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The Effect of Concanavalin A-Stimulated Mononuclear Cells on Low Density Lipoprotein Receptor Activity of Cultured Fibroblasts (42136)

K. HIRAMATSU, A. CHAIT, AND E. L. BIERMAN

Division of Metabolism, Endocrinology, and Nutrition, Department of Medicine, RG-26, University of Washington, Seattle, Washington 98195

Abstract. Secretory products of freshly isolated human circulating blood cells such as platelets, monocytes, and B lymphocytes, but not T lymphocytes, have previously been shown to enhance low density lipoprotein (LDL) metabolism by arterial wall cells. This study was undertaken to evaluate how secretory factor(s) from mononuclear cells that had been stimulated by concanavalin A (Con A) alters LDL receptor activity by cultured human skin fibroblasts. Conditioned medium from Con A-stimulated mononuclear cells produced an increase of ^{125}I-LDL degradation accompanied by increased thymidine incorporation into DNA. The effect of conditioned medium from the Con A-stimulated mononuclear cells was mediated by the LDL receptor pathway. Degradation of HDL and methylated LDL, neither of which is taken up by the classical LDL receptor pathway, was not affected. The conditioned medium from these Con A-stimulated cells also failed to stimulate fluid pinocytosis, as measured by the uptake of [^{14}C]sucrose. Some strains of fibroblasts, deficient in LDL receptors, responded to the conditioned medium from the Con A-stimulated mononuclear cells by increasing the very small amounts of LDL degraded by these cells. Fibroblasts from other homozygous familial hypercholesterolemic cell strains were unresponsive, however. The effect on LDL receptors was characterized by an increase in LDL receptor number without a change in the affinity of LDL for its receptor. Thus stimulated mononuclear cells secrete mitogens that also stimulate LDL receptor activity in human skin fibroblasts.

Low density lipoprotein (LDL), the major carrier of cholesterol in plasma, is catabolized by cells after binding to LDL cell surface receptors (1). The cholesterol provided to the cell by this pathway is used for membrane components and cell division. When cell proliferation occurs, or when cells are exposed to mitogens, LDL receptor activity increases corresponding to the increased demand for cholesterol that is used for cell components (2–4). Several human circulating blood cells can secrete mitogens and therefore may play an important role in modulating cell proliferation and LDL receptor activity (4–7). Unstimulated B lymphocytes and monocytes have the ability to enhance LDL receptor activity whereas T lymphocytes do not (7). Stimulated lymphocytes and monocytes have been reported to enhance fibroblast proliferation (8, 9), although their effect on LDL receptors is unknown. Therefore, to determine whether stimulated mononuclear cells affect LDL metabolism, we collected the conditioned medium from concanavalin A (Con A)-stimulated mononuclear cells, and examined its effect on LDL receptor activity in cultured human skin fibroblasts.

Materials and Methods. *Collection of conditioned medium from mononuclear cells, T- and B-lymphocyte-rich fractions, and monocytes.* Mononuclear cells were separated from heparinized blood by density gradient centrifugation by the method of Böyum (10). Relatively pure populations of lymphocytes and monocytes were prepared using an Elutriator system (Beckman Instruments, Fullerton, Calif.) essentially according to the method of Fogelman *et al.* (11) as described in detail elsewhere (12). Lymphocytes so obtained were further separated into T- and B-rich lymphocyte fractions according to the method reported by the International Union of Immunological Societies (13). In brief, a T-lymphocyte-rich population was collected after adhesion of B lymphocytes to a nylon reticulum and a B-lymphocyte-rich population was collected by Ficoll–Hypaque density gradient centrifugation after formation of rosettes with T lymphocytes and sheep red blood cells. Ninety percent of the cells in the

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T-lymphocyte-rich population formed sheep red blood cell rosettes while 65% of the cells in the B-lymphocyte-rich population had immunoglobulin on their surfaces. Purity of the monocyte preparation was greater than 90% (12).

