Pigeon aortic smooth muscle cells lack a functional low density lipoprotein receptor pathway

R. Keith Randolph[1] and Richard W. St. Clair[2]

Department of Pathology, Arteriosclerosis Research Center, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103

Abstract The low density lipoprotein (LDL) receptor pathway was studied in aortic smooth muscle cells from atherosclerosis-susceptible White Carneau pigeons and compared with rhesus monkey cells whose LDL receptor pathway has been previously characterized. Pigeon LDL was bound with high affinity in a saturable manner to both pigeon and monkey aortic smooth muscle cells. The kinetics of binding were different, however. LDL binding to pigeon cells exhibited positive cooperativity at low LDL concentrations and at least two classes of binding sites. The same pigeon LDL bound to monkey cells in a manner consistent with a single class of binding sites. Thus, these differences were a property of the pigeon cells and not the result of differences in the LDL. On the average, pigeon cells bound less than 50% the amount of LDL as monkey cells. Despite the surface binding to pigeon cells, little of the LDL was internalized, whereas pigeon LDL was actively internalized by monkey cells. Consistent with this observation, choloroquine and leupeptin had no effect on accumulation of LDL or on LDL degradation by pigeon cells, and incubation of pigeon cells with LDL produced no increase in cellular cholesteryl ester content. Binding of LDL to pigeon cells also differed from that of monkey cells by being unaffected by pretreatment with the proteolytic enzyme pronase, and by not requiring calcium. Binding was not specific for LDL since acetyl-LDL, and to a lesser degree HDL, were able to compete for LDL binding. Incubation with lipoprotein-deficient serum decreased LDL binding in pigeon cells while 25-OH cholesterol caused an increase in binding; both effects are opposite of that seen with the same LDL in mammalian cells. Preincubation with LDL or cholesterol dissolved in ethanol were without effect on LDL binding in pigeon cells, even though they produced significant increases in cellular free cholesterol content. In spite of the failure to internalize LDL, there was considerable degradation of LDL. This apparently occurred on the cell surface rather than by internalization and degradation within the lysosomes as occurs in mammalian cells. The functional significance of LDL binding to pigeon smooth muscle cells is unclear. The characteristics of binding resemble that of a nonspecific lipoprotein receptor referred to by others as the "lipoprotein receptor" or the "EDTA-insensitive receptor." It is apparent, however, that White Carneau pigeon aortic smooth muscle cells lack a functional LDL receptor pathway and in this way resemble cells from human beings with homozygous familial hypercholesterolemia or from Watanabe rabbits.—**Randolph, R. K., and R. W. St. Clair.** Pigeon aortic smooth muscle cells lack a functional low density lipoprotein receptor pathway. *J. Lipid Res.* 1984. **25:** 888–902.

Supplementary key words lipoprotein composition • chloroquine • high density lipoprotein • acetyl LDL • 25-OH cholesterol • lipoprotein-deficient serum

Extrahepatic mammalian cells derive the bulk of their cholesterol from the uptake of low density lipoproteins (LDL) through the LDL receptor pathway (1). The LDL binds to specific high affinity receptors located in specialized areas of the plasma membrane called coated pits. The LDL is rapidly internalized and delivered to lysosomes where the protein is degraded and the cholesteryl esters are hydrolyzed. This results in an increase in cellular cholesterol content which in turn inhibits endogenous cholesterol synthesis, stimulates cholesterol esterification, and down-regulates the synthesis of LDL receptors. Thus, by controlling the number of LDL receptors on the plasma membrane, the cell is able to utilize extracellular cholesterol in a tightly regulated fashion in response to changing cellular cholesterol needs.

Although the magnitude of this regulatory response can vary with cell type, a wide variety of normal mammalian cells has been shown to control LDL receptor activity, endogenous cholesterol synthesis, and cholesterol esterification coordinately in response to fluctuations in cellular cholesterol content (2–8). Changes in cellular cholesterol content resulting in changes in the components of the LDL receptor pathway can be produced in mammalian cells in culture by incubation in a medium containing whole serum, isolated lipoproteins containing apolipoproteins B or E (9–12), or in lipoprotein-free

Abbreviations: LDL, low density lipoproteins; HDL, high density lipoproteins; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; LPDS, lipoprotein-deficient serum; FBS, fetal bovine serum; DiI, 3,3′-dioctadecylindocarbocyanine; TLC, thin-layer chromatography.

[1] This work was performed as partial fulfillment of the requirements for the degree of Doctor of Philosophy from the Bowman Gray School of Medicine of Wake Forest University. Present address: Cleveland Clinic Foundation, Cleveland, OH 44106.

[2] To whom reprint requests should be addressed at Department of Pathology, Bowman Gray School of Medicine, 300 South Hawthorne Road, Winston-Salem, NC 27103.

medium containing a variety of sterols dissolved in ethanol (13, 14).

In a previously published report, we observed a lack of stimulation of cholesterol esterification and little increase in cellular cholesteryl ester content in pigeon arterial smooth muscle cells that were incubated with medium containing whole pigeon serum (15). This occurred despite a substantial increase in the content of cellular free cholesterol. Considered in light of what is known about the regulation of cholesterol metabolism in mammalian cells, these data suggested either that pigeon cells lack a functional LDL receptor pathway or that the regulation of cholesterol metabolism in pigeon cells is fundamentally different from that of mammalian cells. The purpose of the present study was to explore the first of these possibilities by studying LDL metabolism in pigeon aortic smooth muscle cells in culture as compared to monkey aortic smooth muscle cells whose LDL receptor pathway we have previously characterized (16).

