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Uptake of exogenous free cholesterol induces upregulation of tissue factor expression in human monocyte-derived macrophages

(thrombosis/atherosclerosis)

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ABSTRACT **Lipid-laden macrophages present as foam cells may contribute to the hyperthrombotic state of human atherosclerotic lesions by the production of tissue factor (TF). We investigated the effect of exogenous nonlipoprotein cholesterol on the expression of TF by human monocyte-derived macrophages in culture. Nonlipoprotein cholesterol at 50 μg/ml increased TF activity 4-fold; TF induction was dose- and time-dependent. Expression of TF activity was positively correlated with the free cholesterol content of monocyte-derived macrophages, was increased upon inhibition of cholesterol esterification, and reflected *de novo* synthesis of TF protein. TF expression in cholesterol-loaded macrophages remained sensitive to stimulation (≈12-fold) by bacterial lipopolysaccharide, indicating that intracellular free cholesterol and lipopolysaccharide act by distinct mechanisms in inducing TF procoagulant activity. Our results suggest that loading human monocyte-derived macrophages with free cholesterol induces upregulation of TF expression, thereby contributing to thrombus formation at sites of plaque rupture.**

One of the earliest events in the atherogenic process is the adherence of circulating blood monocytes to the endothelium and their subsequent infiltration into the intimal space of the arterial wall where they mature into macrophages. Exposure of these cells to modified low density lipoproteins (LDLs) leads to intracellular cholesterol deposition and formation of lipid-laden foam cells characteristic of atherosclerotic lesions (1).

Macrophages may contribute to an inflammatory reaction in the vessel wall and to lesion formation by their capacity to produce a variety of biologically active factors that may exert proatherogenic effects both on the extracellular matrix and on adjacent cells (1–4). Moreover, cellular cholesterol content may have profound influence on the expression of such factors by monocyte-derived macrophages (3–5). Equally, the macrophage may exert procoagulant activity (PCA), thereby contributing to the prothrombotic state frequently associated with the ruptured atherosclerotic lesion. The expression of tissue factor (TF) by monocytes and macrophages is especially relevant in this context.

TF, a membrane-bound glycoprotein of ≈47 kDa, is an essential cofactor for the activation of the extrinsic pathway of coagulation (6). TF is not normally expressed by cells within the vasculature (7). In contrast, monocytes, macrophages, and endothelial cells express TF under certain pathological conditions (6) and in addition may be induced *in vitro* to express TF upon stimulation by endotoxin, immune complexes, lymphokines, and plasma lipoproteins (6, 8, 9). Recently, modified LDL was found to induce TF expression in the human monocytic cell line THP-1 (10) and in monocytes and macrophages (11); in addition, TF antigen and the corresponding mRNA were localized in foam-cell macrophages of the human atherosclerotic plaque (12). Equally, macrophages isolated from human carotid plaque are active and express elevated surface PCA with the functional characteristics of TF (13). These findings clearly suggest that some relationship may exist between either the exposure or the uptake of lipids by macrophages and the cellular expression of TF. To evaluate such a potential relationship, we examined the effect of exogenous cholesterol on TF expression by human monocyte-derived macrophages. As the lipoprotein-associated coagulation inhibitor (14) is present in LDL, we used either free exogenous nonlipoprotein cholesterol (NLC) or acetylated LDL (AcLDL) to induce macrophage cholesterol accumulation and foam cell formation *in vitro*. Our studies reveal a positive correlation between the intracellular concentration of free cholesterol (FC) and TF activity. Moreover, increase in such activity appears to result from newly synthesized TF protein.

METHODS

Lipoproteins and NLC. LDLs (density, 1.024–1.050 g/ml) were isolated from normolipidemic human serum by ultracentrifugation (15) and dialyzed as described (16). LDLs were modified by acetylation (16). The protein content of LDL and AcLDL was determined by the procedure of Lowry *et al.* (17). NLC was prepared by dissolution of cholesterol (Sigma, C7402) in 100% ethanol (12.4 mg/ml) that had been prewarmed to 37°C. Aliquots of ethanol (8 μl) containing 0 (control)–100 μg of cholesterol were used.