Mixed mononuclear cells, T and B lymphocytes, and monocytes were plated into 35-mm-diameter tissue culture dishes at a concentration of 10^6 cells/ml RPMI 1640 medium containing 10% lipoprotein-deficient serum ($d > 1.25$ g/ml) and were cultured for 48 hr in the presence of 25 μg concanavalin A in a humidified atmosphere of 95% air, 5% CO_2. At the end of the 48-hr incubation, the conditioned medium was collected and transferred to dishes containing human skin fibroblasts. Twenty-five micrograms of Con A was found to be the optimal concentration for stimulation, when LDL degradation by fibroblasts was examined after mononuclear cells had been incubated with various concentrations of Con A. The same concentration has been reported to produce maximal [^{3}H]thymidine incorporation into DNA by 3T3 cells (9).

Cultures of human skin fibroblasts. Human skin fibroblasts were grown from explants of skin biopsies from normal volunteers as previously described (4), and four homozygous familial hypercholesterolemic fibroblast strains were purchased from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository. The cells were grown and subcultured in modified Dulbecco's modified Eagle's medium containing 10% fetal calf serum, as previously described (4). Fibroblasts from the 4th to 10th subculture were trypsinized and 5×10^4 cells were plated in 35 mm dishes in 1.5 ml 10% fetal bovine serum. The cells were fed twice weekly with 10% fetal bovine serum. One day before the addition of the conditioned medium, the medium of the fibroblasts was changed to RPMI 1640 containing 10% human lipoprotein-deficient serum. Ten percent mononuclear cell conditioned medium (v/v), collected as described above, was then added to the fibroblast cultures for a further 48-hr incubation. At that time, ^{125}I-labeled LDL was added to the cells for measurement of its binding and degradation.

Measurement of LDL binding and degradation in fibroblasts cultured with conditioned medium. Human LDL (d = 1.019–1.063 g/ml) was prepared by sequential ultracentrifugation from fresh plasma obtained from normal donors. Iodination with ^{125}I was performed using the iodine monochloride method as modified for lipoproteins (14) as previously described (15) to yield specific activities of approximately 100–300 cpm/ng. The ^{125}I-labeled LDL was used within 2 weeks of preparation and was sterilized by passage through a Millipore filter before use. LDL binding was measured according to the method of Goldstein *et al.* (16). Cells were prechilled to 4°C. The medium was removed, the cells were washed, and the medium was replaced by serum-free medium containing Hepes buffer (10 mM) and ^{125}I-labeled LDL (7.5 μg/ml). After incubation at 4°C for 2 hr, the cell layer was washed extensively. Incubation with dextran sulfate (4 mg/ml for 1 hr at 4°C) was used to release the bound ^{125}I-LDL from cell surface receptors. LDL degradation was measured as previously described (4, 14). ^{125}I-labeled LDL was added to dishes containing fibroblasts to give a final concentration of 7.5 μg/ml. Incubations were performed for 24 hr at 37°C. Non cell-associated lipoprotein degradation was measured under identical conditions in cell-free dishes and was subtracted from total LDL degradation to give a measure of cellular LDL degradation. Fibroblast monolayers were dissolved in 0.1 N NaOH for determination of their protein content (17). LDL binding and degradation were expressed per milligram cell protein.

Other methods. [^{3}H]Thymidine incorporation into DNA was measured 24 hr after the addition of conditioned medium by the method of Raines and Ross (18). [^{3}H]Thymidine (2.5 μCi/ml, New England Nuclear, sp act 6–7 Ci/mmole) was added to cells for 2-hr incubation at 37°C. After aspirating off the media, the cells were washed twice with 1 ml of ice-cold 5% TCA. TCA-insoluble material was solubilized in 0.25 N NaOH (0.8 ml) with mixing. Of this solution 0.6 ml was counted in 5 ml Aquasol in a liquid scintillation counter. (U-^{14}C)Sucrose (New England Nuclear, sp act 396 Ci/mole) was

added to cells at 10 μCi/ml for 24 hr for measurement of fluid phase pinocytosis (19). High density lipoprotein$_3$ (HDL_3; density 1.125–1.21 g/ml) was prepared by sequential preparative ultracentrifugation (20). LDL was chemically methylated by the method of Weisgraber *et al.* (21). Iodination of HDL_3 and methylated LDL were performed as for LDL.

