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Minimally Oxidized Low-density Lipoprotein Induces Tissue Factor Expression in Cultured Human Endothelial Cells

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Oxidatively modified low-density lipoprotein is present in atherosclerotic lesions and has been proposed to play an important role in atherogenesis through its biologic effects on vascular cells. This study examined the effects of minimally oxidixed preparations of LDL (MM-LDL) on tissue factor (TF) expression by cultured human endothelial cells. Low-density lipoprotein purified from normal donors was modified by exposure to iron or by prolonged storage, resulting in levels of thiobarbituric acid-reacting substances of approximately 2.5 to 4 nmoles/mg cholesterol. Preparations had less than 2.5 pg of endotoxin per microgram LDL and had no intrinsic procoagulant activity. This form of modified but not native LDL induced TF expression in endothelial cells in a time- and dose-dependent manner. Peak TF coagulant activity in cells exposed to 40 μg/ml MM-LDL were observed at 4 to 6 hours, and ranged from 50 to 500 pg/10^5 cells, compared with less than 10 pg/10^5 cells exposed to native LDL. Northern blot analysis showed TF mRNA levels to increase approximately 30-fold with exposure to MM-LDL for 2 hours. Induction of TF activity was dependent on the concentration of MM-LDL from 1 μg/ml to 80 μg/ml, a range in which cell viability and morphology were unaffected. The findings suggest that minimally oxidized LDL may be a local mediator promoting thrombosis in atherosclerotic lesions. (Am J Pathol 1991, 138:601–607)

In atherosclerosis and inflammation, alterations in endothelial cell functions may occur, including changes in hemostatic properties.[1–3] Among these is the ability to initiate the coagulation protease cascade through the induced expression of tissue factor (TF), an integral cell membrane glycoprotein that serves as the receptor and essential cofactor for coagulation factors VII/VIIa.[4] The TF-FVIIa complex activates coagulation factors X and IX by limited proteolysis, leading ultimately to thrombin formation and the deposition of fibrin. The identification of mediators that induce TF expression by endothelium is important in unraveling the complex pathophysiology of these processes.

In the course of oxidative modification, low-density lipoprotein (LDL) acquires various biologic properties not present in native (unmodified) LDL that make it a potentially important mediator promoting atherogenesis.[5,6] Recent studies with a form of LDL that is minimally oxidized (designated 'minimally modified' or MM-LDL) have demonstrated effects on several functional properties of cultured endothelial cells that involve interactions with blood and vascular cells. These include enhanced production of macrophage and other colony-stimulating factors, and a chemotactic factor for monocytes, increased monocyte binding to endothelium, and inhibition of platelet-derived growth factor (PDGF) synthesis.[7–9] The current study was performed to determine whether MM-LDL is capable of also altering endothelial cell hemostatic function. We show that MM-LDL is a potent inducer of TF mRNA and protein expression in cultured human endothelial cells.

Methods

Lipoprotein Preparation

Low-density lipoprotein from healthy adult donors was isolated by density gradient centrifugation of serum and

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stored in phosphate-buffered 0.15 mol/l (molar) NaCl containing 0.01% ethylenediaminetetra-acetic acid (EDTA). Minimally modified LDL was obtained by storage of LDL at 4°C for 3 to 6 months in plastic tubes or by brief exposure to 1 μmol/l (micromolar) Fe^{++} as previously described; highly oxidized LDL was prepared by exposure to 5 μmol/l Fe^{++} and contained 9 nmoles per mg cholesterol.[10] Thiobarbituric acid–reactive substances (TBARS) were measured as previously described and expressed as nmoles/mg cholesterol.[11] Malondialdehyde-modified LDL was provided by Dr. Margaret Haberland, prepared as described from recently isolated LDL.[12] Lipopolysaccharide (LPS) in preparations was detected using a sensitive chromogenic limulus lysate assay (Sigma Chemical Co., St. Louis, MO; the chromogenic substrate S2423 was from Kabi-Vitrum, Inc., Franklin, OH). Minimally modified LDL preparation 1 (Table 1) used for the majority of the experiments had 0.25 pg of endotoxin per microgram LDL; other preparations had between 1.5 and 2.5 pg/μg. The maximal concentrations of LPS potentially present in both normal and modified LDL preparations (100 pg/ml in preparations used with cardiac valve endothelial cells and 10 pg/ml for those with aortic endothelial cells) were documented not to induce TF expression in endothelium from either source.

