RAPID COMMUNICATION

Monocyte Adherence to Endothelial Cells in Vitro *Is Increased by β-VLDL*

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The adherence of blood monocytes to the arterial endothelium is an early event in the development of atherosclerotic lesions. The possibility was investigated that alterations in the level and composition of plasma lipoproteins may contribute to this phenomenon. The adherence of human mononuclear cells to primary bovine aortic endothelial cells was measured in an *in vitro* monolayer collection assay. Preincubation of endothelial cells with β-very low density lipoprotein (β-VLDL) from cholesterol-fed rabbits or with very low density lipoprotein (VLDL) from cholesterol/saturated fat-fed cebus monkeys resulted in a significant increase in the subsequent adherence of monocytes to the endothelial cells. The effect of β-VLDL was maximal at 100 μg protein/ml. The response increased with time when endothelial cells were incubated with β-VLDL for 0–120 minutes, then remained maximal for up to 4 hours. The adherence of a human monocytic cell line (U937) to endothelial cells was also increased by β-VLDL. These results suggest that diet-induced alterations in lipoprotein composition may contribute to the development of atherosclerotic lesions by affecting the adherence of monocytes to the arterial endothelium. (Am J Pathol 1987, 126:1–6)

ONE OF THE initial events in the development of atherosclerotic lesions is the adherence of blood monocytes to arterial endothelial cells. This has been demonstrated in animal models in which atherosclerosis is induced by cholesterol feeding[1,2] and in Watanabe rabbits, which have a defect in the low-density lipoprotein (LDL) receptor gene (analogous to human familial hypercholesterolemia).[3] Evidence exists that monocyte adhesion is followed temporally by the accumulation of lipid-rich foam cells of monocytic origin in the arterial intima, disruption and denudation of the endothelium, platelet adherence and degranulation, and extensive proliferation of intimal smooth muscle cells.[4–6]

The adherence of monocytes to the aortic endothelium could be modulated by the secretion of chemotactic factors by the arterial wall or by alterations in the expression of endothelial cell or monocyte adhesion molecules. Medium conditioned by aortic endothelial cells has recently been shown to contain factors that are chemotactic for mouse peritoneal macrophages[7] and human monocytes.[8] Interestingly, chemotactic factor(s) for blood monocytes have been found in extracts of aorta from cholesterol-fed, but not control, swine.[9] The adherence of human monocytes to endothelial cells appears to be mediated in part by a monocyte surface complex termed CDw18.[10] A related complex termed Mac-1 has been implicated in the adherence of mouse monocytes to endothelial cells.[11] Endothelial cell surface molecules that mediate the adherence of monocytes have not been identified.

Both diet-induced atherosclerosis and LDL receptor-defective models are characterized by alterations in both the level and composition of plasma lipoproteins. We tested the possibility that certain lipoproteins might interact with the arterial wall to enhance the adherence of monocytes. We have recently dem-

Supported by NIH Postdoctoral Fellowship 5 F32 HL06702-02 to Dr. Endemann and Best Foods, C.P.I. International.

Accepted for publication October 24, 1986.

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onstrated that exposure of cultured aortic endothelial cells to certain human LDL influences the subsequent adhesion of monocytes.[12] We currently describe studies with VLDL from hypercholesterolemic cebus monkeys and rabbits indicating that these particles promote the adhesion of monocytes to endothelial cells in our *in vitro* monolayer collection assay.

Materials and Methods

Lipoprotein Preparation and Analysis

Rabbits were fed chow (controls) or chow plus 3% corn oil and 0.5% cholesterol (cholesterol-fed) for 2–4 weeks. The control group of cebus monkeys was fed a purified diet containing 12% corn oil and 17% lactalbumin for 2 years.[13] The experimental group of cebus monkeys was given the same diet except for the substitution of casein for lactalbumin, coconut oil for corn oil, and the addition of 0.5% cholesterol. Casein and coconut oil are the protein and fat which act together with dietary cholesterol to maximally increase plasma lipoprotein levels in cebus monkeys and other species[13,14] (K. C. Hayes, unpublished observations).