Results. *Effect of conditioned media on LDL degradation and on thymidine incorporation into DNA.* LDL degradation by fibroblasts was compared using conditioned media from either unstimulated or Con A-stimulated T lymphocytes and mononuclear cells. Both T lymphocyte and mixed mononuclear cell-conditioned media failed to enhance LDL degradation when unstimulated. However, Con A stimulation of T lymphocytes or mixed mononuclear cells for 48 hr increased LDL degradation by fibroblasts by 31–38% (Table I). Five different combinations of cells that had been stimulated by Con A were used to collect conditioned media. All the conditioned media stimulated both LDL degradation and [^{3}H]thymidine incorporation to a greater extent than did control medium that had not been exposed to mononuclear cells (Table III; $P < 0.02$). To evaluate the effect of Con A itself on LDL degradation and DNA synthesis by fibroblasts, 2.5 μg/ml Con A (i.e., the same final concentration of Con A to which fibroblasts exposed to conditioned media were subjected) was added directly to fibroblast cultures. No significant difference in DNA synthesis and LDL degradation was observed between 2.5 μg/ml Con A and control (data not shown). In other experiments, methyl-α-D-mannoside, which binds Con A, was added at the end of the mononuclear cell incubation with Con A. The conditioned media which contained methyl-α-D-mannoside led to the same stimulation of LDL degradation as that without methyl-α-D-mannoside.

A positive relationship was observed be-

TABLE I. EFFECT OF CONDITIONED MEDIA FROM UNSTIMULATED AND CONCANAVALIN A (CON A)-STIMULATED CELLS ON LDL DEGRADATION BY CULTURED SKIN FIBROBLASTS

Experiment	Conditioned medium source	^{125}I-LDL degradation (μg/mg protein 24 hr)			
		Unstimulated	Con A stimulated	% Stimulation	% Increase from Control
1	Control	1.84 ± 0.17			
	T lymphocyte	1.96 ± 0.19	2.20 ± 0.08	+12	+20
	Mononuclear cell	1.88 ± 0.02	2.47 ± 0.21*	+31	+34
2	Control	4.31 ± 0.07			
	T lymphocyte	4.33 ± 0.09	5.36 ± 0.14**	+24	+24
	Mononuclear cell	4.99 ± 0.52	4.81 ± 0.21	−4	+12
3	Control	1.63 ± 0.22			
	T lymphocyte		2.42 ± 0.02	—	+48
	Mononuclear cell		2.76 ± 0.25	—	+69
	Mean % change (control) vs. stimulated)				
	T lymphocyte				+31
	Mononuclear cell				+38

Note. Blood cells were plated into 35-mm-diameter tissue culture dishes at a concentration of 10^6 cells/ml RPMI 1640 medium containing 10% lipoprotein-deficient serum and were cultured for 48 hr in the absence (unstimulated) or presence (stimulated) of 25 μg/ml Concanavalin A. Control medium was not exposed to T lymphocytes or mononuclear cells. Fibroblasts were incubated for 24 hr with various conditioned media (10%) before measurement of ^{125}I-LDL degradation over the following 24 hr; all values are means ± SD from triplicate samples.

* $P < 0.01$; unstimulated versus Con A-stimulated mononuclear cell.

** $P < 0.001$; unstimulated versus Con A-stimulated T lymphocyte.

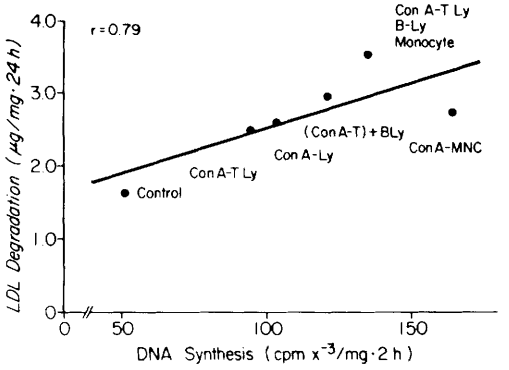

FIG. 1. Relationship between LDL receptor activity and DNA synthesis in the presence of conditioned media from various cell sources. The procedures for the collection of conditioned media from the cell sources indicated were as described in Table I: (B-Ly = B lymphocyte; T-Ly = T-lymphocyte; MNC = mixed mononuclear cells; Con A = concanavalin A stimulated). For mixtures of conditioned media, 1 ml of conditioned medium from each blood cell source was combined and added to fibroblasts at a final concentration of 10%. Fibroblasts were incubated in the presence of the various conditioned media for 24 hr before the measurement of [^{3}H]thymidine incorporation over the ensuing 2-hr, and for 48 hr before the measurement of ^{125}I-LDL degradation during the following 24 hr.