The results indicate that pigeon cells bind LDL in a saturable manner but that this binding does not result in internalization of the LDL or in delivery of exogenous cholesterol to the cells. Despite the inability to efficiently internalize LDL, pigeon cells are capable of degrading LDL at rates that exceed that which can be attributed to nonspecific processes.

MATERIALS

Sodium [^{125}I]iodide (17.0 Ci/mg, carrier-free low pH) and [1,2-^{3}H]cholesterol (40 Ci/mmol) were purchased from New England Nuclear Corporation, Boston, MA. The fluorescent probe 3,3′-dioctadecylindocarbocyanine (DiI) was obtained from Molecular Probes Inc., Plano, TX. Chloroquine, leupeptin, and pronase were purchased from Sigma Chemical Co., St. Louis, MO. All tissue culture supplies were obtained from Flow Laboratories, Rockville, MD.

METHODS

Cell cultures

Pigeon (15) and monkey aortic smooth muscle cells (17) were obtained and cultured by methods that have been previously described. Skin fibroblasts (GM-2000) from a patient with the receptor-negative form of familial hypercholesterolemia were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ. The pigeon cells were grown from aortic explants from young (less than 6 months of age), grain-fed random-bred White Carneau (WC) pigeons. The monkey cells were grown from aortic explants from adult rhesus monkeys (*Macaca mulatta*) fed Monkey Chow (Ralston Purina Co., St. Louis, MO). Stock cultures of pigeon and human cells were maintained in medium consisting of Eagle's Minimum Essential Medium supplemented with Eagle's vitamins, fetal bovine serum (FBS) (10% final concentration), glucose (1.5 mg/ml), 23 mM sodium bicarbonate, 200 mM L-glutamine, 100 I.U. penicillin/ml, and 100 mg streptomycin/ml, and will be referred to as medium containing FBS. Cholesterol concentrations in this medium averaged 40 μg cholesterol/ml. Monkey cells were cultured in identical medium except that the FBS was replaced by calf serum.

Cells from stock cultures were harvested from 75-cm^2 flasks with 0.05% trypsin–0.2% ethylenediaminetetraacetic acid (EDTA) and plated into 60-mm tissue culture dishes at densities of 2.5×10^5 cells/dish for monkey and human cells and 5.0×10^5 cells/dish for pigeon cells. Prior to initiation of experiments, cells were grown to confluence.

Medium containing lipoprotein-deficient serum (LPDS) was prepared by replacing the FBS with the d > 1.21 g/ml fraction of calf serum to give a final protein concentration of 2.5 mg/ml (17). This medium contained less than 1 μg of cholesterol/ml.

Measurement of cellular free cholesterol and cholesteryl ester content

After incubation with the appropriate medium, cells were harvested from dishes with trypsin-EDTA and washed twice with phosphate-buffered saline (PBS) (16). The cells were suspended in 1.0 ml of deionized water and disrupted by sonication. An aliquot was taken for protein determination (18) and another was extracted for lipids by the method of Bligh and Dyer (19). Free cholesterol and cholesteryl ester mass were quantified as described previously (16) by gas–liquid chromatography using the method of Ishikawa et al. (20).

Lipoprotein isolation, labeling, and characterization

Lipoproteins were isolated from grain-fed WC pigeons and from rhesus monkeys consuming Monkey Chow (Ralston Purina Co.). Both of these diets were essentially cholesterol-free. Blood from fasted animals was collected in tubes containing EDTA at a final concentration of 1 mg/ml and kept at 4°C during subsequent procedures. Low density lipoproteins were separated as described previously using a combination of ultracentrifugation and agarose column chromatography (16). Pigeon high density lipoproteins (HDL) were isolated by adjusting the d > 1.080 g/ml infranatant solution, from which the LDL had been isolated, to 1.21 g/ml with solid KBr and centrifuging for 40 hr at 36,000 rpm in a Beckman SW-40 rotor. The isolated lipoproteins were

exhaustively dialyzed against 0.9% NaCl and 0.01% EDTA. LDL and HDL migrated on agarose electrophoresis as single bands with beta- and alpha-mobility, respectively. Pigeon LDL, however, migrated only a few millimeters from the point of origin while monkey LDL migrated 10–15 mm from the origin in a manner similar to human LDL.

Pigeon and monkey LDL were labeled with ^{125}I using the iodine monochloride method as described previously (16). After iodination, the lipoproteins were dialyzed extensively against PBS containing 0.01% EDTA and sterilized by passage through a 0.45-μm filter (Millipore Corp., Bedford, MA). Less than 2% of the radioactivity in the final LDL preparation was soluble in chloroform–methanol 2:1, and greater than 97% of the radioactivity was precipitable in (10%) trichloroacetic acid (TCA). Iodinated LDL preparations had beta-mobility when separated by electrophoresis on agarose, and virtually all radioactivity was localized to the LDL band as determined by radioautography. Specific activities of the ^{125}I-labeled LDL preparations were typically 300–600 cpm/ng protein.