Preparation of Human Monocyte-Derived Macrophages. Isolation of monocytes from the blood of healthy normolipidemic volunteers and their subsequent culture were performed as described (3). At day 8 of culture, monocyte-derived macrophages (henceforth denoted as "macrophages") were washed twice with serum-free RPMI 1640 medium containing polymyxin B (60 units/ml; GIBCO/BRL) and 1% Nutridoma HU (Boehringer Mannheim); cells were then incubated with various agents in this latter medium. In experiments in which bacterial lipopolysaccharide (LPS) was added, polymyxin B was omitted. Cycloheximide, dextran sulfate, and LPS were purchased from Sigma. The acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitor CI-976 was the kind gift of J. Cornicelli (Parke–Davis). The endotoxin content of all culture materials and reagents used was measured with the *Limulus* amebocyte lysate assay (KabiVitrum Diagnostica, Mölndal, Sweden) and only those

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Abbreviations: TF, tissue factor; LPS, lipopolysaccharide; LDL, low density lipoprotein; AcLDL, acetylated LDL; oxLDL, oxidized LDL; FC, free cholesterol; CE, cholesteryl ester; NLC, nonlipoprotein cholesterol; PCA, procoagulant activity; ACAT, acyl-coenzyme A:cholesterol acyltransferase; mU, milliunit(s).

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free of endotoxin or containing <3 pg/ml were used. This level was lower than that required for activation of macrophages (18). In each experiment, cell viability was typically >95% by the trypan blue exclusion method.

Filipin Staining and Cholesterol Analysis. Isolated monocytes were plated in 1 ml of culture medium in single-well slide chambers (Nunc), instead of Primaria dishes, and then cultured as above. After cell stimulation by either AcLDL (100 μg of protein per ml) or NLC (50 μg/ml) for 48 h, cellular FC and cholesteryl ester (CE) were separately stained with filipin (19). Intracellular cholesterol content was determined as described (16) and involved use of a cell lysis solution (0.5 ml per dish) containing 10 mM Tris·HCl and 1 mM EDTA at pH 7.4.

Tissue Factor Assay. The PCA of the cell preparations was determined from the one-step recalcification clotting time as reported (8). Briefly, samples of sonicated cells (80 μl) were transferred to 96-well microplates. To each well, 80 μl of normal human citrated platelet-free plasma and 80 μl of 30 mM $CaCl_2$ were added. Clot formation in the well was monitored by determination of the optical density at 540 nm. The clotting time was determined as the time required to double the initial optical density. Milliunits (mU) of TF were arbitrarily defined by conversion from standard curves (log–log plot) developed with a preparation of rabbit brain thromboplastin (Sigma). PCA was totally TF-dependent as assessed by use of factor VII-deficient plasma. In addition, such PCA was abolished when crude samples were incubated with a specific polyclonal antibody to human TF (the kind gift of J. M. Morrissey, Oklahoma Medical Research Foundation, Oklahoma City). Similarly, addition of an aliquot of NLC, ethanol, or AcLDL did not interfere with TF PCA.

Immunoprecipitation of ^{35}S-Labeled TF. TF synthesized *de novo* in 8-day-adherent macrophages was radiolabeled with [^{35}S]methionine (16) in the presence of NLC (100 μg/ml) or AcLDL (100 μg of protein per ml). Control cells received only methionine-free medium or this medium supplemented with ethanol; all dishes were subsequently incubated at 37°C. After a 36-h incubation, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and then lysed with 0.5 ml of PBS containing 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (50 μg/ml). Cell lysates were centrifuged for 10 min at 12,000 × *g* at 4°C to remove nuclei and the amounts of trichloroacetic acid-precipitable [^{35}S]methionine-labeled proteins were then measured. For immunoprecipitation, aliquots of the cell lysates were incubated with a goat polyclonal antibody directed against human TF, and the TF–IgG complex was precipitated with protein A (Pharmacia Fine Chemicals). Immunoprecipitates were treated as outlined (16), and protein content was analyzed by SDS/PAGE in a 10% gel. Radiolabeled TF of 46 kDa was visualized by autoradiography; for calibration, radioactively labeled protein markers (Amersham) were electrophoresed in parallel.