tween DNA synthesis and LDL degradation ($r = 0.79$, $n = 6$) (Fig. 1). Of the conditioned media, Con A-stimulated mononuclear cell conditioned medium showed the greatest stimulation of thymidine incorporation (Table II). Thus, to test the mechanism by which LDL degradation was stimulated, conditioned medium from Con A-stimulated mononuclear cells was used in the following experiments.

Response of LDL receptor activity to Con A-stimulated mononuclear cell conditioned medium. To examine whether Con A-stimulated mononuclear cell conditioned medium stimulated LDL receptor number or affinity, increasing concentrations of ^{125}I-LDL were added. At all concentrations of ^{125}I-LDL, ^{125}I-LDL binding (Fig. 2A) and degradation (Fig. 2B) was greater in the fibroblasts cultured with Con A-stimulated mononuclear cell conditioned medium. Linearization techniques (22) indicated that the major effect of Con A-stimulated mononuclear cell conditioned medium was to increase the "apparent B_{max}" from 24.5 to 39.1 ng/mg, while the "apparent K_m" remained unchanged (Fig. 2C). To test the specificity of these effects of the conditioned medium for the LDL receptor, other lipoproteins which do not bind to the LDL receptor were studied. HDL_3, which had essentially no Apo E (23), was tested, as was LDL that was methylated to prevent its binding to LDL receptors (21). Activated mononuclear cell conditioned medium stim-

TABLE II. EFFECT OF CONDITIONED MEDIA FROM CONCANAVALIN A-STIMULATED CELLS ON DNA SYNTHESIS AND LDL DEGRADATION BY CULTURED SKIN FIBROBLASTS

Conditioned medium source	[^{3}H]Thymidine incorporation (cpm/μg protein 2 hr)	^{125}I-LDL degradation (μg/mg protein 24 hr)
Control	50.6 ± 12.1	1.63 ± 0.22
T lymphocyte	95.5 ± 15.9**	2.42 ± 0.02†
Mixed B and T lymphocyte	104.1 ± 30.6*	2.62 ± 0.04†
T lymphocyte plus unstimulated B lymphocyte	124.5 ± 23.0***	2.85 ± 0.17†
T lymphocyte plus unstimulated B lymphocytes and monocytes	135.7 ± 14.5†	3.55 ± 0.29†
Mononuclear cell	164.7 ± 20.6†	2.76 ± 0.25†

Note. Procedures for collection of conditioned media were as described in Table I. For mixtures, 1 ml of conditioned medium from each blood cell source was combined and added to fibroblasts at a final concentration of 10%. Fibroblasts were incubated for 24 hr with various conditioned media before [^{3}H]thymidine incorporation over the ensuing 2 hr, and for 48 hr before ^{125}I-LDL degradation over the following 24 hr were determined; all values are means ± SD from triplicate samples.

* $P < 0.02$, ** $P < 0.01$, *** $P < 0.005$, † $P < 0.001$; all values were compared with control.

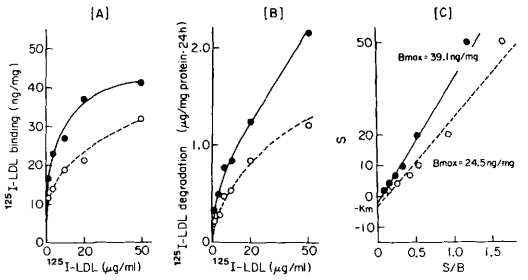

FIG. 2. Effect of Con A-stimulated mononuclear cell conditioned medium on LDL receptor activity. Fibroblasts were incubated without (○) or with (●) mononuclear conditioned medium for 48 hr. ^{125}I-LDL was then added at the concentrations indicated for determination of LDL binding for 2 hr (A) and degradation for 24 hr (B). (C) Linearization plot of the data in Fig. 2A. The slope of the line = "apparent B_{max}," and the point of intersection with the y axis = "apparent $-K_m$." Averages of duplicate values are shown.