Lipoproteins were prepared from fasting plasma by density gradient ultracentrifugation.[15] Fractions containing VLDL ($d<1.006$ g/ml), LDL ($1.02<d<1.04$), and lipoprotein-deficient serum ($d>1.21$) were dialyzed against phosphate-buffered saline (PBS) containing the antitoxidants butylated hydroxytoluene (0.1 mM) and EDTA (1 mM). Cholesterol and triglyceride concentrations were determined enzymatically (Sigma diagnostic kits 350 and 90) and expressed relative to protein concentration.[16] Electrophoresis of plasma lipoproteins was carried out on cellulose acetate plates (Helena Laboratories) that were stained with oil red O. The relative migration of the lipoprotein classes was determined by using isolated lipoprotein fractions as standards and by comparison with published data.[17]

Endothelial Cell Culture

Endothelial cells were isolated from bovine aortas by exposing the lumen of the vessel to 1 mg/ml collagenase (Worthington) for 20 minutes and then dislodging cells by flushing the vessel with growth medium (Opti-MEM, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin; Gibco).[18] Cells were grown at 37 C in 5% CO_2 with fresh medium every 2 days until reaching confluence (5–7 days). These primary cultures were incubated with 10% lipoprotein-deficient serum for 18 hours before the adherence assay.

Mononuclear Cell Preparation

Mononuclear leukocytes were isolated from the peripheral blood of normocholesterolemic adult human males by centrifuging blood (diluted 1:1 with PBS containing 1 mM EDTA and 0.5 mM sodium citrate) through Lymphocyte Separation Medium (Litton Biometics) for 40 minutes at 570*g* at room temperature.[19] The mononuclear cell layer was washed twice with cold diluent to remove platelets. The human monocytic cell line U937 (provided by M. Bevilacqua) was maintained in culture as previously described.[20] Cells were labeled by a 60-minute incubation at 4 C with 0.1 mCi/ml ^{51}Cr, washed, and resuspended in Opti-MEM at 2×10^6 mononuclear cells/ml or 1×10^6 U937 cells/ml.[21]

Adherence Assay

Adhesion of mononuclear leukocytes to endothelial cells was quantitated with a monolayer collection assay.[22] Two-square centimeter endothelial cell cultures were incubated with 300 μl Opti-MEM and 100 μl PBS or lipoprotein in PBS for 4 hours unless otherwise specified. The final concentrations of LDL or VLDL were 100 μg protein/ml unless otherwise specified. One hundred microliters ^{51}Cr-labeled mononuclear cells were then added to each well. After 30 minutes nonadherent cells were removed, and the cultures were washed once. The cultures were solubilized in 0.2 M NaOH, and adherence was quantitated by liquid scintillation counting. The two-sided Student *t* test was used to determine the significance of differences between lipoprotein and PBS-treated wells.

Results

Composition of Rabbit and Monkey Lipoproteins

The alterations in plasma lipoprotein levels and composition that result when rabbits are fed cholesterol have been well documented.[23] The rabbits used in this study responded as expected to cholesterol feeding with increased plasma total cholesterol (Table 1) due to increased levels of all lipoprotein classes (data not shown). Cholesterol feeding also resulted in a large increase in the cholesterol/protein ratio in VLDL and a smaller increase in the cholesterol/protein ratio in LDL (Table 1). The abnormal composition of VLDL from cholesterol-fed rabbits was reflected by a β mobility upon electrophoresis (Figure 1), an alteration that has been previously observed.[23] Because cebus monkeys are known to respond less to cholesterol feeding than do rabbits, we elevated

Table 1 — Plasma Cholesterol Levels and Composition of Lipoproteins From Normocholesterolemic and Hypercholesterolemic Rabbits and Monkeys

Lipoprotein source	Plasma cholesterol (mg/dl)	Lipid/protein ratios (mg lipid/mg protein)					
		Cholesterol/protein		Triglyceride/protein		Cholesterol + triglyceride/protein	
		VLDL	LDL	VLDL	LDL	VLDL	LDL
Cebus monkeys							
Control							
1	178	0.78	1.28	1.63	0.23	2.41	1.51
2	156	1.11	1.32	1.06	0.23	2.17	1.55
3	144	0.58	1.47	0.67	0.09	1.25	1.56
Cholesterol-fed							
1	550	1.83	1.54	2.79	0.25	4.62	1.79
2	384	1.02	2.46	2.52	0.22	3.54	2.66
3	423	1.35	1.92	2.42	0.13	3.77	2.05
Rabbits							
Control							
1	43	0.31	0.62	0.82	0.39	1.13	1.01
2	59	0.61	0.54	0.80	0.24	1.41	0.78
Cholesterol-fed							
1	709	5.25	1.89	0.24	0.08	5.49	1.97
2	831	3.96	2.03	0.24	0.08	4.20	2.11