RESULTS

Effect of NLC and AcLDL on Cholesterol Accumulation. Exposure of macrophages to increasing concentrations of NLC for 48 h at 37°C resulted in substantial elevation in the cellular content of both FC and CE. Maximal loading in the absence of cytotoxicity was observed at ≈100 μg/ml (Fig. 1*a*), representing an ≈4-fold increase in total cellular cholesterol content as compared to control preparations. The levels of FC and CE were, therefore, determined in control macrophages and in cells incubated with NLC (100 μg/ml) at the indicated time intervals (Fig. 1*b*). In control cells, CE was not detectable whereas the level of FC was constant, representing ≈20 μg/mg of cell protein. However, the cellular content of CE increased linearly from nondetectable levels to ≈30 μg/mg of cell protein at 50 h of incubation of macrophages in the presence of NLC. Similarly, the free sterol induced a rapid increase in the cellular FC level (≈30 μg/mg of cell protein at 1 h of macrophage incubation). Maximal accumulation of FC was observed at ≈30 h (≈55 μg/mg of cell protein) and was followed by a progressive decrease to 50 h of incubation (Fig. 1*b*).

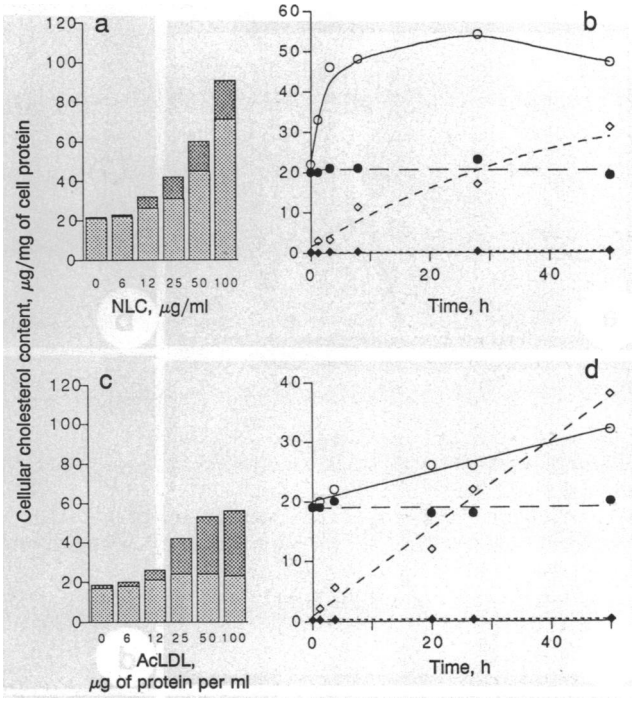

FIG. 1. Effect of NLC and AcLDL on cholesterol accumulation. Cultured macrophages were washed three times with serum-free RPMI 1640 medium supplemented with polymyxin B (40 μg/ml) and 1% Nutridoma HU and, subsequently, incubated in this latter medium with increasing concentrations of NLC (*a*) or AcLDL (*c*) for 48 h at 37°C. The cells were then washed and lysed, and aliquots were taken for quantitation of intracellular FC and CE. Values for FC (light shading) and CE (dark shading) are the means of duplicate determinations from three experiments. (*b*) Time course of the effect of NLC (100 μg/ml) on the accumulation of FC (○) and CE (◇) in macrophages cultured for 8 days. Solid symbols (●, ◆) represent control values for FC and CE, respectively. (*d*) Corresponding time course for AcLDL (100 μg of protein per ml). ○, FC; ◇, CE. Solid symbols represent corresponding control values. Values represent the means of duplicate determinations from three experiments.

We also used AcLDL as a source of exogenous cholesterol to compare the levels of FC and CE in cultured macrophages with those observed after incubation with NLC (Fig. 1 *c* and *d*). Incubation of increasing concentrations of AcLDL (0–100 μg of protein per ml) with macrophages at 8 days of culture revealed that a concentration of ≈50 μg of AcLDL protein per ml leads to maximal elevation of cellular cholesterol content (60 μg/mg of cell protein) and that >50% was present in the esterified form (Fig. 1*c*). In addition, quantitation of FC and CE in control cells or in macrophages exposed to AcLDL (100 μg of protein per ml) was performed at the indicated time intervals (Fig. 1*d*). Again, in control cells, the level of FC was constant (20 μg/mg of cell protein) and the level of CE was undetectable. However, in cells incubated with AcLDL, the CE content increased from nondetectable levels to ≈40 μg/mg of cell protein from 1 to 50 h; in addition, FC increased from 20 to 30 μg/mg of cell protein over the same period.

