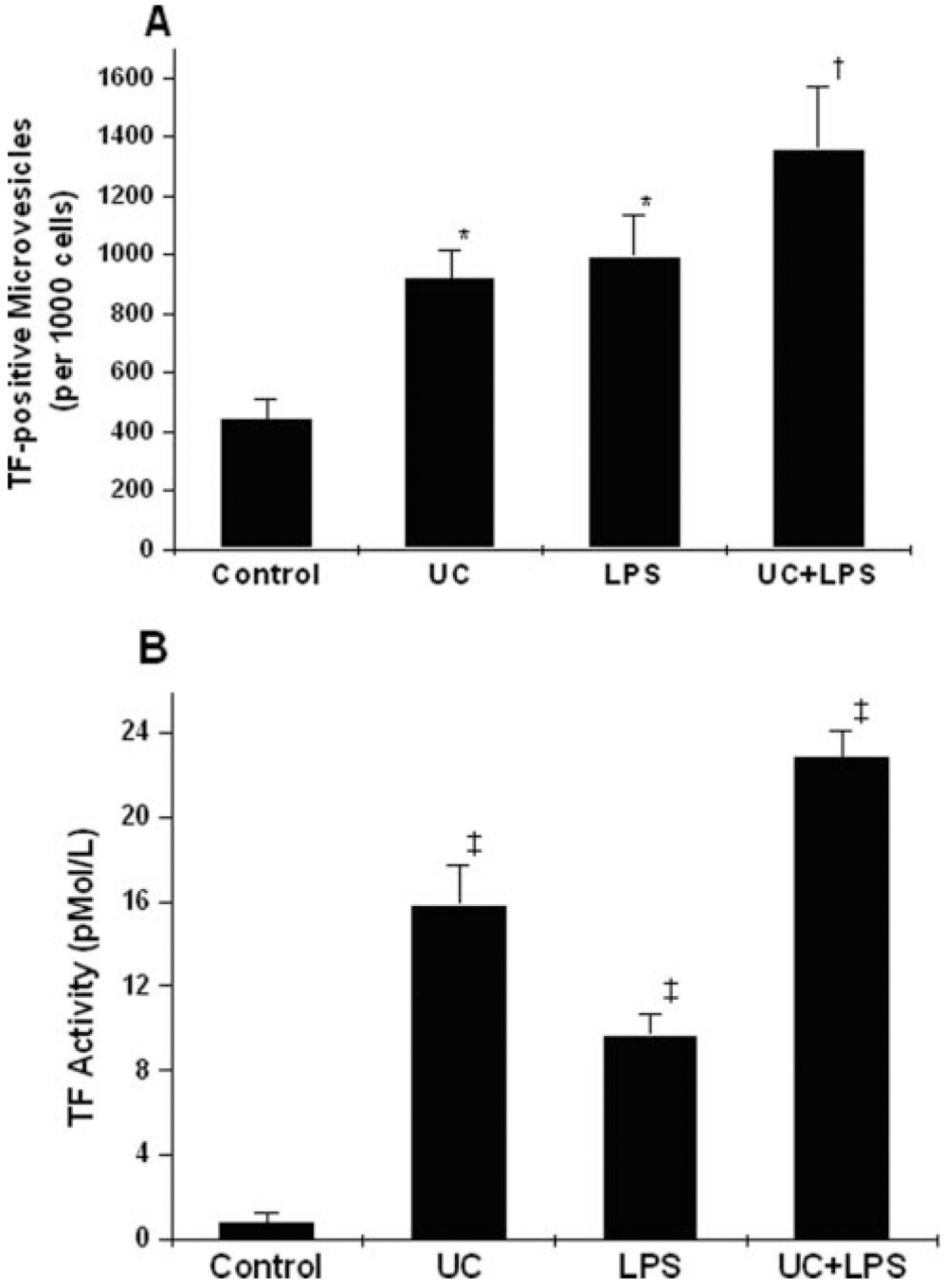

Figure 2.

Cholesterol enrichment of monocytic cells increases the generation of biologically active TF-positive MVs. Suspensions of THP-1 monocytes were treated as in Figure 1. A, Quantitative counts at 4 hours of PS-positive MVs that were also TF-positive. B, TF procoagulant activity of MV-containing supernatants in an assay using exogenous factor VIIa and collagen-activated platelets. $P<0.01$ by ANOVA. *$P<0.05$, †$P<0.01$, and ‡$P<0.001$ vs the control group by the Student-Newman-Keuls test.