plasma cholesterol maximally by feeding the monkeys a diet that contained cholesterol and saturated fat.[13,14] This resulted in increased levels of all lipoprotein classes (data not shown), a slightly higher cholesterol content in VLDL and LDL, and a marked elevation in the triglyceride/protein ratio in VLDL (Table 1). In contrast to the cholesterol-enriched VLDL from rabbits, the triglyceride-enriched VLDL from cholesterol/saturated fat-fed monkeys migrated with normal (pre-β) mobility (Figure 1).

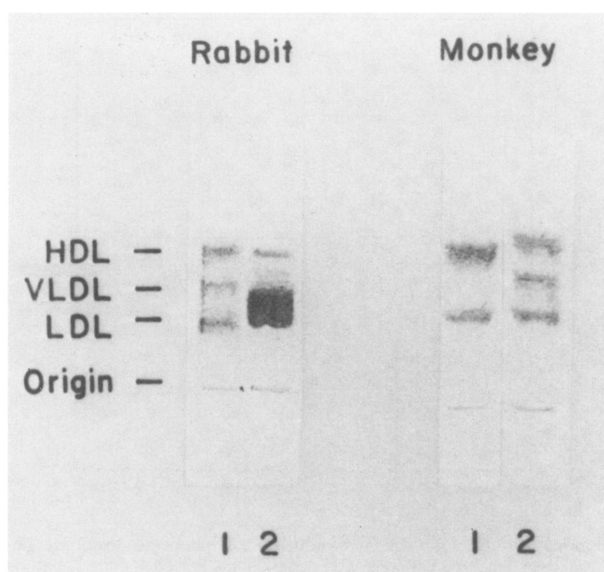

Figure 1 — Electrophoresis of plasma lipoproteins. Electrophoresis of plasma was carried out on cellulose acetate plates, which were stained with oil red O. Rabbit plasma: Lane 1, control; Lane 2, cholesterol-fed. Monkey plasma: Lane 1, control; Lane 2, cholesterol-fed.

Effect of Lipoproteins on Adherence of Monocytes to Endothelial Cells

We preincubated primary cultures of bovine aortic endothelial cells with various lipoproteins and measured the subsequent adhesion of human mononuclear cells to the endothelial cell monolayer. As reported previously,[12] more than 95% of the adherent cells were shown to be esterase-positive, which indicated that they were monocytes.[24] Incubation of endothelial cells with VLDL from hypercholesterolemic rabbits (Figure 2A) or hypercholesterolemic monkeys (Figure 2B) caused a significant increase in the adherence of monocytes. Neither the VLDL from control animals nor the LDL from any group had a significant effect on monocyte adhesion. VLDL from normocholesterolemic fasting humans was without significant effect in this assay.

The effect of β-VLDL on the adherence of monocytes to endothelial cells was time and concentration-dependent. The maximal response was obtained by preincubating the endothelial cells with 100 μg protein/ml (Figure 3A). The magnitude of the response increased with time when β-VLDL was incubated with endothelial cells for 0–120 minutes. After 2 hours the response remained maximal for up to 4 hours of incubation (Figure 3B).

In order to assess the effect of β-VLDL on monocyte adherence in the absence of other mononuclear

