American Journal of Pathology, Vol. 132, No. 3, September 1988

Perturbation of Cultured Human Endothelial Cells by Atherogenic Levels of Low Density Lipoprotein

JAMES A. HOLLAND, MD,
KIRKWOOD A. PRITCHARD, PhD,
NANCY J. ROGERS,
and MICHAEL B. STEMERMAN, MD

From the Department of Medicine, New York Medical College, Valhalla, New York

Cultured human umbilical vein endothelial cells (EC) exposed to atherogenic levels of low density lipoprotein (LDL) for protracted periods demonstrated no measurable evidence of overt cytotoxicity, but were perturbed as indicated by an increase in prostacyclin (PGI2) production. Confluent EC were incubated with high LDL concentrations (240 or 330 mg/dl cholesterol) for 1 to 12 days. LDL was added to culture media containing 25% human lipoprotein-deficient serum to determine the effects of LDL independent of other lipoproteins. LDL did not injure EC as assessed by cell count, vital dye exclusion, ^{51}chromium release, and lactate dehydrogenase release. Although high concentrations of LDL did not cause EC cytotoxicity, such LDL concentrations did result in increased PGI2 generation. PGI2 accumulation in postincubation media was increased two-to-fivefold in otherwise unstimulated cells as measured by radioimmunoassay of the stable PGI2 breakdown product, 6-keto-PGF1-alpha. This elevation persisted for the entire 12-day exposure to high LDL concentrations. These results indicate that prolonged exposure to atherogenic concentrations of LDL does not effect EC viability, but does cause an endothelial perturbation as demonstrated by an increased PGI2 production. (Am J Pathol 1988, 132:474–478)

HIGH BLOOD concentrations of low density lipoprotein (LDL) are a major risk factor predicting development of atherosclerosis.[1] Likewise, attention has been focused on the endothelium's role in the pathobiology of atherogenesis.[2] *In vitro* studies examining the effect of high LDL concentrations on endothelial cell (EC) function have been hampered due to an apparent toxicity of the lipoprotein on the cells.[3,4] Morel[3] and van Hinsbergh[5] have demonstrated that LDL-induced toxicity results from an oxidized LDL product that forms during LDL preparation and culture with human EC. As a result of *in vitro* oxidized LDL formation, examination of LDL-induced changes in EC metabolism have been limited to studies that maximally used LDL levels less than 60 mg/dl cholesterol for incubation periods less than 48 hours. These levels are much lower than those associated with the development of atherosclerosis. This report describes the culture of endothelial cells in high concentrations of LDL for protracted periods. Such cultures use LDL prepared with minimal oxidation and are not associated with measurable EC cytotoxicity. Cells were incubated in LDL concentrations up to five times that previously reported, which corresponds to LDL levels associated epidemiologically with the premature development of atherosclerosis. Constant exposure to these LDL concentrations causes an early and persistent change in EC eicosanoid metabolism.

Materials and Methods

Endothelial Cell Cultures

EC were isolated from human umbilical veins by a modified method of Jaffe et al.[6] Briefly, the vein lumen was perfused with Hank's balanced salt solution containing antibiotic/antimycotic (penicillin, streptomycin, and fungizone), and incubated at 4 C. After 1 hour, the vein was drained, filled with collagenase, and incubated at 37 C for 15 minutes. The collagenase solution with cells was flushed from the vessel, then seeded in 25 sq cm flasks (Corning, Corning, NY) on human fibronectin coated surfaces (10 μg/cc).[7] Fibro-

Supported by NIH grant #HL33742 and HL02026.
Accepted for publication April 13, 1988.
Address reprint requests to James A. Holland, MD, New York Medical College, Vosburgh 302, Valhalla, NY 10595.

nectin was prepared by the method of Ruoslahti.[8] Fresh culture media was added, which consisted of Medium 199 supplemented with 25% fresh pooled human serum (HS), antibiotic/antimycotic, L-glutamine, HEPES buffer (21 mM), heparin (90 μg/ml), and endothelial cell growth factor (ECGF) (300 μg/ml), and cells incubated in an atmosphere containing 5% CO_2. ECGF was prepared by the method of Maciag et al.[9] The cultured cells were identified as EC by immunofluorescent staining for von Willebrand's factor.[10] Culture media was changed every 48 hours. Cells were maintained at confluence, as determined by morphologic appearance, for approximately 5 days before experimental use.