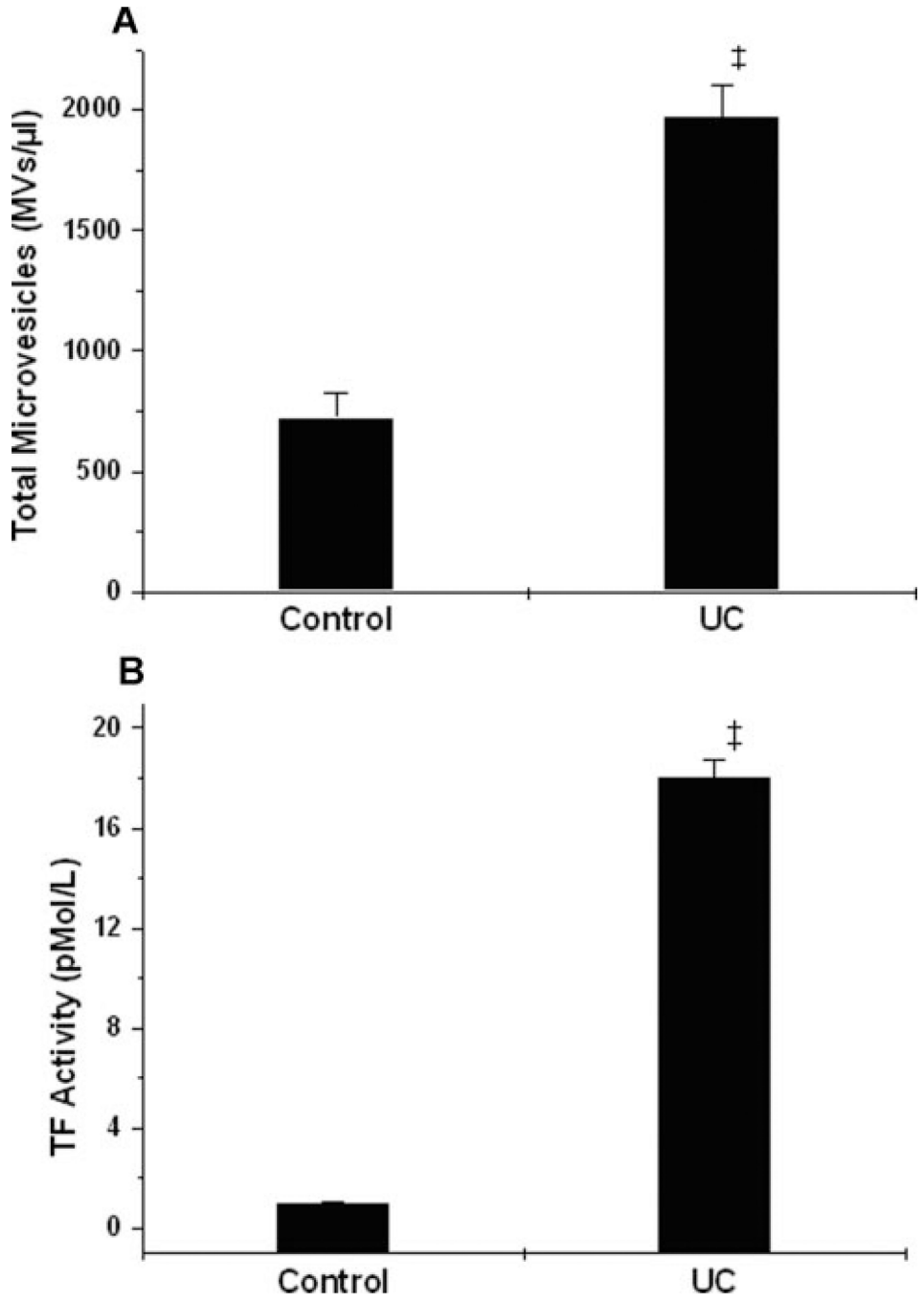

Figure 3.

Cholesterol enrichment of primary human monocyte-derived macrophages increases the generation of total as well as biologically-active TF-positive MV. Adherent primary human MDMs in 6-well plates were treated without (Control) or with UC for 20 hours. A, Total concentrations of PS-positive MVs in the conditioned medium, as assessed by flow cytometry. B, TF procoagulant activity of MV-containing culture supernatants. ‡P<0.001 vs the control group by Student unpaired t test.