Endothelial Cell Culture

Human aortic endothelial cells (a gift from Dr. Alan Fogelman) and human cardiac valve endothelial cells were isolated and propagated as described.[13] Cells were used at passages 4 to 9, and were maintained in medium 199 supplemented with 20% fetal bovine serum (FBS), 25 mmol/l (millimolar) HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), glutamine (292 μg/ml), pyruvate (110 μg/ml), penicillin (100 U/ml), endothelial cell growth supplement (50 μg/ml), and heparin (90 μg/ml) in an atmosphere of 5% CO_2 at 37°C. Fetal bovine serum was from Hyclone, Logan, UT; other tissue culture media and supplements were from GIBCO Laboratories, Grand Island, NY.

Endothelial Cell Tissue Factor Expression

Experiments were performed with confluent cell monolayers in 24-well tissue culture dishes (Costar, Van Nuys, CA), containing approximately 200,000 cells per well. Each experimental condition was set up in duplicate and was repeated on at least one separate occasion. For each experiment, monolayers were washed three times in serum-free medium then incubated in 0.5 ml M199 with 10% heat-inactivated FBS containing the relevant agonist. In the competition experiments, MM-LDL (in 5 μl M199) was added to wells after addition of medium with normal or MDA-LDL. After varying times, wells were washed twice in chilled Dulbecco's phosphate-buffered saline (PBS) and once with TRIS saline albumin (TSA) assay buffer (20 mmol/l TRIS, 130 mmol/l NaCl, 0.1% bovine serum albumin, pH 7.4), then mechanically scraped into 200 μl TSA assay buffer and frozen at −70°C. Before assay, cell suspensions were freeze-thawed three times in a dry ice–ethanol bath. Tissue factor was measured by a one-stage citrated plasma recalcification time assay as described,[13] and quantitated by reference to a standard curve prepared from dilutions of purified relipidated human brain TF[14]; 50 pg of this reference preparation yielded a clotting time of 55 seconds. For this assay, 60 μl of sample was incubated for 30 seconds in a fibrometer cup with 60 μl each of pooled human plasma and diluted rabbit brain cephalin, then the clotting time determined after the addition of $CaCl_2$ using a fibrometer. Specificity of this assay for TF was documented by incubating samples with an inhibitory murine IgG_1 monoclonal antibody (MAb) to human TF (clone TF9-9C3 at 10 μg/ml) for 10 minutes before assay.[15] In limited studies, a two-stage assay for TF was used, in which 5 μl of cell suspension was added to 20 ng FVIIa

Table 1. *Tissue Factor (TF) Coagulant Activity in Human Endothelial Cells Incubated with 40 μg/ml Normal or Modified LDL (MM-LDL) for 6 Hours*

LDL added	TBARS* (nmol/mg cholesterol)	TF activity† n	TF activity† (pg/10^5 cells)
None	—	7	5 ± 2
Normal LDL‡	<1.0	8	7 ± 2
MM-LDL #1	3.0	3	299 ± 116
#2	3.5	2	586 ± 89
#3	2.8	2	74 ± 35
#4	3.5	1	61
#5	3.7	1	141

* TBARS, Thiobarbituric acid-reacting substances.
† Mean ± 1 SD.
‡ Data are combined results for five separate preparations.

and 10 μg FX in 390 μl TSA assay buffer. $CaCl_2$ (28 μl of 100 mmol/l) was then added (stage 1). After 10 minutes, 60 μl of assay medium was withdrawn and Xa generated during the first stage was measured by a clotting time procedure with factors VII– and X–deficient bovine plasma (stage 2). Purified factors VIIa and X, TF, and anti-TF MAbs were provided by Drs. James Morrissey and Thomas Edgington of the Research Institute of Scripps Clinic, La Jolla, CA.