To verify whether the CE and FC derived from either AcLDL or NLC were accumulated within macrophages, we used a fluorescent probe, the polyene antibiotic filipin, to detect the presence of intracellular FC or CE (Fig. 2); this

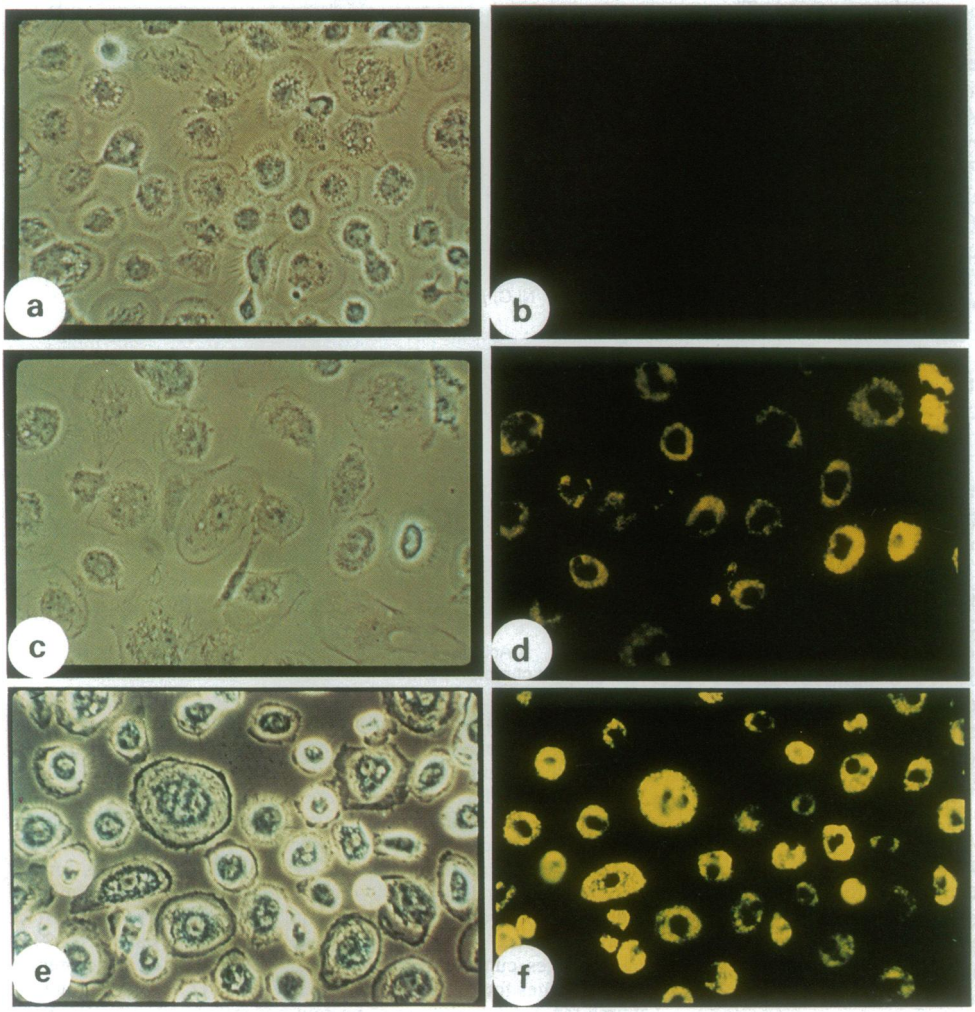

FIG. 2. Filipin-stained CE in cultured macrophages after incubation with AcLDL or NLC. Human monocytes were isolated and plated in single-well slide chambers. After 14 days of culture, the cells were rinsed three times with serum-free RPMI 1640 medium supplemented with antibiotics. Certain cell dishes were then incubated in this latter medium as controls (*a* and *b*), and others received AcLDL (100 μg of protein per ml) (*c* and *d*) or NLC (50 μg/ml) (*e* and *f*). After 48 h, the cells were rinsed three times. The FC (data not shown) and CE (*b*, *d*, and *f*) were stained with filipin as described (19). Filipin dye was excited (UG1 filter) by a 100-W mercury arc lamp and fluorescence was viewed through a 510-nm barrier filter. (*a*, *c*, and *e*) Macrophages examined by phase-contrast microscopy.