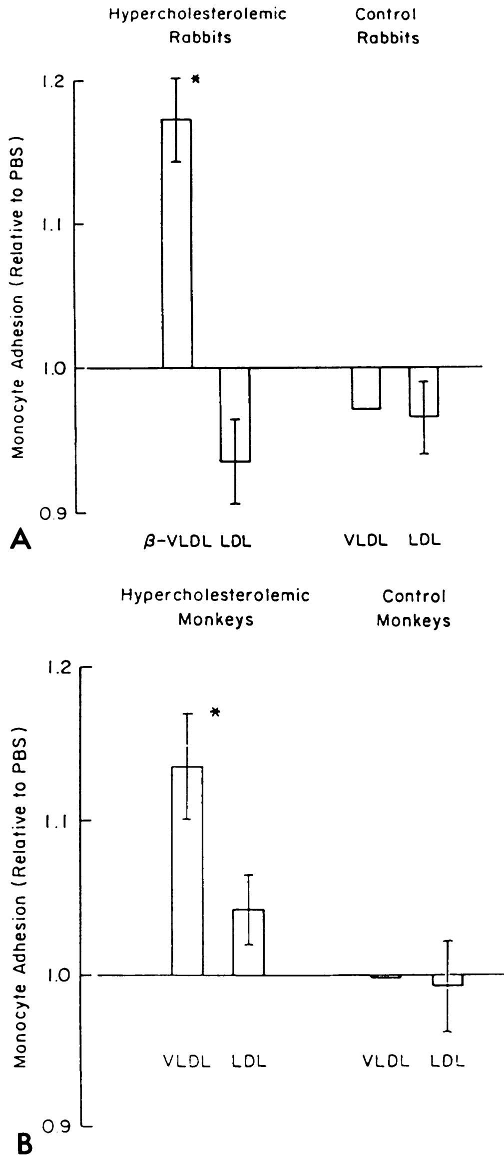

Figure 2—The effects of VLDL from normocholesterolemic and hypercholesterolemic rabbits and monkeys on the adherence of monocytes to primary aortic endothelial cells *in vitro*. Confluent primary cultures of bovine aortic endothelial cells were incubated for 4 hours with lipoproteins from rabbits **(A)** or cebus monkeys **(B)**. The concentration of VLDL or LDL was 100 μg protein/ml. The adherence of human mononuclear cells to lipoprotein- or PBS-treated endothelial cells was quantitated in a monolayer collection assay as described in Materials and Methods. The average of the counts per minute from six replicate lipoprotein-treated wells was expressed as a ratio to the average of six replicate PBS-treated wells. Each experiment was repeated three to five times with lipoproteins from separate donors for a mean ± SE except in the case of VLDL from control animals. Because the plasma level of VLDL was extremely low in both rabbits and monkeys fed control diets, VLDL was pooled from control animals. Significant difference between lipoprotein- and PBS-treated wells, ($P<0.05$).

cells (lymphocytes), we measured the adherence of U937 cells, a human monocytic tumor cell line,[20] to endothelial cell monolayers. Pretreatment of endothelial cells with 100 μg/ml β-VLDL resulted in a 52% ± 3.4% (mean ± SE, n=15) increase in the subsequent adherence of U937 cells relative to PBS-treated endothelial cells.

Discussion

The ability of VLDL from cholesterol-fed, but not control, animals to increase the adherence of monocytes to endothelial cells *in vitro* is consistent with the *in vivo* finding that cholesterol feeding results in the adherence of monocytes to the arterial endothelium.[1,2,4] Both VLDL from cholesterol-fed rabbits and that from cholesterol/saturated fat-fed cebus