Northern Blot Analyses for TF mRNA

The cDNA probe for TF (provided by Drs. James Morrissey and Thomas Edgington of the Research Institute of Scripps Clinic, La Jolla, CA[16]) was labeled with ^{32}P by the random primer method[17] to approximately 10^8 cpm/μg. Endothelial cell monolayers were exposed to 40 μg/ml MM-LDL, normal LDL, or medium alone for 2 hours as described above, then total cellular RNA isolated by lysis of cells in guanidinium isothiocyanate, phenol-chloroform extraction, and precipitation.[18] Twenty micrograms of each RNA preparation was denatured and electrophoresed in 1.2% formaldehyde agarose gels, then blotted onto nylon filters and UV cross-linked.[19] Filters were prehybridized in 7% sodium dodecyl sulfate (SDS), 0.5 mol/l sodium phosphate, 1 mmol/l EDTA, 1% bovine serum albumin (BSA), pH 7.2, with 100 μg/ml salmon sperm DNA. Hybridization was performed in the same buffer for 16 hours at 65°C. Blots were washed with 0.5× saline sodium citrate, 0.1% SDS at 65°C, then autoradiographed for 5 days. As an internal control, filters were also hybridized and autoradiographed as described using a ^{32}P-labeled CHO-B cDNA probe. CHO-B is an abundant constitutively expressed mRNA originally detected in Chinese hamster ovary cells, the expression of which in endothelial cells is unaltered by agonists such as LPS.[20]

Endothelial Cell Viability

Cells were detached by brief exposure to trypsin-EDTA and counted in a hemocytometer after incubation with trypan blue as described.[13]

Statistics

Comparisons between groups were analyzed by using Student's *t*-test.[21]

Results

Five separate preparations of minimally modified LDL induced TF expression in cultured human endothelial cells (Table 1). These included one preparation oxidized by prolonged storage (#1 in Table 1) and four prepared by limited exposure to iron (#2 through 4 in Table 1), all having increased levels of TBARS. Measured as TF coagulant activity, 40 μg/ml MM-LDL induced levels at 6 hours that were 8- to over 50-fold greater than those observed in cells exposed to medium alone or normal LDL. For two of the five preparations, these levels were in the range observed for maximal stimulation with endotoxin (430 ± 147 pg/10^5 cells). Similar results were obtained with cultured human aortic endothelial cells, excluding the possibility that the response was unique to cultured cardiac valve endothelium. Minimally modified LDL preparation 1 (40 μg/ml) induced 469 ± 211 pg TF/10^5 aortic endothelial cells (mean ± 1 standard deviation [SD]), *versus* 8 ± 2 for the same concentration of normal LDL in two representative experiments.

Control experiments demonstrated specificity of the response (Table 2). Minimally modified LDL alone or mixed with lysed endothelial cells suspended in assay buffer had no detectable coagulant activity. Specificity of the assay for TF was documented by incubating lysed cell suspensions (ie, total cellular TF) with inhibitory MAb to human TF (10 μg/ml) for 10 minutes before assay. Procoagulant activity was inhibited by more than 98% in all cases. Additionally, induced procoagulant activity was dependent on factors VIIa and X in a two-stage assay, demonstrating that it had the functional characteristics of TF. In these experiments, cells were incubated with excess factors VIIa and X in the presence of Ca^{++} (stage 1). Factor X activation (dependent on the presence of TF:VIIa complexes) was subsequently detected by a clotting time procedure using factors VII- and X-depleted

Table 2. *Control Experiments Demonstrating Specificity of MM-LDL Induction of Tissue Factor in Endothelial Cells (EC)*