antibiotic specifically binds to 3β-hydroxysterols (20). Incubation of macrophages with AcLDL (100 μg of protein per ml; Fig. 2*d*) or NLC (50 μg/ml) for 48 h led to significant increase in cellular fluorescence, which corresponded to the accumulation of FC (data not shown; H.S.K., S.S., P.L., M.R., and M.J.C., unpublished data). In addition, and under the same experimental conditions as above, the filipin-stained CE visualized after hydrolysis was dramatically increased (Fig. 2 *d* and *f*; macrophages were incubated, respectively, with AcLDL and with NLC). In control cells, the CE was below the level of detection (Fig. 2*b*), whereas the FC was visible (data not shown).

Induction of TF PCA in Macrophages by AcLDL and NLC. Fig. 3*a* shows the time course of the effect of NLC on the activity of TF in macrophages. NLC induced an initial increase in TF activity representing a 2-fold elevation over basal levels that was almost maximal by 6 h; subsequently, a transient decrease in TF activity occurred after ≈24 h of incubation, followed by a progressive increase (4-fold more than basal level) to 70 h. The basal level of TF activity in macrophages at 8 days of culture was ≈80 mU/mg of cell protein; such activity may be typical of mature human macrophages. To investigate the effect of cholesterol loading on macrophage TF activity, therefore, we chose 48 h for cell incubations with NLC, LDL, or AcLDL for the following studies. The capacity of NLC to increase TF activity in macrophages was dose-dependent with a maximal effect at ≈50 μg/ml. The activity of TF under these conditions amounted to 355 mU/mg of cell protein over a 48-h period and represented an ≈4-fold stimulation over basal levels at 8 days of culture (Fig. 3*b*).

Incubation of AcLDL with adherent human monocytes (8–48 h of culture) was reported to result in a substantial increase in TF activity whereas native LDL was without effect (11); we have now extended these studies to mature monocyte-derived macrophages (data not shown for native LDL). Furthermore, incubation of macrophages with increasing concentrations of AcLDL revealed that a concentration range of 25–50 μg of protein per ml maximally induced TF activity, representing an ≈2-fold stimulation over basal levels. In addition, a positive correlation was found between cellular FC content and TF activity ($r = 0.834$), but no correlation was observed between TF activity and cellular CE ($r = 0.349$).

To investigate the effect of ACAT-catalyzed esterification of cellular FC on TF activity, we treated macrophages (that had been preincubated with NLC at 100 μg/ml for 48 h) with an ACAT inhibitor (CI-976) at 10 μg/ml. This resulted in an elevation in FC content and a concomitant decrease in the amount of CE as compared to cholesterol-loaded control cells. Under these conditions, the activity of TF was significantly elevated (160% vs. control) (Table 1).

Cellular protein synthesis was required for the generation of TF activity as shown by the virtually complete suppression of such activity when macrophages were incubated with NLC in the presence of cycloheximide at 50 μg/ml (Fig. 3*a*). In addition, after incubation of macrophages with [^{35}S]methionine, radioactively labeled TF (46 kDa) was immunoprecipitated from the cell lysate. As shown in Fig. 4, lysates of NLC-treated cells (Fig. 4, lane 3) and AcLDL-treated macrophages (Fig. 4, lane 2) contained large amounts of immunoprecipitable TF at 36 h, whereas smaller amounts of