The chemical composition of pigeon and monkey LDL was determined as previously described (21). Molecular weight of LDL was determined using a ^{125}I-labeled LDL of known molecular weight as an internal standard (22). Electrophoresis in 12% polyacrylamide gels (PAGE) containing 0.1% sodium dodecyl sulfate was performed on delipidated apolipoproteins (23). Apolipoprotein A-I was isolated from rhesus monkey plasma (23) and used as a standard for PAGE.

Low density lipoproteins were double labeled with [^3H]cholesteryl ester and ^{125}I (^3H-CE/^{125}I-LDL) exactly as described previously (24). Specific activities for ^{125}I-labeled protein and [^3H]cholesteryl ester averaged 38 cpm/ng LDL protein and 12 cpm/ng LDL cholesteryl ester, respectively. The ratio of ^{125}I to ^3H in the final preparation was 2.5. Low density lipoproteins were labeled with DiI as described by Pitas et al. (25).

Determination of ^{125}I-labeled LDL binding, internalization, and degradation

Cells were grown to confluence in medium containing FBS in 60-mm dishes, washed twice with PBS, and fresh medium containing FBS or LPDS was added. After the indicated period of incubation, the appropriate concentrations of ^{125}I-labeled LDL, in the presence or absence of a 20-fold excess of homologous unlabeled LDL, were added to the dishes in medium containing FBS or LPDS, and incubated for 5 hr at 4°C or 37°C. Specific binding, internalization, and degradation of ^{125}I-labeled LDL were determined after subtracting the radioactivity obtained in the presence of a 20-fold excess of unlabeled LDL from that obtained in its absence (26). Total cell associated ^{125}I-labeled LDL (bound plus internalized) was determined as described by Goldstein and Brown (26) by digesting the washed cells with 1 N NaOH. In some experiments we measured the amount of LDL bound and internalized as heparin-releasable or resistant ^{125}I-labeled LDL as described by Goldstein et al. (27) or as trypsin-releasable or resistant ^{125}I-labeled LDL as described by Bierman, Stein, and Stein (7). Cells were washed exhaustively as indicated previously (16) before the above-described methods were applied.

Proteolytic degradation of ^{125}I-labeled LDL was determined on 1 ml of post-incubation medium by measurement of TCA (10%)-soluble (non-iodide) ^{125}I radioactivity as described by Goldstein and Brown (26). Results were corrected for TCA-soluble, non-iodide ^{125}I found in control dishes incubated without cells.

Internalization of ^3H-CE/^{125}I-LDL was measured after release of surface-bound material with trypsin. The cell pellet was suspended in 1.0 ml of deionized H_2O; 100 μl was used for protein determination and 100 μl was counted for internalized ^{125}I in the gamma spectrometer, (Tracor model 1185 Autogamma System). Lipids were extracted from the remaining 0.8 ml by the method of Bligh and Dyer (19). An aliquot of the total lipid extract was counted for total internalized ^3H. The remaining lipid extract was dried under a stream of nitrogen and neutral lipids were separated by thin-layer chromatography (TLC) as described previously (21). The free cholesterol and cholesteryl ester bands were visualized with iodine, scraped into scintillation vials, and counted for ^3H radioactivity. With this method, the total lipid and TLC-separated fractions were essentially free of ^{125}I radioactivity which simplified the ^3H counting, as there was no contamination of ^3H with ^{125}I.

Degradation products of ^{125}I-labeled LDL were characterized exactly as described by Brown and Goldstein (26) using the pooled media from groups of ten dishes for each experimental treatment. Prior to characterization of the degradation products the pooled media were centrifuged to remove cell debris and lyophilized.

All experiments shown in the tables and figures were repeated at least once with similar results.

RESULTS

The chemical composition of the pigeon and monkey LDL and pigeon HDL used in these studies is shown in **Table 1.** Monkey and pigeon LDL were remarkably similar in composition with the exception that pigeon LDL contained somewhat less cholesteryl ester and more triglyceride. The proportion of total mass representing core lipids (cholesteryl esters plus triglycerides) was similar for both species, comprising approximately 45% of total lipoprotein mass.

TABLE 1. Chemical composition of pigeon and monkey LDL and pigeon HDL

	N[a]	Molecular Weight	% of Total Lipoprotein Mass					FC	TC	FC
			Protein	FC	CE	TG	PL	Prot	Prot	PL
		$\times 10^{-6}$			$\bar{x} \pm SD$					
LDL										
Pigeon	3	3.1 ± 0.4	20.1 ± 1.4	9.8 ± 0.6	30.4 ± 4.9	14.9 ± 7.9	24.8 ± 2.4	0.49 ± 0.04	1.38 ± 0.15	0.40 ± 0.02
Monkey	3	2.9 ± 0.2	22.0 ± 0.4	9.0 ± 0.4	37.5 ± 1.0	8.2 ± 1.1	23.3 ± 0.5	0.41 ± 0.03	1.41 ± 0.02	0.39 ± 0.02
HDL										
Pigeon	1	N.D.[b]	39.4	5.2	17.5	5.1	32.8	0.13	0.40	0.16

FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid; TC, total cholesterol.
[a] N denotes the number of pooled lipoprotein preparations that were characterized.
[b] N.D., not determined.