Condition	TF activity (pg/10^5 cells) Exp. 1	Exp. 2	Exp. 3
EC alone (6 hours incubation)	9.7	6.9	6.9
EC + nl LDL (40 μg/ml for 6 hours)	7.8	6.0	7.1
EC + MM-LDL (40 μg/ml for 6 hours)*			
+ control MAb	681	528	49.5
+ anti-TF MAb	<6.7	<2.8	<2.8
MM-LDL alone†	<6.7	<2.8	<2.8
MM-LDL + lysed control EC‡	ND	4.7	4.6

* MM-LDL used for Exp. 1 was preparation #1 in Table 1; for Exp. 2, preparation #2; for Exp. 3, preparation #3. Lysed cell suspensions in assay buffer were incubated with 10 μg/ml of control or inhibitory anti-TF monoclonal for 10 minutes before prior to assay for TF activity.

† MM-LDL (20 μg) was added to 60 μl buffer and assayed directly for TF activity.

‡ MM-LDL (20 μg) was added to 60 μl of lysed control EC and assayed directly for TF activity.

plasma (stage 2). In two such determinations, assay of MM-LDL–stimulated cells lead to second-stage clotting times of 31.6 and 36.9 seconds. Substitution of either factors VIIa or X with buffer in the first stage, however, yielded clotting times of more than 200 seconds, indicating that TF was the cellular procoagulant activity being measured. (These clotting times corresponded to TF activity equivalent to 737 and 420 pg/10^5 cells, respectively, in the complete assay and less than 5 pg/10^5 cells in the factor-depleted assays.)

Northern blot analysis demonstrated that TF mRNA expression was also induced in cells exposed to MM-LDL (preparation 1) but not normal LDL for 2 hours. Figure 1 shows a major band of approximately 2.3 kb in length, consistent with the mature TF message. Densitometric comparison of this band showed MM-LDL–exposed cells to have TF mRNA levels approximately 30-fold higher than normal LDL-treated cells. A somewhat larger minor band is also present that has been observed by other investigators as well.[22–24] A small amount of TF mRNA is detectable in control and normal LDL-treated cells when autoradiographs are exposed for 5 days as shown here. Hybridization for the internal control (CHO-B) showed bands of equivalent intensity (not shown), indicating that comparable amounts of RNA had been loaded and that the increase in TF mRNA was specific. Comparable results were obtained from additional experiments with MM-LDL preparation 2. Both actinomycin D (5 μg/ml) and cyclohexamide (10 μg/ml) blocked induction of TF coagulant activity (Table 3). These findings indicate that the induction of TF activity by MM-LDL was primarily dependent on increases in cellular TF mRNA levels, with resulting increased TF protein synthesis, similar to the reported effects of tumor necrosis factor.[22–24]

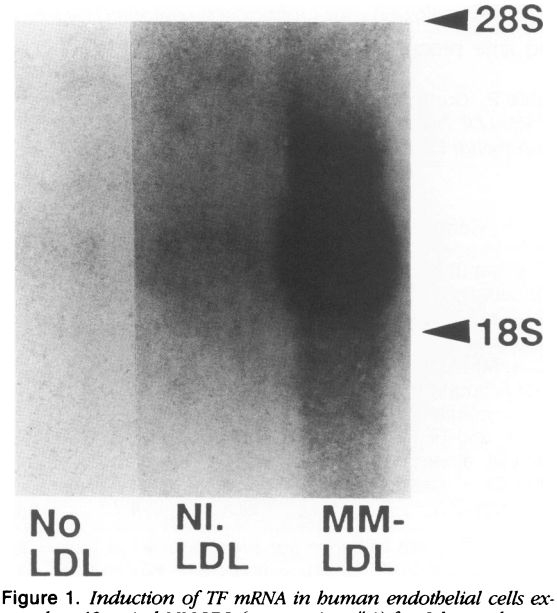

Figure 1. *Induction of TF mRNA in human endothelial cells exposed to 40 μg/ml MM-LDL (preparation #1) for 2 hours, but no induction with 40 μg/ml normal (N1) LDL, demonstrated by Northern blot analysis; ("No LDL" lane represents cells incubated with medium but without added LDL).*