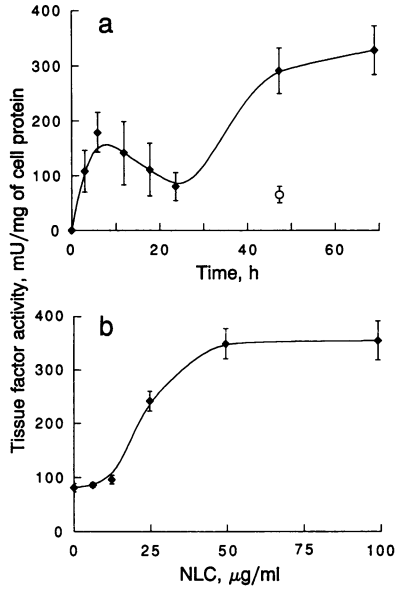

FIG. 3. Effect of NLC on cell-associated TF activity. Cultured macrophages were incubated with NLC (100 μg/ml) at 37°C for the indicated time intervals (*a*). The cells were subsequently rinsed, lysed, and sonicated (3) and aliquots (80 μl) were taken for the TF assay. Values are the means ± SD of TF activity minus the corresponding control values from triplicate determinations in three experiments. ○, TF PCA in macrophages incubated for 48 h in the presence of cycloheximide (50 μg/ml). (*b*) Dose–response curve of the effect of increasing concentrations of NLC on TF activity in macrophages at 48 h. Values are means ± SD of triplicate determinations in two experiments; all control dishes received 1 ml of medium containing 8 μl of ethanol.

immunoprecipitable TF could be identified in the lysate from [^{35}S]methionine-labeled control cells (Fig. 4, lane 1). Use of a nonimmune IgG control serum demonstrated the specificity of TF immunoprecipitation (Fig. 4, lane 4); minor bands, apparently reflecting immunoprecipitation specific to this serum, were detected with molecular masses of 64.0, 61.9, 46.7, and 44.4 kDa.

To assess the capacity of lipid-laden macrophages to modulate expression of TF in response to stimulation by LPS, we performed studies of TF activity on macrophages whose cellular cholesterol content had been markedly increased (4-fold) by exposure to NLC. Cholesterol-loaded macrophages remained responsive to LPS at 10 μg/ml (in preliminary experiments, this concentration corresponded to maximal stimulation of cellular TF activity in control non-cholesterol-enriched macrophages), displaying a significant (11.7-fold) elevation in TF activity as compared to their cholesterol-loaded nonstimulated counterparts (231 ± 81 mU/mg of cell protein, $P \leq 0.001$) after 48 h of incubation.

Table 1. Effect of an ACAT inhibitor (CI 976) on the CE and FC content and TF activity of human macrophages

	TF activity, % of control	CE, % of total cholesterol	FC, % of total cholesterol
Control	100	19.3 ± 2.9	79.5 ± 5.1
+ ACAT inhibitor	160 ± 36	2.6 ± 0.3	97.3 ± 11.4

Macrophages (8 days of culture) were incubated with NLC (100 μg/ml) in the presence or absence of an ACAT inhibitor (CI 976; 10 μg/ml) for 48 h. After incubation, the cells were washed and lysed. Aliquots of cell lysates were used for TF, FC, and CE quantitation. Values are the means ± SD of triplicate determinations from three experiments. TF activity in control cells was taken as 100%.

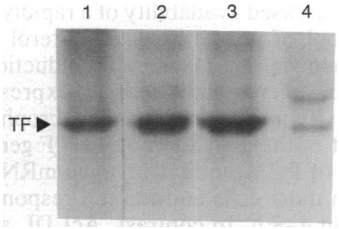

FIG. 4. Autoradiogram of ^{35}S-labeled TF immunoprecipitated from macrophage lysates. TF was immunoprecipitated from cultured macrophages with an antibody to TF from control cells (lane 1) and from cells incubated with AcLDL (lane 2) or NLC (lane 3); in lane 4, immunoprecipitation was performed using a nonimmune IgG control serum.

DISCUSSION

Our data suggest that cultured macrophages are activated to express TF activity by NLC and may provide an explanation for the elevated surface TF activity observed by others in macrophages isolated from atheromatous lesions (13). In contrast to native LDL, which fails to enhance PCA in peripheral blood mononuclear cells, AcLDL and malondialdehyde LDL are potent inducers of TF activity (30-fold elevation) in human monocytes adherent for up to 48 h (11). In similar experiments in which AcLDL was incubated with macrophages cultured for 8 days, we found a smaller (2-fold) increase in TF activity. The discrepancy between our results and those of Schuff-Werner *et al.* (11) may, therefore, be related to both the conditions and period of cell culture, and thus to differences in the capacities of adherent monocytes as compared to mature monocyte-derived macrophages to express TF activity. It is equally relevant that cholesterol-loaded macrophages remained sensitive to the action of LPS, thereby suggesting that intracellular FC and LPS may induce TF activity by different mechanisms.