Lipoprotein Preparation

LDL was prepared under sterile conditions by the diafiltration/ultracentrifugation methods recently developed in the authors' laboratory.[11] Briefly, 1800 to 2000 ml of fresh plasma was obtained from healthy adults and within 1.5 hours, 20 μM butylated hydroxytoluene (BHT) and 0.01% EDTA were added and sealed under argon. Diafiltration was performed using a Pellicon Ultrafiltration System (Millipore, Bedford, MA) with three membranes (SK1P188A6, Millipore) using operational parameters: 1) cross flow 500 ml/min, 2) filtration rate 8 ml/min, 3) inlet pressure 4 psi, 4) outlet pressure 1 psi, 5) filtration pressure 1 psi, and 6) operating temperature 4 C. Wash solution contained 0.15 M NaCl with 20 μM BHT and 0.01% EDTA, pH 7.4. Total plasma protein and fluid volume was reduced by greater than 75% while it retained greater than 90% of the total LDL-cholesterol content.[11] LDL (1.019 to 1.063 g/ml) was then isolated by preparative ultracentrifugation in a Beckman L8 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) using a Beckman Ti-55.2 rotor at 50,000 rpm for 14 hours at 10 C.[12] The isolated lipoprotein was dialyzed against 4 volumes of Medium 199, pH 7.45, using a Minitan Ultrafiltration System (Millipore, Bedford, MA). LDL was characterized[11] by: 1) agarose gel electrophoresis, 2) 3 to 27% SDS-polyacrylamide gel electrophoresis, 3) protein content, 4) phospholipid content, 5) cholesterol content, 6) thiobarbituric acid reactive substances (TBARS) content, and 7) negative-staining electron microscopy.

Cytotoxicity Studies

A series of confluent 1st passage human EC plated on fibronectin-coated 35 mm wells (Costar, Cambridge, MA) were incubated in media containing 25% human lipoprotein deficient serum (LPDS) (d = 1.25) with LDL (240 or 330 mg/dl cholesterol) for 1 to 12 days. Control incubations were performed in parallel using 25% human serum in place of LPDS and LDL. During these studies, human serum for LDL and control media was prepared from the same source. All experiments were carried out in triplicate. Media was changed every 48 hours. Cytotoxicity studies were performed on days 4, 8, and 12 using cell count and vital dye exclusion and on day 8 by ^{51}chromium release[13] and lactate dehydrogenase release (LDH).[3] LDL oxidation during EC culture was determined by TBARS concentration in pre- and postculture media.[18]

Prostacyclin Production

Culture medium aliquots were collected at varying time intervals for measurement of PGI2 content. The PGI2 medium accumulation during successive 48-hour incubations with high LDL concentrations was measured in medium aliquots by radioimmunoassay (RIA) of 6-keto-PGF-1-alpha, a stable degradation product of PGI2, performed using a radioimmunoassay kit (Seragen Inc., Boston, MA). Briefly, this assay was performed by mixing 100 μl of sample or standard with 100 μl of [3H]6-keto-PGF-1-alpha and 100 μl of antiserum. After a 16-hour incubation at 4 C, 500 μl of dextran coated charcoal in buffer was added. The tube was centrifuged at 4 C and the supernatant was combined with Scint-A (Packard, Sterling, VA). The radioactivity in the supernatant was measured in a liquid scintillation spectrophotometer (Packard, Sterling, VA).

Statistical Analysis

All data were expressed as a mean ± SE and analyzed using Duncan's test.

Results

Cytotoxicity Assays

Results of cytotoxicity assays showed no injurious effect caused by high LDL concentrations up to 330 mg/dl when assessed by cell count, vital dye exclusion, ^{51}chromium release, and LDH release. Cell count and vital dye exclusion remained stable for EC incubated in high LDL concentrations (240 or 330 mg/dl cholesterol) throughout the 12 days. ^{51}Chromium and LDH release were not increased during exposure to high LDL concentrations (Figures 1A and B).

The presence of oxidized LDL was monitored by TBARS concentration in pre- and postculture me-