Induction of TF coagulant activity was time and dose dependent. Maximal induction was reached by 4 hours, with levels declining after 8 hours, reaching less than 20% of peak activity by 24 hours (Figure 2). In contrast, endothelial cell (EC) monolayers incubated with normal LDL (40 μg/ml) for up to 48 hours showed no induction of TF activity (TF levels less than 10 pg/10^5 cells at 0, 6, 24, and 48 hours). In dose-response experiments with three preparations, the minimal effective concentration ranged from 0.625 μg/ml to 10 μg/ml (Figure 3). Low concentrations of MM-LDL (0.2, 1, and 5 μg/ml) were tested in combination with comparably active concentrations of recombinant interleukin-1 (0.2, 1, and 5 U/ml), and additive effects observed (Figure 4). Similar experiments of MM-LDL combined with concentrations of LPS that alone did not induce TF expression (less than 100 pg/ml) showed no enhancement of MM-LDL–induced TF coagulant activity.

Evaluated in addition were one preparation each of malondialdehyde-modified LDL (MDA-LDL) and highly oxidized LDL. Neither preparation at 20 or 40 μg/ml induced TF coagulant activity in endothelial cells after 6 or 24 hours' exposure (TF levels less than 2 pg/10^5 cells). Competition studies were also performed in which cells were exposed to 20 μg/ml MM-LDL in the presence of 200 μg/ml of normal LDL or MDA-LDL. Tissue factor coagulant activity in MM-LDL–treated cells after 6 hours was no different when 10 times greater concentrations of normal or MDA-LDL were present (Table 4), suggesting that the biologic activity of MM-LDL was not dependent on its binding to or uptake by the LDL or scavenger receptors.

Neither normal LDL, MM-LDL, MDA-LDL, nor the

Table 3. *Inhibition of MM-LDL–Induced TF Coagulant Activity in Cultured Endothelial Cells by Actinomycin D and Cyclohexamide*

Condition	TF activity (pg/10^5 cells) Exp. 1	Exp. 2
EC alone (6 hours incubation)	≤2.5	≤5.2
EC + nl LDL (40 μg/ml for 6 hours)	≤2.5	ND
EC + MM-LDL (40 μg/ml for 6 hours)	226	130
+ actinomycin D*	≤2.5	≤5.2
+ cyclohexamide*	≤2.5	ND

* MM-LDL used was preparation #1 in Table 1. Endothelial cell monolayers were incubated with 10 μg/ml of cyclohexamide or 5 μg/ml of actinomycin D in medium 199 plus 10% FBS for 30 minutes before addition of agonist. Cell viability assessed by trypan blue dye exclusion was more than 90% for all assay conditions.

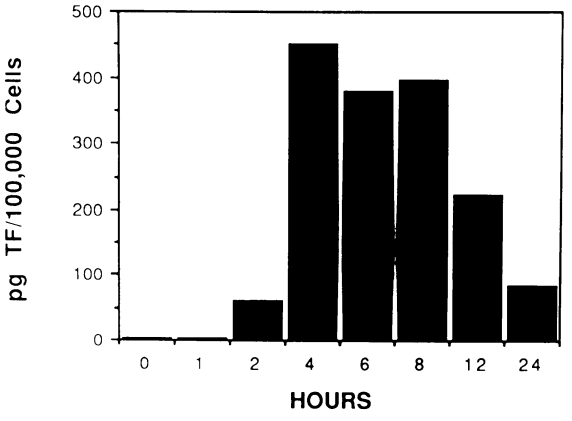

Figure 2. *Time course of TF coagulant activity induced in human endothelial cells exposed to 40 μg/ml MM-LDL. Results are from one of three representative experiments with preparation 1 in Table 1. Endothelial cells incubated concurrently with medium alone or with 40 μg/ml normal LDL for 6 hours showed no TF induction (levels < 10 pg/10^5 cells).*