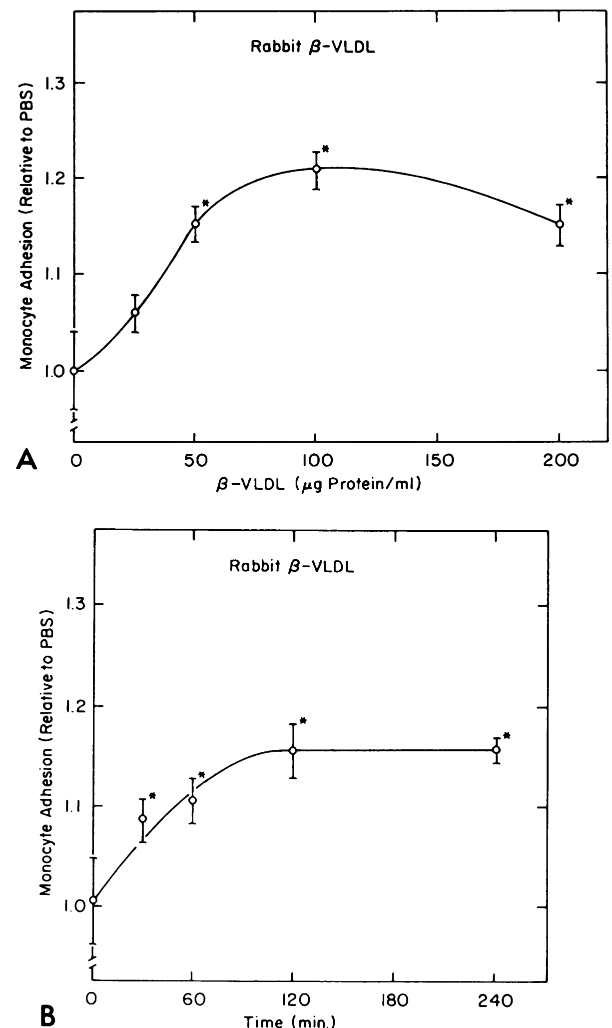

Figure 3—Effect of β-VLDL on the adherence of monocytes to aortic endothelial cells; time and concentration dependence. For each experiment the counts per minute from six lipoprotein-treated wells were expressed as ratios to the average counts per minute of PBS-treated wells. For each figure ratios were pooled from two to four experiments for a mean ± SE. **A**—Endothelial cells were incubated with the indicated levels of rabbit β-VLDL for 4 hours before measurement of mononuclear cell adhesion. **B**—Endothelial cells were incubated with 100 μg protein/ml rabbit β-VLDL for the indicated periods of time before measurement of mononuclear cell adhesion.

monkeys appear to be equally effective in inducing monocyte adherence relative to PBS. VLDL normally contain a triglyceride-rich core, which in β-VLDL is replaced largely by cholesteryl esters. The VLDL from the cholesterol-fed rabbits used in these studies appears to be β-VLDL, as judged by composition and electrophoretic migration. The VLDL from the cholesterol/saturated fat-fed cebus monkeys was enriched in triglyceride and to a lesser extent cholesterol, compared with VLDL from control monkeys, but did not show β mobility upon electrophoresis. Thus the best predictor of lipoprotein-induced increases in monocyte adherence appears to be the ratio of cholesterol + triglyceride/protein (Table 1), a finding in agreement with our previous data concerning the effect of human LDL on monocyte adherence.[12] The use of an antioxidant in all of our lipoprotein preparations makes it unlikely that the lipoproteins used in these studies contained peroxidized lipids.

The increase in monocyte adherence to endothelial cells induced by β-VLDL requires a 30-minute preincubation of β-VLDL with endothelial cells, suggesting that the β-VLDL must interact specifically with the endothelial cells. A receptor on endothelial cells for β-VLDL has recently been described which is saturated at approximately 60–100 μg/ml protein,[25] which is the approximate concentration of β-VLDL at which we observed maximal monocyte adherence. Triglyceride-enriched VLDL from hypertriglyceridemic patients has been shown to interact with the β-VLDL receptor of marcophages.[26] Thus it is likely that the triglyceride-enriched VLDL from cholesterol/saturated fat-fed cebus monkeys is also taken up by endothelial cells via the β-VLDL receptor. Normal VLDL does not compete for uptake by this receptor and has no effect in our monocyte adherence assay.

It is possible that binding of the lipoprotein to the endothelial cell surface is sufficient to promote binding of monocytes, e.g., by subsequent binding of a monocyte to the bound VLDL particle, as suggested previously.[2] Monocytes express high levels of β-VLDL receptors within 1 day of culture.[27] Lipoproteins also form complexes with extracellular proteoglycans; these complexes are readily internalized by macrophages.[28] An alternate explanation would be that internalization of the VLDL by the endothelial cell is required, resulting in one of several possible consequences. First, β-VLDL may induce the secretion of a chemotactic factor by the endothelial cells. Aortic endothelial cells are known to be sources of fibronectin,[29] collagen,[30] and the B chain of platelet-derived growth factor,[31] all of which are chemotactic for monocytes.[32–34] Others have recently reported that β-VLDL causes endothelial cells to secrete increased amounts of a factor that is chemotactic for monocytes.[8] However, this required a much longer incubation of lipoprotein with endothelial cells (3 days) than is the case for our effect. In addition, the factors produced by endothelial cells which are chemotactic for monocytes are constitutively secreted by endothelial cells.[7,8] We do not observe constitutive secretion of factors by endothelial cells which affect monocyte adhesion in our assay.