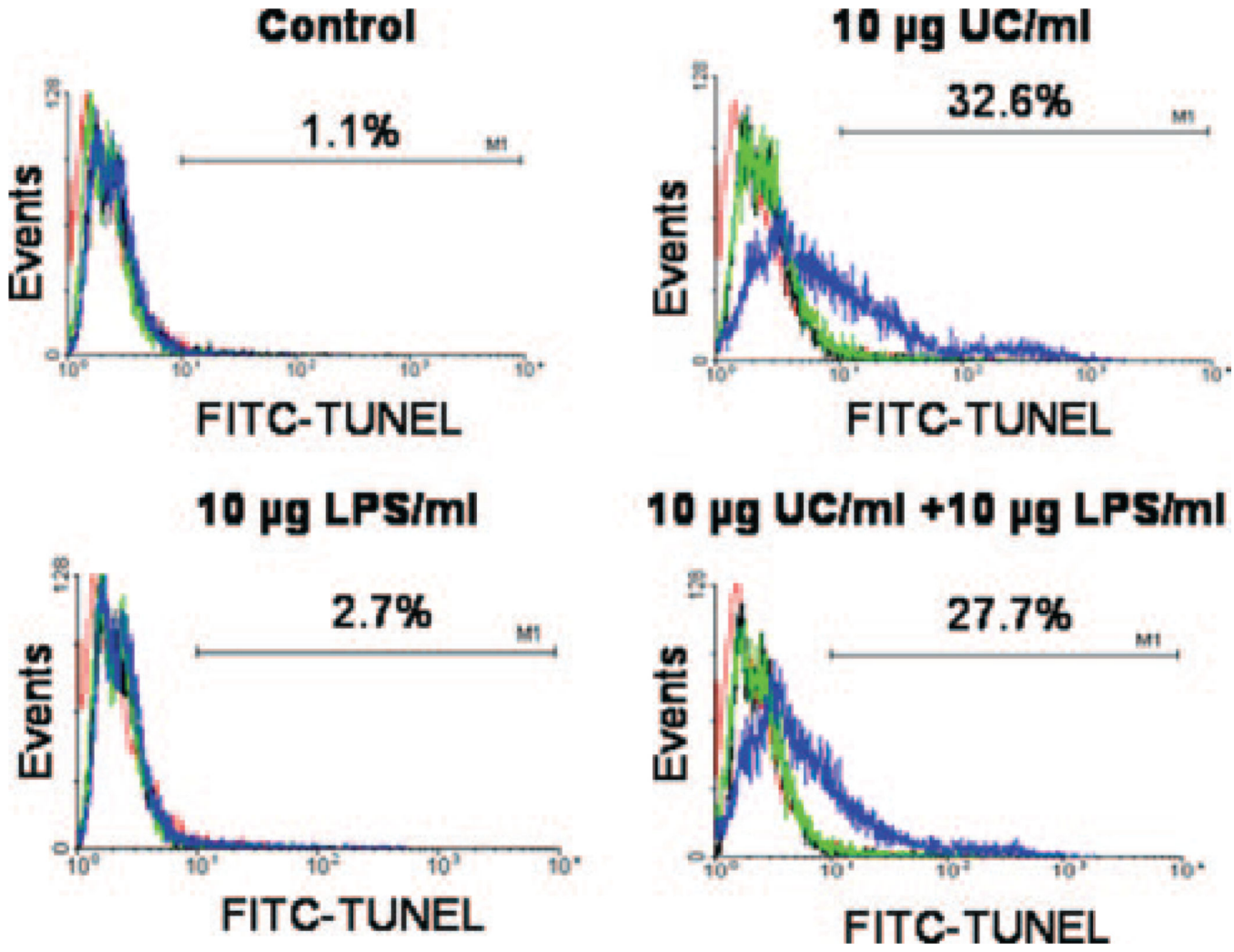

Figure 4.

Apoptosis during cholesterol-induced MV generation. THP-1 monocytes were incubated at 37°C with or without UC, LPS, or combinations, as indicated, then analyzed by flow cytometry. Displayed are representative histograms of TUNEL staining after 0 hours (red), 2 hours (black), 4 hours (green), and 20 hours (blue). Each histogram includes approximately 1000 cells. Percentages indicate the proportion of cells that were TUNEL positive at 20 hours.