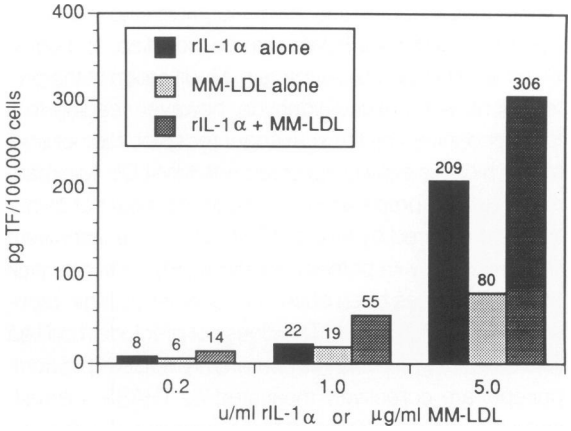

Figure 4. *Synergy study of MM-LDL (preparation 1) with rIL-1α on induction of TF coagulant activity in cultured human endothelial cells. Cells were incubated with each agonist alone or in combination at sub-maximal concentrations as indicated for 6 hours. Concurrently run controls showed no induction of TF.*

highly oxidized LDL preparations at the concentrations used affected endothelial cell morphology, attachment, or viability, which is consistent with other investigations of these preparations for human endothelial cells.[7,8]

Discussion

This study has identified minimally oxidized LDL as a potent stimulus for tissue factor expression in cultured human endothelial cells. Minimally modified LDL induced TF mRNA and protein synthesis in a manner similar to that observed for interleukin-1, tumor necrosis factor, and endotoxin.[13,22] The time course of tissue factor expression in response to MM-LDL is comparable to that observed for MM-LDL induction of colony-stimulating factors, but differs from that of increased monocyte adhesion, which, although peaking at 4 hours, was sustained for up to 48 hours.[7,8]

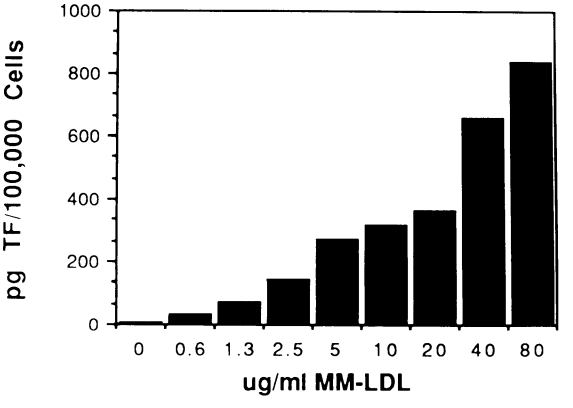

Figure 3. *Dose–response of TF coagulant activity induced in human endothelial cells exposed to varying concentrations of MM-LDL for 6 hours. Results are from one of two representative experiments with preparation 2 in Table 1. Endothelial cells incubated concurrently with medium alone or with 20, 40, and 80 μg/ml normal LDL for 6 hours showed no TF induction (levels < 10 pg/10^5 cells).*

The preparations of minimally oxidized LDL examined contained levels of thiobarbituric acid reactive substances, hydroperoxide, and cholesterol epoxide that were 2.5- to 4-, 1.5-, and 4-fold greater, respectively, than are present in freshly isolated LDL.[8] In contrast with most 'oxidized LDL' preparations reported in the literature, however, the preparations used here were still recognized by the LDL receptor, indicating minimal if any modification of the apoprotein (hence the use of the term 'minimally modified'). Binding of MM-LDL to the LDL receptor did not appear to be essential for its biologic activity, as competition studies with 10-fold excess normal LDL showed no reduction in activity. Similar findings have been observed for the effects of oxidized LDL on toxicity and monocyte adhesion.[25,26] The specific components