A second possibility is that β-VLDL induces the secretion by endothelial cells of a factor that affects the expression or properties of monocyte surface adhesion molecules. The monocyte surface complex CDw18 appears to mediate in part the adherence of human monocytes to endothelial cells.[10] Phorbol ester, which enhances the adherence of monocytes to endothelial cells, increases within minutes expression of the epitope on this molecule which is thought to interact with endothelial cells. However, this molecule is present only at very low levels on U937 cells,[10] which we and others[35] have found to adhere to primary cultures of endothelial cells. The adherence of both U937 cells and monocytes to endothelial cells is increased by β-VLDL.

A third possibility is that lipoproteins affect the expression or properties of endothelial cell adhesion molecules. Expression of a cell surface binding site for monocytes and U937 cells is increased by treatment of endothelial cells with interleukin-1.[35] It has recently been demonstrated that interleukin-1 also induces endothelial cell expression of ICAM-1, which has been implicated in cell–cell adherence mechanisms.[36] It remains to be determined whether the expression of endothelial cell or monocyte adhesion molecules is modulated by treatment of the endothelial cell with atherogenic lipoproteins.

References

1. Gerrity RG: The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol 1981, 103:181–190.
2. Joris I, Zand T, Nunnari JJ, Krolikowski FJ, Majno G: Studies on the pathogenesis of atherosclerosis: I. Adhesion and emigration of mononuclear cells in the aorta of hypercholesterolemic rats. Am J Pathol 1983, 113:341–358
3. Rosenfeld ME, Ross R: Comparative morphology of fatty streak formation in Watanabe and fat-fed rabbits (Abstr). Arteriosclerosis 1985, 5:498a
4. Faggiotto A, Ross R, Harker L: Studies of hypercholesterolemia in the nonhuman primate: I. Changes that lead to fatty streak formation. Arteriosclerosis 1984, 4:323–340
5. Brown MS, Goldstein JL: Lipoprotein metabolism in the macrophage: Implications for cholesterol deposition in atherosclerosis. Ann Rev Biochem 1983, 52:223–261