ulated only LDL degradation without affecting either the degradation of HDL_3 or of methylated LDL (Table III). [^{14}C]Sucrose uptake, which provides a measure of fluid-phase pinocytosis, also was not altered by Con A-stimulated mononuclear cell conditioned medium (Table III).

Whether Con A-stimulated mononuclear cell conditioned medium has the ability to modulate LDL catabolism in the absence of the LDL receptor also was tested in familial hypercholesterolemic fibroblast strains (Table IV). The amount of LDL degradation by familial hypercholesterolemic fibroblasts was approximately 2–10% of that by fibroblasts from normal donors. As a result of 48-hr exposure to conditioned medium from Con A-stimulated mononuclear cells, a slight but significant increase in LDL degradation was observed in three receptor negative fibroblast strains (GM 488, GM 1915, GM 2000), while no detectable response was seen in the receptor negative strain (GM 486) with lowest LDL receptor activity.

Discussion. Conditioned medium obtained from unstimulated monocytes and B lymphocytes is able to enhance LDL degradation by fibroblasts while conditioned medium from unstimulated T lymphocytes does not have a similar effect (7). However, results in the present study indicate that T lymphocytes also can secrete a factor that promotes LDL catabolism after activation of these cells with Con A, since LDL degradation by fibroblasts was increased by Con A-stimulated lymphocyte conditioned medium.

It is possible that lymphocytes and monocytes interact, thereby modulating production of factors that increase LDL catabolism. Also, it is possible that factors produced by these different cells can interact with each other to influence LDL receptor activity. To test whether Con A-stimulated lymphocytes could modulate the secretion by other mononuclear cells of factors that stimulate LDL degradation by fibroblasts, conditioned medium was collected after B lymphocytes or monocytes were cocultured in the presence of Con A-stimulated lymphocytes and their effect on the LDL degradation was determined. The conditioned media of cocultured cells tended to decrease the LDL degradation compared to a mixture of conditioned media from the same cellular sources. To clarify the effect of Con A-stimulated T lymphocytes on the secretion by other mononuclear cells, con-

TABLE III. EFFECT OF CON A-STIMULATED MONONUCLEAR CELL CONDITIONED MEDIUM ON ^{125}I-LABELED LIPOPROTEIN DEGRADATION AND [^{14}C]SUCROSE UPTAKE BY NORMAL FIBROBLASTS

Addition	^{125}I-lipoprotein degradation (μg/mg protein)	
	Without conditioned medium	With conditioned medium
^{125}I-LDL	0.86 ± 0.03	1.69 ± 0.04
^{125}I-HDL_3	0.02 ± 0.001	0.02 ± 0.001
^{125}I-Methylated LDL	0.06 ± 0.001	0.06 ± 0.001
	Uptake (cpm/mg protein)	
[^{14}C]Sucrose	32057 ± 237	29400 ± 2963

Note. Fibroblasts were incubated for 48 hr without or with Con A-stimulated mononuclear cell conditioned medium at a final concentration of 10%. ^{125}I-LDL, ^{125}I-HDL_3, and ^{125}I-methylated LDL were added at 7.5 μg/ml for measurement of their degradation during the next 24 hr. [^{14}C]Sucrose was added at a concentration of 10 μCi/ml and uptake into cells was counted after 24 hr. All values are expressed as means ± SD of triplicate samples.