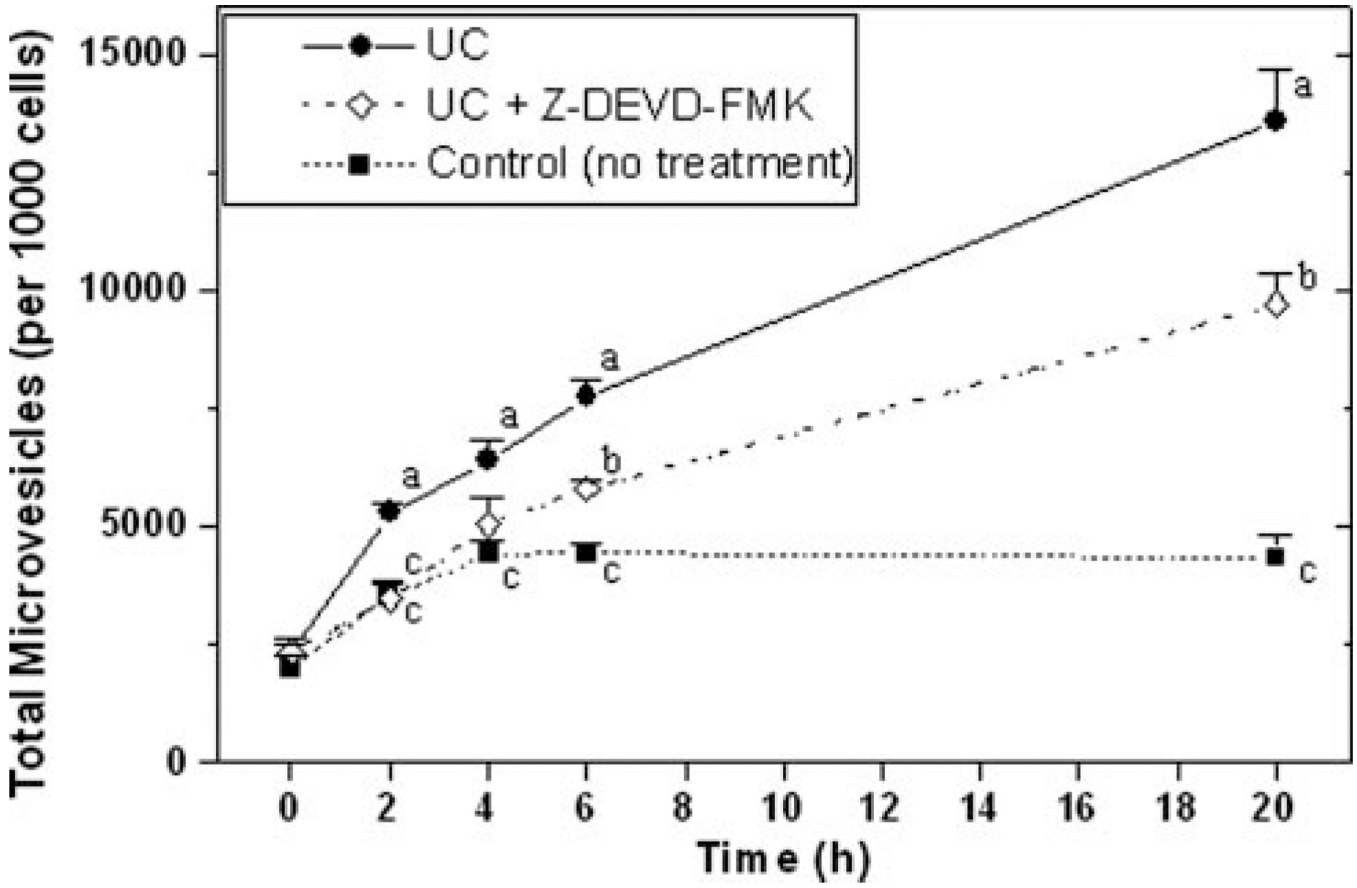

Figure 5.

Role for apoptosis in cholesterol-induced MV generation. THP-1 monocytic cells were incubated at 37°C without or with UC, Z-DEVD-FMK (a caspase-3 inhibitor), or combinations, as indicated, then analyzed by flow cytometry. Displayed are quantitative counts of PS-positive MVs over time after the indicated treatments. $P<0.01$ by ANOVA. Symbols labeled with different lowercase letters are statistically different by the Student-Newman-Keuls test ($P<0.05$).