The chemical composition of pigeon HDL differed somewhat from that of previously published values for rhesus monkey HDL. The percentage of protein in pigeon HDL was lower than for rhesus monkey HDL (28), 39 vs 46%, respectively, while the percentage of phospholipid was higher, 33 vs 27%, respectively. Cholesteryl ester was the predominant component of core lipids in pigeon HDL (approximately 77%) and monkey HDL (approximately 85%).

The apolipoproteins present in pigeon and monkey HDL and LDL are shown in **Fig. 1.** The major apoprotein of pigeon and monkey LDL was apoB. Of the other apoproteins the only consistent difference was the presence in pigeon apoLDL of a band comigrating with monkey apoA-I, and the absence of a band migrating in the position of monkey apoE. ApoA-I was the predominant apoprotein of both pigeon and monkey HDL. The identities of the unlabeled larger molecule weight proteins in pigeon HDL and LDL, that migrated between apoB and apoA-I, are not known.

Since previous studies have shown that pigeon cells fail to increase cholesterol esterification or cholesteryl ester content when incubated with whole serum (15), initial experiments were designed to determine whether isolated pigeon LDL would promote cholesterol accumulation in pigeon and monkey cells (**Fig. 2**). When incubated with the same medium containing FBS or LPDS, pigeon cells contained less free cholesterol and cholesteryl ester than monkey cells. With increasing concentrations of pigeon LDL there was an increase in the cellular free and esterified cholesterol content of monkey cells similar to previous observations using monkey LDL (21). In contrast, there was no increase in cellular cholesteryl ester content in pigeon cells even though the free cholesterol content increased to a degree similar to that seen in the monkey cells.

Prior to the initiation of studies on the LDL receptor pathway, experiments were carried out to determine the optimal conditions for measurement of LDL binding by pigeon cells. Low density lipoprotein binding was measured by three different methods in cells that were preincubated with medium containing either FBS or LPDS (**Table 2**). Monkey cells, as do other mammalian cells, increase the number of LDL receptors on their surface when cellular cholesterol concentrations are depleted by incubation with LPDS (2). Pigeon cells, in contrast, bound more LDL when preincubated with medium containing FBS as compared to cells preincubated with medium containing LPDS. This difference was observed in incubations carried out at 4°C and 37°C and occurred regardless of whether binding was measured as total cell-associated ^{125}I or as heparin- or trypsin-releasable ^{125}I. As a result, in subsequent experiments with pigeon cells, ^{125}I-labeled LDL was added directly to medium containing FBS and the cells were not preincubated with LPDS.

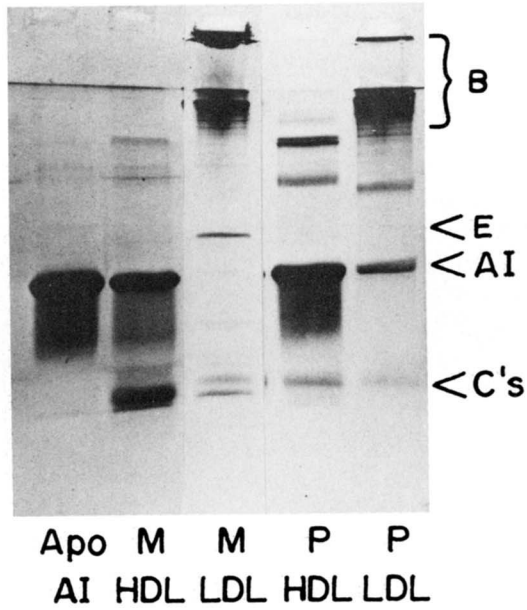

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of monkey and WC pigeon apoHDL and apoLDL. B, E, A-I, and C refer to the location of apoproteins B, E, A-I, and C, respectively, from rhesus monkeys. Gels were intentionally overloaded with 100 μg of protein applied to each gel in order to identify all bands present. MHDL, MLDL, PHDL, PLDL refer to monkey HDL and LDL and pigeon HDL and LDL, respectively.

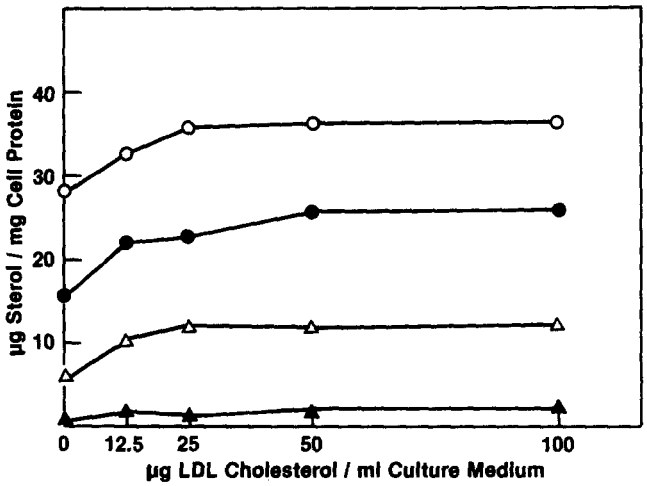

Fig. 2. Effect of pigeon LDL on cholesterol content of WC pigeon (●, ▲) and monkey (○, △) smooth muscle cells. Cells were grown to confluence in medium containing serum. All dishes then received fresh medium containing FBS plus the indicated concentrations of pigeon LDL cholesterol. Cells were incubated for an additional 24 hr and were harvested by trypsinization. Free cholesterol (○, ●) and cholesteryl ester (△, ▲) content were measured as described under Methods. Cholesterol content was also measured in two separate sets of dishes that were incubated for 24 hr in medium in which the FBS was replaced with LPDS. Under these conditions free cholesterol and cholesteryl ester content of monkey cells was 26.9 and 2.2 µg/mg cell protein and for pigeon cells was 16.0 and 0.4 µg/mg cell protein, respectively. All results are the average of duplicate cultures.