6. Faggiotto A, Ross R: Studies of hypercholesterolemia in the nonhuman primate: II. Fatty streak conversion to fibrous plaque. Arteriosclerosis 1984, 4:341-356
7. Quinn MT, Parthasarathy S, Steinberg D: Endothelial cell-derived chemotactic activity for mouse peritoneal macrophages and the effects of modified forms of low density lipoprotein. Proc Natl Acad Sci USA 1985, 82:5949-5953
8. Berliner JA, Territo M, Almada L, Carter A, Shafonsky E, Fogelman AM: Monocyte chemotactic factor produced by large vessel endothelial cells in vitro. Arteriosclerosis 1986, 6:254-258
9. Gerrity RG, Goss JA, Soby L: Control of monocyte recruitment by chemotactic factors in lesion-prone areas of swine aorta. Arterioscerosis 1985, 5:55-66
10. Wallis WJ, Beatty PG, Ochs HD, Harlan JM: Human monocyte adherence to cultured vascular endothelium: Monoclonal antibody-defined mechanisms. J Immunol 1985, 135:2323-2329
11. Sastre L, Kishimoto TK, Gee C, Roberts T, Springer TA: The mouse leukocyte adhesion proteins Mac-1 and LFA-1: Studies on mRNA translation and protein glycosylation with emphasis on Mac-1. J Immunol 1986, 137:1060-1065
12. Alderson LM, Endemann G, Lindsey S, Pronczuk A, Hoover RL, Hayes KC: LDL enhances monocyte adhesion to endothelial cells *in vitro*. Am J Pathol 1986, 123:334-342
13. Ershow AG, Nicolosi RJ, Hayes KC: Separation of the dietary fat and cholesterol influences on plasma lipoproteins of rhesus monkeys. Am J Clin Nutr 1981, 34:830-840
14. Lichtenstein AH, Nicolosi RJ, Hayes KC: Dietary fat and cholesterol effects on plasma lecithin: cholesterol acyltransferase activity in cebus and squirrel monkeys. Atherosclerosis 1980, 37:603-616
15. Redgrave TG, Robert DCK, West CE: Separation of lipoproteins by density gradient ultracentrifugation. Anal Biochem 1975, 65:42-49
16. Kashyap ML, Hynd BA, Robinson K: A rapid and simple method for measurement of total protein in very low density lipoproteins by the Lowry method assay. J Lipid Res 1980, 21:491-495
17. Mahley RW, Weisgraber KH, Innerarity T: Canine lipoproteins and atherosclerosis: II. Characterization of the plasma lipoproteins associated with atherogenic and nonatherogenic hyperlipidemia. Circ Res 1974, 35:722-733
18. Booyse FM, Sedlak B, Rafelson N: Culture of arterial endothelial cells: Characterization and growth of bovine aortic cells. Thromb Diath Haemorrh 1975, 34:825-839
19. Boyum A: Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 1968, 97(Suppl 21):77-89
20. DiCorleto PE, de la Motte CA: Characterization of the adhesion of the human monocytic cell line U937 to cultured endothelial cells. J Clin Invest 1985, 75:1153-1161
21. Gallin J, Clark RC, Kimball HP: Granulocyte chemotaxis: An improved *in vitro* assay employing ^{51}Cr-labeled granulocytes. J Immunol 1973, 110:233-240
22. Walther BT, Ohman R, Roseman S: A quantitative assay for intercellular adhesion. Proc Natl Acad Sci USA 1973, 70:1569-1573
23. Mahley RW, Innerarity TL, Brown MS, Ho YK, Goldstein JL: Cholesteryl ester synthesis in macrophages: stimulation by β-very low density lipoproteins from cholesterol-fed animals of several species. J Lipid Res 1980, 21:970-980
24. Yam LT, Li CY, Crosby WH: Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971, 55:283-290
25. Baker DP, Van Lenten BJ, Fogelman AM, Edwards PA, Kean C, Berliner JA: LDL, scavenger, and β-VLDL receptors on aortic endothelial cells. Arteriosclerosis 1984, 4:248-255
26. Gianturco SH, Bradley WA, Gotto AM, Morriset JD, Peavy DL: Hypertriglyceridemic very low density lipoproteins induce triglyceride synthesis and accumulation in mouse peritoneal macrophages. J Clin Invest 1982, 70:168-178
27. Van Lenten BJ, Fogelman AM, Hokom MM, Benson L, Haberland ME, Edwards PA: Regulation of the uptake and degradation of β-very low density lipoprotein in human monocyte macrophages. J Biol Chem 1983, 258:5151-5157
28. Salisbury BGJ, Falcone DJ, Minick CR: Insoluble low-density lipoprotein-proteoglycan complexes enhance cholesteryl ester accumulation in macrophages. Am J Pathol 1985, 120:6-11
29. Birdwell CR, Gospodarowicz D, Nicolson GL: Identification, localization and role of fibronectin in cultured bovine endothelial cells. Proc Natl Acad Sci USA 1978, 75:3272-3277
30. Kramer RH, Bensch KG, Davison PM, Karasek MA: Basal lamina formation by cultured microvascular endothelial cells. J Cell Biol 1894, 99:692-698
31. Collins T, Ginsburg D, Boss JM, Orkin SH, Pober JS: Cultured human endothelial cells express platelet-derived growth factor B chain: cDNA cloning and structural analysis. Nature 1985, 316:748-750
32. Norris DA, Clark RAF, Swigart LM, Huff JC, Weston WL, Howell SE: Fibronectin fragments are chemotactic for human peripheral blood monocytes. J Immunol 1982 129:1612-1618
33. Postlethwaite AE, Kang AH: Collagen and collagen peptide-induced chemotaxis of human blood monocytes. J Exp Med 1976, 143:1299-1307
34. Deuel TF, Senior RM, Huang JS, Griffen GL: Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. J Clin Invest 1982, 69:1046-1049
35. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA: Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related cell lines. J Clin Invest 1985, 76:2003-2011
36. Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA: Induction by Il 1 and Interferon-γ: Tissue distribution, biochemistry and function of a natural adherence molecule (ICAM-1). J Immunol 1986, 137:245-254