TABLE IV. CHANGES OF ^{125}I-LDL DEGRADATION BY NORMAL AND FOUR HOMOZYGOUS FAMILIAL HYPERCHOLESTEROLEMIC FIBROBLAST STRAINS AFTER INCUBATION WITH CON A-STIMULATED MONONUCLEAR CELL CONDITIONED MEDIUM FOR 48 hr

* Fibroblast strain	^{125}I-LDL degradation (μg/mg 24 hr)		
	(CM−)	(CM+)	% Change
Normal fibroblasts	0.625 ± 0.068	1.208 ± 0.029	+93
GM 488	0.063 ± 0.004	0.076 ± 0.011	+21
GM 1915	0.047 ± 0.007	0.107 ± 0.018	+128
GM 2000	0.018 ± 0.001	0.026 ± 0.003	+44
GM 486	0.012 ± 0.001	0.012 ± 0.001	0

Note. ^{125}I-LDL degradation by one normal and four homozygous familial hypercholesterolemic fibroblasts was determined as described in Table I, in the absence (CM−) or presence (CM+) of Con A-stimulated mononuclear cell-conditioned medium. All values are shown as means ± SD of triplicate samples.

* The numbers refer to the repository number of the cell culture obtained from the Human Genetic Mutant Cell Repository.

ditioned media were collected after various numbers of T lymphocytes were cocultured with other mononuclear cells during stimulation with Con A. However, no changes were observed in LDL degradation related to numbers of stimulated T lymphocytes added (data not shown). Therefore, our data do not provide evidence for the existence of cooperation or interaction among mononuclear cells for production and secretion of factors that stimulate LDL degradation by fibroblasts.

The promotion of LDL degradation appears to be mediated by the LDL receptor pathway, as observed with other mitogens. Among the conditioned media from different cell combinations of mononuclear cells, medium from Con A-stimulated mixed mononuclear cells showed the highest [^{3}H]thymidine incorporation. For all combinations, stimulation of LDL receptor activity was correlated with stimulation of DNA synthesis. These findings suggest that the stimulation of LDL receptor activity in fibroblasts was due to mitogens secreted by the mononuclear cells, since similar results have been reported to occur with other growth factors. One of these mitogens, platelet-derived growth factor (PDGF) has been shown to increase LDL degradation by fibroblasts and smooth muscle cells (3, 4); since our mixed mononuclear preparation was contaminated with some platelets, it is conceivable that some of the effects observed were due to contamination with PDGF. Of purified PDGF 1.5 ng has been estimated to be released by 20×10^7 platelets after complete aggregation (24). Thus the maximum amount of PDGF that could have contaminated the cell preparations in our sample was calculated to be less than 5 pg on the basis of 20 platelets/100 cells, a concentration well below that which can stimulate LDL receptor activity (Chait, Bierman, and Ross, unpublished). Therefore, it is highly unlikely that the effect of Con A-stimulated mononuclear cell conditioned media on LDL receptors in fibroblasts reflects the effect of PDGF from contaminating platelets.

Results of experiments using increasing amounts of ^{125}I-LDL added to fibroblasts exposed to Con A-stimulated mononuclear cell conditioned medium demonstrate that the effect of the conditioned medium is to increase the number of LDL receptors without changing the receptor affinity. Increase of LDL receptor number also has been reported with PDGF (4) and macrophage conditioned medium (6) which also is likely to contain mitogens. The increase in degradation was specific for LDL, but did not occur with HDL_3 or methylated LDL, neither of which are recognized by the LDL receptor. Fluid

phase pinocytosis also was not affected by conditioned medium. Therefore, the promotion of LDL degradation by Con A-stimulated mononuclear cell conditioned medium appears to be due to enhancement of LDL receptor activity.

The effect of Con A-stimulated mononuclear cell conditioned medium was examined in four familial hypercholesterolemic fibroblast strains. Although LDL degradation was much less than in control cells, the low levels of LDL degradation that were observed in the unstimulated state in some strains suggested that some residual LDL receptor was present in these cell strains. Fibroblasts from three of these receptor negative strains responded to the conditioned medium from the Con A-stimulated mononuclear cells by increasing the very small amounts of LDL degraded by these cells. LDL degradation by the strain with the lowest LDL degradation in the unstimulated state did not respond to the addition of conditioned medium. These findings are consistent with the suggestion that stimulation is an LDL receptor-mediated process.

In conclusion, mononuclear cells, especially when stimulated, secrete a factor which specifically enhances LDL receptor activity in recipient cells, in concert with mitogenic activity as reflected by thymidine incorporation into DNA.

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