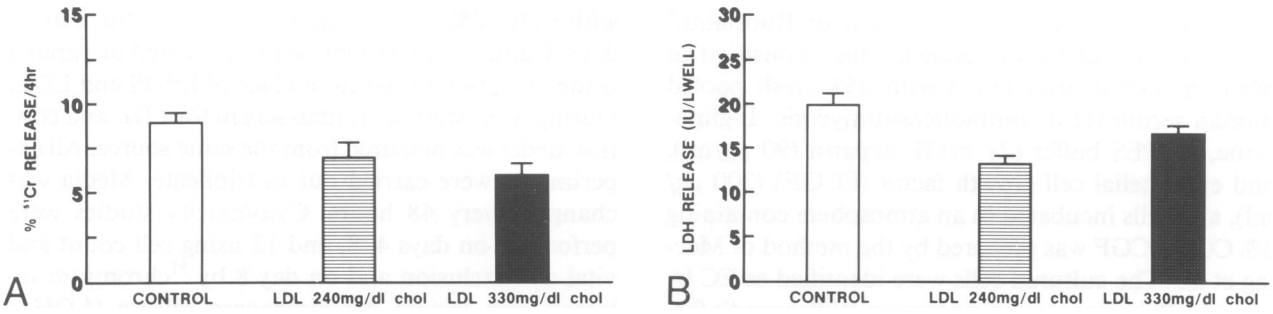

Figure 1A, B—The effect of high LDL concentrations on cell viability. Confluent human endothelial cells were incubated in 25% LPDS and LDL 240 or 330 mg/dl cholesterol with media changes every 48 hours. Control cultures were carried out in parallel and contained 25% human serum substituted for LPDS and LDL. Cell viability was determined by ^{51}Cr (**A**) and LDH release (**B**). Each value is the mean ± SE of results obtained from three cultures each from the same passage and incubated with the same lipoprotein preparation. Values for control *vs.* high LDL cultures were not statistically different.

dia.[3,4] TBARS concentrations[18] in postculture media from control cells was similar to that of cells incubated with high LDL concentrations (Table 1).

Prostacyclin Production

An increase in endothelial PGI2 production was observed when confluent human umbilical vein EC were incubated with high LDL concentrations. The time-dependent accumulation of PGI2 was observed during successive 48-hour LDL incubations (Figure 2A). Endothelial PGI2 production was not affected by LDL exposure during the initial 60-minute incubation. Maximum productivity for the control of the LDL-incubated EC occurred between 1 and 8 hours after each media change. During this interval, PGI2 production was consistently greater in the LDL-treated cells.

PGI2 accumulation following 4, 8, and 12 days' exposure to 330 mg/dl LDL cholesterol concentration was increased 225, 270, and 490%, respectively (Figure 2B), compared with controls. LDL-exposed EC persistently produced increased amounts of PGI2 throughout the 12 days in culture, whereas PGI2 generation by control EC diminished over the 12 day culture. EC produced on average, 30% more PGI2 at 330 mg/dl compared with 240 mg/dl LDL cholesterol.

Table 1—Average Concentration ± SEM of TBARS in Culture Medium After 48 hours of Incubation*

Incubation medium	TBARS (nmol MDA/ml medium)	TBARS (nmol MDA/mg LDL chol)
Control	0.29 ± 0.11	0.97 ± 0.37
High LDL	0.19 ± 0.5	0.06 ± 0.15

* LDL concentration was 330 mg/dl cholesterol. TBARS results for control and LDL media were not statistically different.

Discussion

This study demonstrates that exposure of EC to concentrations of LDL often associated with the premature development of atherosclerosis does not cause EC cytotoxicity. Exposure of EC to such levels of LDL does cause an early and persistent increase in PGI2 production. These findings indicate that LDL may promote atherogenesis by mechanisms other than direct EC injury or death.[19] The results also support studies by Morel[3] and van Hinsbergh[5] demonstrating that LDL-induced cytotoxicity results from toxic lipid peroxides formed by LDL oxidation and imply that injury is due to oxidized LDL. Oxidation of the lipoprotein can occur during LDL preparation and culture with human EC.[3,5] The preparative LDL procedure in the present study minimized LDL oxidation. Lipid peroxide formation in media containing high LDL concentrations is similar to that of controls, which have low LDL concentrations (approximately 30 mg/dl cholesterol).

The ability to perform protracted incubation of EC with high LDL concentrations probably is due to the lack of LDL oxidation. LDL was prepared by the diafiltration/ultracentrifugation method[11] developed with consideration of Morel's[3,4] and Steinbrecher's[20] observations. The addition of anti-oxidants such as BHT was used to limit LDL oxidation, thus diminishing EC cytotoxicity.[3,4] Peroxidative changes in LDL are promoted by the presence of certain divalent cations (ie, copper).[20] LDL oxidation is contained during isolation and culture by rapid processing plasma, adding anti-oxidants, and sealing under argon; rapid dialysis of divalent cations; and using the LDL within 1 week after preparation. With this approach, EC can be maintained in LDL concentrations in excess of five times those reported previously.[3-5,17] These data demonstrate that such culture conditions inhibit LDL oxi-