TABLE 2. Effect of preincubation with FBS or LPDS on binding of ^{125}I-labeled LDL by pigeon smooth muscle cells

Treatment	ng ^{125}I-labeled LDL Bound/mg Cell Protein
NaOH[a]	
FBS	507
LPDS	261
Heparin[b]	
FBS	282
LPDS	118
Trypsin[c]	
FBS	313
LPDS	207

Confluent pigeon smooth muscle cells were preincubated in medium containing 10% FBS or LPDS for 24 hr. Dishes were washed and medium containing FBS or LPDS plus the indicated concentrations of ^{125}I-labeled pigeon LDL were added to dishes and incubation was continued for 5 hr at 37°C or 4°C. Cells incubated at 37°C were then chilled on melting ice, washed extensively, and binding was determined as indicated. Results are corrected for nonspecific binding as described under Methods and are the average of duplicate dishes. Total cholesterol concentration in cells incubated with FBS or LPDS for 24 hr was 23.0 and 22.5 µg/mg cell protein, respectively. Less than 1 µg of CE was detected under both conditions.

[a] Cells were incubated with 20 µg of ^{125}I-labeled LDL protein/ml for 5 hr at 37°C.

[b] Cells were incubated with 25 µg of ^{125}I-labeled LDL protein/ml for 5 hr at 37°C.

[c] Cells were incubated with 25 µg of ^{125}I-labeled LDL protein/ml for 5 hr at 4°C.

The binding, internalization, and degradation of ^{125}I-labeled LDL by pigeon and monkey cells are shown in **Table 3.** The sum of the ^{125}I-labeled LDL bound and internalized (NaOH treatment) by monkey smooth muscle cells was more than fourfold greater than for pigeon cells. Heparin removed 23% of the cell-associated ^{125}I from the pigeon cells and trypsin removed 50%. Under the same conditions, heparin removed 9% of the cell-associated ^{125}I from monkey cells, and trypsin removed 14%. After removal of surface-bound LDL by trypsin, the amount of cell-associated LDL remaining was only about 10% (119 ng/mg protein) as much in pigeon cells as monkey cells (1188 ng/mg protein). Despite these large differences in the amount of LDL internalized by pigeon and monkey cells, pigeon cells degraded considerable amounts of LDL.

The concentration dependence of LDL binding, internalization, and degradation by pigeon cells is shown in **Fig. 3.** Binding of ^{125}I-labeled LDL displayed saturation kinetics that could be separated into "specific" and "nonspecific" components. At low LDL concentrations, binding was predominantly "specific." Saturation occurred at concentrations greater than 50 µg LDL protein/ml, with one-half maximum binding occurring at approximately 15 µg LDL protein/ml. Total binding capacity of pigeon cells was calculated to be 35,000 LDL particles/cell as compared with 80,000 LDL particles/cell for monkey cells (16), and 70,000 LDL particles/cell for human cells (27) under similar conditions. There was again very little ^{125}I-labeled LDL internalized, and the amount that was measurable did not increase in a concentration-dependent manner, as the binding data would have predicted had LDL binding been coupled with internalization (29). The amount of ^{125}I-labeled LDL degraded was again surprisingly large compared to the amount of ^{125}I-labeled LDL internal-

TABLE 3. Comparison of measures of bound, internalized, and degraded ^{125}I-labeled LDL in pigeon and monkey smooth muscle cells

Cell Type	Treatment	Bound	Internalized	Degraded
		ng ^{125}I-labeled LDL/mg cell protein ± SD		
Pigeon	NaOH	303 ± 14		1169 ± 54
	Heparin	72 ± 12	242 ± 6	1159 ± 84
	Trypsin	148 ± 8	119 ± 9	1183 ± 72
Monkey	NaOH	1409 ± 226		1807 ± 137
	Heparin	116 ± 11	1238 ± 75	1697 ± 71
	Trypsin	167 ± 11	1188 ± 167	1725 ± 91

Confluent pigeon and monkey smooth muscle cells were preincubated for 24 hr in medium containing 10% FBS and LPDS, respectively. Dishes were washed and experimental medium containing 20 µg of ^{125}I-labeled pigeon LDL protein/ml was added. After 5 hr incubation at 37°C, binding and internalization were measured by the indicated methods. Results are corrected for nonspecific processes as described under Methods and are the average of triplicate dishes.