Table 4. *Competition Experiments Between MM-LDL and Normal and MDA-LDL*

Condition*	TF activity (pg/10^5 cells) Exp. 1	Exp. 2
No LDL	<2	<2
MM-LDL (20 μg/ml)	80	26
MM-LDL (20 μg/ml) + nl. LDL (200 μg/ml)	76	25
MM-LDL (20 μg/ml) + MDA-LDL (200 μg/ml)	65	26
Normal LDL (200 μg/ml)	2	ND
MDA-LDL (200 μg/ml)	<2	ND

* Cells were incubated for 6 hours in M199 plus 10% FBS containing the LDL preparations indicated. The MM-LDL used was a stored preparation not listed in Table 1, having 2.5 nmoles TBARS per mg cholesterol.
ND, not detected.

of MM-LDL that are responsible for triggering the endothelial cell responses have yet to be identified, and could be either modified lipid or protein. Modification of the protein alone with malondialdehyde, however, leading to a form recognized by the scavenger receptor, did not confer the biologic activity observed with MM-LDL. Minimally modified LDL preparations varied in the extent of oxidation as measured by levels of TBARS, but the latter were not correlated with potency in inducing TF; a similar lack of correlation has been observed for other biologic properties of modified LDL.[10] This does not preclude modified lipid as a likely mediator, as numerous oxidized lipid components are collectively measured as TBARS. Interestingly, the one preparation of highly oxidized LDL (having the highest level of TBARS of all LDL preparations tested) had no TF-inducing activity. Endotoxin was excluded as a spurious cause of these results by directly determining that the maximum levels that could have been present (less than 2.5 pg/μg LDL) had no direct or synergistic effect on endothelial cell TF expression, and by demonstrating that low concentrations of LPS (less than 100 pg/ml) did not enhance MM-LDL–induced TF expression. Normal LDL preparations were comparable to MM-LDL preparations in having very low to undetectable levels of endotoxin, and none of these induced TF expression, even when incubated with endothelial cells for up to 48 hours. In addition to providing further evidence that contaminating LPS was not inducing TF expression, these results show that endogenous modification of LDL by endothelial cells was not a factor under the conditions used.

Reports of the influence of native or modified lipoproteins on TF expression by vascular cells are limited, and none have examined endothelial or smooth muscle cells. Among circulating cells, only the monocyte is capable of expressing TF, and only after induction by specific agonists. In one report, chemically modified LDL (acetyl- and malondialdehyde-LDL) but not normal LDL was found to induce TF in human monocyte/macrophages.[27] Earlier studies by Edgington and coworkers[28,29] of normal plasma lipoprotein fractions identified very-low-density lipoprotein as a stimulus for TF expression by peripheral blood mononuclear cells, an effect dependent on T cells and suppressed by LDL. Preliminary studies in our laboratory suggest that MM-LDL also induces TF expression in isolated human monocytes.

There is strong evidence that modified LDL is present *in vivo* in atherosclerotic lesions and is an important mediator of atherogenesis.[30–32] The development of atherosclerotic lesions in genetically hyperlipidemic rabbits can be substantially reduced by treatment with potent antioxidants such as probucol, which may act by inhibiting formation of modified LDL.[33] These observations suggest that one or more of the biologic properties demonstrated for modified LDL occur *in vivo* and have an important role in atherogenesis. Although it has yet to be demonstrated that TF is expressed by endothelial cells *in vivo*, current methods may not be sufficiently sensitive to detect low but functionally significant levels of expression.[34–36] Activation of the coagulation protease cascade by endothelium, thus generating thrombin, would be expected to have important consequences for lesion development in atherosclerosis. While cleavage of fibrinogen with thrombus formation is one major consequence of thrombin generation, thrombin has additional potent biologic effects, which include platelet activation with release of growth factors, stimulation of mitogenesis and monocyte chemotaxis, and induction of endothelial-dependent relaxing factor release from endothelium.[37] These findings should stimulate further investigation of TF expression *in vivo* in atherosclerosis, and of the role of the coagulation protease cascade in atherogenesis.

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