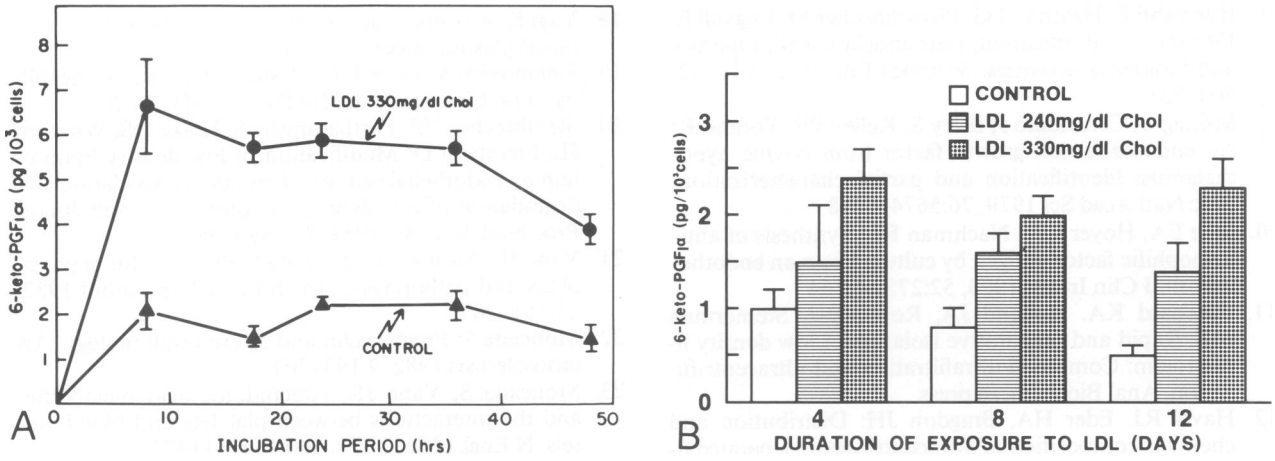

Figure 2A—Representative time-dependence of PGI2 production by confluent human umbilical vein endothelial cells during 96-hour incubations with high LDL concentrations. Cultures of endothelial cells were incubated with M-199 based medium containing 25% LPDS and LDL (330 mg/dl cholesterol) or 25% human serum with medium change every 48 hours. Aliquots of culture media were taken at varying time intervals for PGI2 determinations by radioimmunoassay of 6-keto-PGF1a. Each point represents the mean ± SE of results obtained from three cultures. Statistical differences were obtained from 8 to 48 hours ($P < 0.03$). **B**—The effect of high LDL concentrations on PGI2 production by cultured human endothelial cells. PGI2 accumulation in 48-hour postculture media was determined by radioimmunoassay of 6-keto-PGF1a following 4, 8, and 12-day incubations. The data represents the mean ± SE of results obtained from three experiments. PGI2 accumulation by LDL-exposed cells (330 mg/dl cholesterol) was significantly greater than control cells at each time interval ($P < 0.01$). Studies have been performed measuring PGI2 accumulation in 48 hour postculture media containing 60 and 160 mg/dl LDL cholesterol. These results were 1.19 ± 0.18 and 1.28 ± 0.06 pg of 6-keto-PGF1a/10^3 cells, respectively.

dation, which is probably a consequence of excessive free radical generation caused by EC injury.

Eicosanoid generation is perturbed in EC exposed to high LDL concentrations for long periods. PGI2 production is altered without otherwise stimulating these cells. The endothelium is a major source of PGI2, an eicosanoid product that inhibits platelet aggregation[21] and promotes arterial vasodilation.[22] PGI2 is thought to protect against coronary artery occlusion secondary to thrombosis and/or vasospasm.[23] Cultured endothelial cells can be stimulated to produce PGI2 by agents such as oxidized LDL, thrombin, calcium ionophore A23187, and arachidonic acid.[24–26] In contrast to oxidized LDL, LDL does not induce rapid PGI2 production. Oxidized LDL causes an immediate release of PGI2 that plateaus within 30 minutes.[24] High LDL concentrations cause a more gradual increase in PGI2 production that remains constant after 8 hours. Spector et al demonstrated that PGI2 release progressively increases in bovine aortic and human umbilical vein EC during 18-hour incubations with low LDL concentrations (60 mg/dl cholesterol).[17] The minimal increases in PGI2 production led to the suggestion that LDL does not play an important role in EC prostaglandin formation. In contrast, human ECs exposed to high LDL concentrations (240 or 330 mg/dl cholesterol) continuously produce excessive amounts PGI2. This alteration in eicosanoid generation may denote a modulation of EC metabolism which could influence atherogenesis. How such cells respond to vasoactive stimuli may provide insights regarding the mechanism(s) of this process.

In conclusion, human vascular EC can be exposed to high LDL concentrations for protracted periods. Incubation with these atherogenic LDL levels does not affect EC viability, but does result in an endothelial cell perturbation.