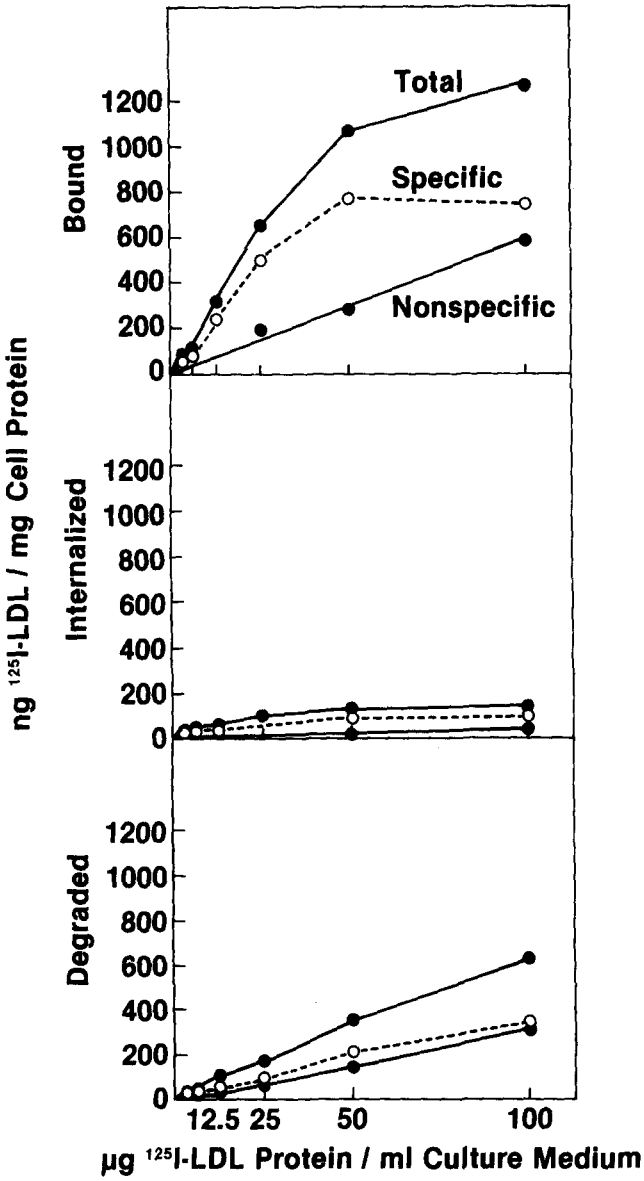

Fig. 3. Binding, internalization, and degradation of ^{125}I-labeled LDL by WC pigeon smooth muscle cells. Cells were incubated for 5 hr at 37°C with fresh medium containing FBS plus the indicated concentrations of ^{125}I-labeled pigeon LDL in the presence and absence of a 20-fold excess of unlabeled pigeon LDL. Binding and internalization were measured after removal of surface-bound material with trypsin. Specific binding, internalization, and degradation were calculated as described under Methods. Results are the average of duplicate dishes.

ized. Degradation was similar to internalization in that it was not saturable.

Among the apparent differences in the binding, internalization, and degradation of LDL by pigeon cells that distinguished them from mammalian cells was the shape of the binding curve (**Fig. 4**). The shape of the binding curve for pigeon cells was sigmoidal (at both 4°C and 37°C), not hyperbolic as has been described in human (26), monkey (16), and rat cells (30). This suggested some cooperativity of binding of LDL to pigeon cells. When the binding data were analyzed according to the method of Scatchard (31), there was a striking difference between monkey (Fig. 4A) and pigeon