More recently, oxidized LDL (oxLDL) has been reported to induce TF expression in cultured human arterial and venous endothelial cells (21). Moreover, the interaction of oxLDL with the AcLDL (scavenger) receptor is partially involved in the procoagulant response since fucoidin and dextran sulfate, which are known competitors for binding to the AcLDL receptor, partially blocked the oxLDL-induced increase in TF activity (21). In addition, no induction of TF activity was observed in endothelial cells in the presence of either fucoidin or dextran sulfate alone (21). This finding may indicate that activation of the scavenger receptor is not involved in the process leading to expression of TF activity. We performed similar experiments with dextran sulfate alone in human macrophages and found no change in TF activity as compared to control cells (results not shown). This observation indicates that, as seen earlier in endothelial cells (21), the scavenger receptor is not implicated in the upregulation of TF activity in our cell system. Another argument that excludes the possibility of scavenger receptor involvement in the generation of TF activity is that minimally modified LDL, which binds to the LDL receptor, induces TF expression in cultured human endothelial cells (22).

The increase in TF activity observed with either monocytes/macrophages or endothelial cells incubated with modified lipoproteins could then arise as a consequence of the release of lipoprotein–cholesterol after internalization and degradation. Our present study involving NLC supports this hypothesis. However, when Weis *et al.* (21) coincubated endothelial cells with oxLDL and chloroquine, a lysosomal inhibitor, they did not observe a reduction in the induction of TF activity, indicating that degradation of oxLDL is not necessary for the procoagulant response. In light of our results on the effect of NLC on TF activity, we can hypoth-

esize that the increased availability of a rapidly exchangeable intracellular pool of unesterified cholesterol may trigger a signaling mechanism that leads to TF induction.

A variety of agents may induce expression of clot-promoting activity in monocytes and macrophages, presumably as a result of the activation of the TF gene (6). Indeed, the generation of PCA requires *de novo* mRNA and protein biosynthesis in most cells and the full response is typically observed within 4–8 h. In contrast, AcLDL and malondialdehyde LDL maximally induced TF activity on cultured human monocytes within 24 h (11). NLC induced a biphasic response in TF activity (Fig. 3*a*), although the mechanism by which NLC induced TF activity remains to be established. We can speculate that the first peak may correspond to the direct effect of cholesterol on the synthesis of TF protein and the second may correspond to synthesis of a substance that may in turn act on TF synthesis. A biphasic effect on TF activity in cultured human phorbol ester-stimulated monocytes has been observed (E. Nicodeme, personal communication). In addition, cholesterol enrichment of macrophages induces a variety of effects including induction of tumor necrosis factor (23), which may in turn induce TF in endothelial cells (5) and monocytes (24).

A number of findings obtained with various cell types in culture indicate the existence of multiple mechanisms that may lead to modulation of the expression of TF PCA. The constitutive production of TF by extravascular cells and the induction of its expression by the endothelial cell and the monocyte/macrophage indicate that mRNA and protein synthesis are required (6). Indeed, previous studies on monocytes have indicated that these cells do not possess a preformed pool of TF protein (6) and that, in the presence of bacterial LPS, the primary level of control is transcriptional (25), with possibly a minor contribution at the level of mRNA stability (26). However, HL-60, a human promonocyte cell line, has been reported to possess a pool of inactive TF (6). Mobilization of latent PCA might occur by a posttranslational modification that activates the TF protein or, alternatively, by the disappearance of an inhibitor. In addition, acidic phospholipids such as phosphatidylserine may facilitate the activator–enzyme association and may increase the proteolytic activity of the complex (6). Indeed, the appearance of phosphatidylserine on the cell surface in response to injury represents a potential mechanism for regulation of TF cell surface expression (6). In our cell system, NLC could not act by this latter mechanism, as experiments were performed on sonicated cell preparations. However, consideration of our findings on the *de novo* production of TF (Fig. 4) and on the initiation of TF expression by cycloheximide (Fig. 3) suggests an effect of cholesterol on the translational control of the synthesis of TF protein, rather than an effect mediated by an alternative mechanism.

In conclusion, our results indicate that an increase in the content of cholesterol in macrophages is alone sufficient to induce a significant elevation in TF PCA. This observation is clearly of relevance to the transformation of macrophages into foam cells in the arterial wall and to the role of such cells in contributing to local thrombin generation and fibrin deposition as occurs in atherogenesis and its thrombotic complications.

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