References

1. Arteriosclerosis. Report by National Heart, Lung, and Blood Institute Task Force on Arteriosclerosis. DHEW Publication No. 72-219, 13, 1972
2. Stemerman MB, Colton CK, Morell EM. Perturbation of the endothelium, Progress in Hemostasis and Thrombosis, Vol. 1. Edited by TH Spaet. New York, Grune and Stratton, 1984, pp 289–324
3. Morel DW, Dicorleto PE, Chisolm GM: Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. Arteriosclerosis 1984, 4:357–364
4. Morel DW, Hessler JR, Chisolm GM: Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. J Lipid Res 1983, 24:1070–1076
5. van Hinsbergh VWM, Scheffer M, Havekes L, Kempen HJM: Role of endothelial cells and their products in the modification of low-density lipoproteins. Biochim Biophys Acts 1986, 878:49–64
6. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins: Identification by morphologic and immunologic criteria. J Clin Invest 1973, 52:2745–2756
7. Maciag T, Hoover GA, Stemerman MB, Weinstein R: Serial propagation of human endothelial cells in vitro. J Cell Biol 1981, 91:420–426

8. Ruoslahti E, Hayman EG, Pierschbacher M, Engvall E: Fibronectin: Purification, immunochemical properties and biological activities. Methods Enzymol 1982, 82: 803–831
9. Maciag T, Cerundolo J, Ilsley S, Kelley PR, Forand R: An endothelial cell growth factor from bovine hypothalamus: Identification and partial characterization. Proc Natl Acad Sci 1979, 76:5674–5678
10. Jaffe EA, Hoyer LW, Nachman RL: Synthesis of antihemophilic factor antigen by cultured human endothelial cell. J Clin Invest 1973, 52:2757–2764
11. Pritchard KA, Holland JA, Rogers NJ, Stemerman MB: Rapid and preparative isolation of low density lipoprotein: Combined ultrafiltration and ultracentrifugation. Anal Biochem, in press
12. Havel RJ, Eder HA, Bragdon JH: Distribution and chemical composition of ultracentrifugally separated lipoproteins and chylomicrons. J Clin Invest 1955, 34: 1345–1353
13. Fellit HM, Jaffe EA, Sabriskie JB: In vitro correlates of endothelial injury and repair. Lab Invest 1982, 46:1–9
14. Muller KR, Slammer R: Studies on the toxicity of LDL. Abstracts of the Sixth International Symposium on Atherosclerosis. Berlin, International Atherosclerosis Society, 1982, p 533
15. Gimbrone MA, Cotran RS, Folkman J: Human vascular endothelial cells in culture: Growth and DNA synthesis. J Cell Biol 1974, 60:673–684
16. Davies PF, Selden SC III, Schwartz SM: Enhanced rates of fluid pinocytosis during exponential growth and monolayer regeneration by cultured arterial endothelial cells. J Cell Phys 1980, 102:119–127
17. Spector AA, Scanu AM, Kaduce TL, Figard PH, Fless GM, Czervionke RL: Effect of human plasma lipoproteins on prostacyclin production by cultured endothelial cells. J Lipid Res 1985, 26:288–297
18. Yagi K: A simple fluorometric assay for lipoperoxide in blood plasma. Biochem Med 1976, 15:212–216
19. Stefanovich V, Gore I: Cholesterol diet and permeability of rabbit aorta. Exp Mol Pathol 1971, 14:20
20. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D: Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc Natl Acad Sci 1984, 81:3883–3887
21. Vane JR, Bunting S, Moncada S: Prostacyclin in physiology and pathophysiology. Int Rev Exp Pathol 1982, 23:161–207
22. Moncada S: Prostacyclin and arterial wall biology. Arteriosclerosis 1982, 2:193–207
23. Moncada S, Vane JR: Arachidonic acid metabolites and the interactions between platelets and blood vessels. N Engl J Med 1979, 300:1142–1147
24. Triau JE, Meydani SN, Meydani M, Libby P, Schaefer EJ: Oxidized low density lipoproteins stimulate prostacyclin (PGI2) production by adult human vascular endothelial cells. Fed Proc 1986, 45:347
25. Weksler BB, Ley CW, Jaffe EA: Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and the ionophore A23187. J Clin Invest 1978, 62: 923–930
26. Marcus AJ, Weksler BB, Jaffe EA: Enzymatic conversion of prostaglandin endoperoxide H2 and arachidonic acid to prostacyclin by cultured human endothelial cells. J Biol Chem 1978, 253:7138–7141

Acknowledgments

The authors thank the Hudson Valley Blood Service for their support and Sue Murphy for assisting in preparation of this manuscript.