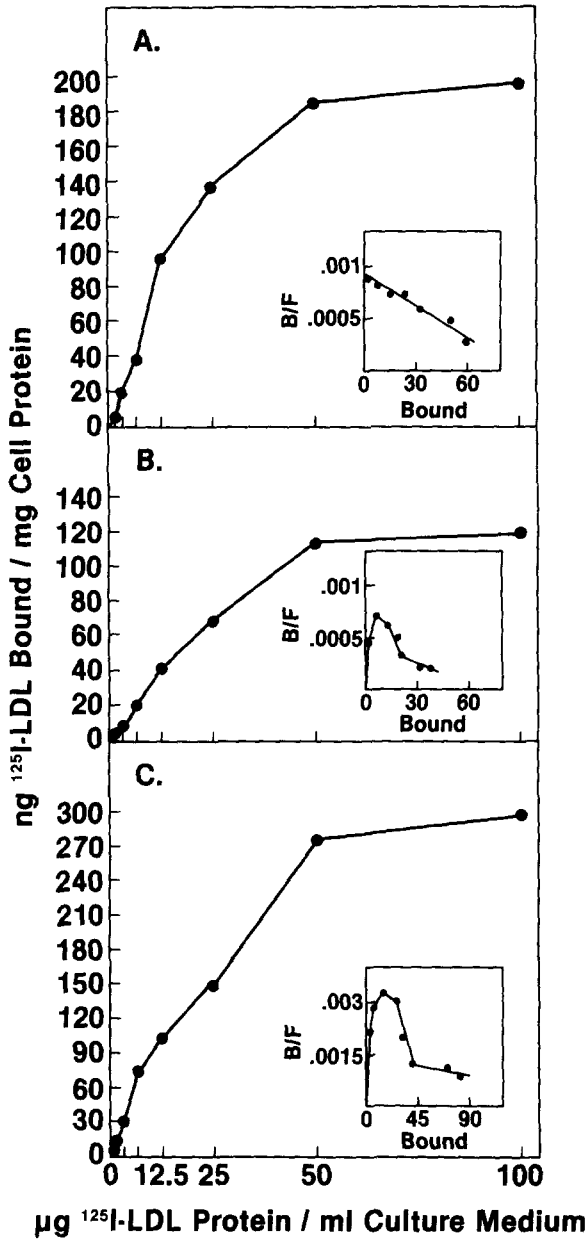

Fig. 4. Comparison of binding ^{125}I-labeled pigeon LDL by monkey and WC pigeon smooth muscle cells. Confluent monkey (panel A) and pigeon cells (panels B, C) were preincubated with medium containing LPDS or FBS, respectively, for 24 hr. Fresh medium containing LPDS (for monkey cells) or FBS (for pigeon cells) plus the indicated concentrations of ^{125}I-labeled pigeon LDL were added and the cells were incubated for 5 hr at 37°C (panel A, B) or 4°C (panel C). Cells were washed extensively as described in the Methods and binding was measured by heparin release (panel A, B) or by NaOH digestion (panel C). The inserts display the Scatchard plot for each experiment. B/F represents the lipoprotein bound (ng protein/dish), divided by the lipoprotein free in the medium (ng/2.0 ml).

cells (Fig. 4B and 4C). The complex nature of binding of pigeon LDL to pigeon cells appeared to be a function of the cells themselves and not the LDL, since binding of the same pigeon LDL to monkey cells resulted in a linear Scatchard plot (Fig. 4A).

Unlike the binding of LDL to LDL receptors on human skin fibroblasts, binding of LDL to pigeon smooth muscle cells did not require calcium and was unaffected by pronase treatment (**Table 4**).

The specificity of LDL binding is shown in **Fig. 5.** Both monkey and pigeon LDL competed effectively with ^{125}I-labeled pigeon LDL for binding to pigeon

TABLE 4. Effect of calcium and pronase on LDL binding to pigeon and monkey smooth muscle cells

	Pronase		Calcium			
			4°C		37°C	
	−	+	0	2 mM	0	2 mM
	ng ^{125}I-labeled LDL/mg cell protein					
Monkey SMC						
Bound	199	69	55	137		
Cell-associated	1259	303				
Degraded	607	108			13	369
Pigeon SMC						
Bound	24	39	67	64		
Cell-associated	79	57				
Degraded	204	227			30	25

Cells were grown to confluence in medium containing serum. The monkey cells were incubated for an additional 48 hr in medium containing LPDS and the pigeon cells were incubated for 48 hr with fresh medium containing FBS. For the pronase experiment, the cells were incubated for 45 min with 3 μg pronase/ml of HEPES-buffered medium without serum. The medium was discarded and the cells were washed two times with albumin (2 mg/ml)-PBS solution and the cells were incubated for 4 hr at 37°C with ^{125}I-labeled pigeon LDL (10 μg/ml) in LPDS containing HEPES-buffered medium. Surface-bound LDL was released with trypsin, and cell-associated and degraded LDL were determined as described in Methods. For the calcium experiments, bound LDL was determined at 4°C as follows. Cells were preincubated for 30 min at 4°C, washed three times with ice-cold PBS containing 100 μM EDTA, and incubated for 1 hr in HEPES-buffered saline containing 100 μM EDTA, 2.5 mg glucose/ml, 2.5 mg LPDS/ml, and 10 μg/ml of ^{125}I-labeled pigeon LDL. Cells were washed extensively as described in Methods and surface-bound LDL was released with heparin. Cells remained attached to the dish during the 1-hr incubation. The effect of calcium on LDL degradation was determined at 37°C as follows. At 37°C without Ca^{2+} in the medium, cells released from the dishes within 30–60 min. Thus, in order to compare LDL degradation on cells in the same growth conditions, we first washed cells three times with PBS containing 100 μM EDTA and incubated the cells for 1 hr at 37°C in HEPES-buffered saline containing 50 μM EDTA, 2.5 mg glucose/ml, and 2.5 mg LPDS/ml. The detached cells were transferred to 60-mm petri dishes (not tissue culture dishes) in the same medium. To this was added ^{125}I-labeled pigeon LDL (final concentration 10 μg/ml) with or without Ca^{2+}. After 3 hr incubation at 37°C, the cells were pelleted by centrifugation and an aliquot of the cell-free supernatant fluid was analyzed for ^{125}I-labeled LDL degradation as described in Methods. During the 3-hr incubation, all cells remained unattached to the dishes. Results are the mean of three dishes for all experiments except the 37°C calcium experiments, in which results are the mean of duplicate dishes.

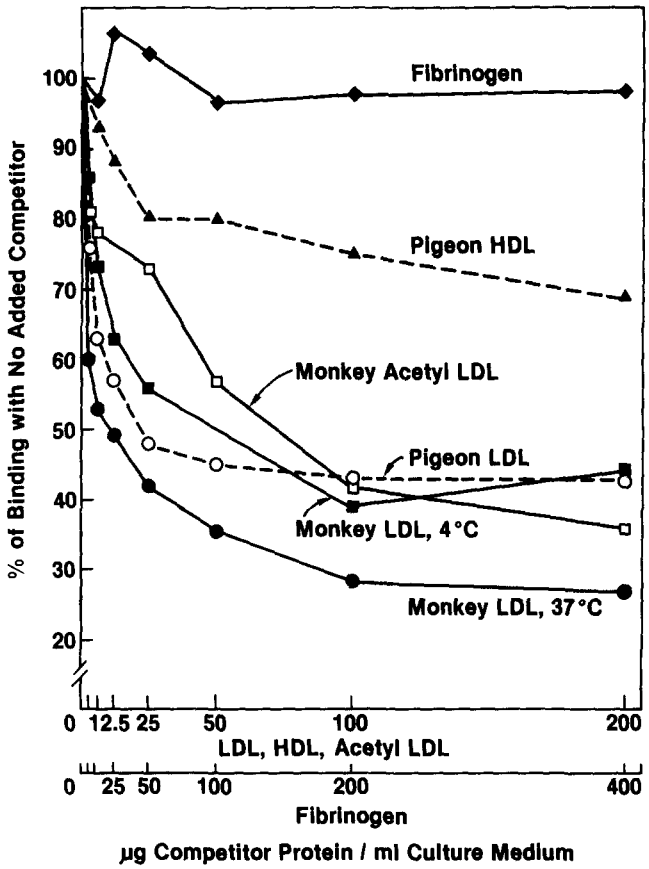

Fig. 5. Competition of pigeon HDL (▲) and LDL (○), monkey LDL (■, ●) and acetyl LDL (□), and fibrinogen (♦) for binding of ^{125}I-labeled pigeon LDL by pigeon smooth muscle cells. Cells were grown to confluence in medium containing serum. Fresh medium containing FBS was added along with 10 μg of protein of ^{125}I-labeled pigeon LDL and the indicated concentrations of unlabeled proteins. Monkey LDL (37°C), pigeon LDL, and pigeon HDL were incubated with the cells for 5 hr at 37°C; cells were washed extensively and binding was measured as heparin-releasable ^{125}I. Monkey LDL (4°C), monkey acetyl LDL, and fibrinogen were incubated with the cells at 4°C for 3 hr; cells were washed extensively and binding was measured as total cell-associated ^{125}I after digestion of the cells with NaOH. Results are the mean of duplicate cultures at each point and include data from two separate experiments.

cells. A similar result was obtained using monkey cells and ^{125}I-labeled monkey LDL (data not shown). At equivalent protein concentrations, monkey LDL was consistently 10–15% more effective as a competitor for ^{125}I-labeled LDL binding than was pigeon LDL. Acetyl LDL was nearly as effective a competitor as native LDL, while pigeon HDL competed less effectively, and fibrinogen not at all. In parallel studies with monkey cells, pigeon HDL did not compete for binding with ^{125}I-labeled monkey LDL (data not shown).

To confirm the apparent lack of high affinity LDL internalization suggested by the data in Table 4 and Fig. 3, cells were incubated with LDL whose protein moiety was labeled with ^{125}I and whose cholesteryl ester

moiety was labeled with [^{3}H]cholesteryl oleate (**Fig. 6**). In monkey cells, with increasing time of incubation, LDL was internalized and reached a plateau by approximately 3 hr. The [^{3}H]cholesteryl ester was also internalized and rapidly hydrolyzed to [^{3}H]free cholesterol. Both ^{125}I-labeled degradation products and [^{3}H]free cholesterol were lost from the cells and accumulated in the medium with time.

The internalization of ^{125}I by pigeon cells was less than 20% of that seen in monkey cells. Even this was probably an overestimate since a negligible amount of ^{3}H was found in the cells. The small amounts of ^{125}I found in the cells probably represent uptake of either ^{125}I-labeled degradation products present in the culture medium or exchange with small amounts of ^{125}I-labeled phospholipids of the original LDL, rather than uptake of the intact LDL particle. The failure to find ^{3}H in the cells was not the result of uptake, hydrolysis, and efflux of [^{3}H]cholesterol since only trace amounts of [^{3}H]free cholesterol were found in the culture medium of pigeon cells even after 24 hr in incubation.

In order to determine whether lysosomes were involved in the degradation process, cells were incubated with the ^{3}H-CE/^{125}I-labeled LDL in the presence and absence of chloroquine, a known inhibitor of lysosomal function in both mammalian (32) and avian (33) species. As shown in **Table 5,** and consistent with previous findings from this laboratory (2) and those of Goldstein, Brunschede, and Brown (32), chloroquine markedly inhibited the degradation of ^{125}I-labeled LDL protein and the hydrolysis of LDL [^{3}H]cholesteryl esters in monkey cells. Consistent with a lysosomal site of LDL degradation, there were large accumulations of both ^{125}I and ^{3}H-cholesteryl esters in monkey cells incubated with chloroquine. Chloroquine, however, had no effect on either degradation or accumulation of LDL in pigeon cells, suggesting that degradation of LDL by pigeon cells did not occur in the lysosomal compartment. These results further support the conclusion that LDL was not internalized.

In other studies, leupeptin (a thiol-cathepsin inhibitor (34)) was compared with chloroquine for its ability to inhibit LDL degradation by pigeon smooth muscle cells (**Table 6**). Neither the general lysosomotropic agent

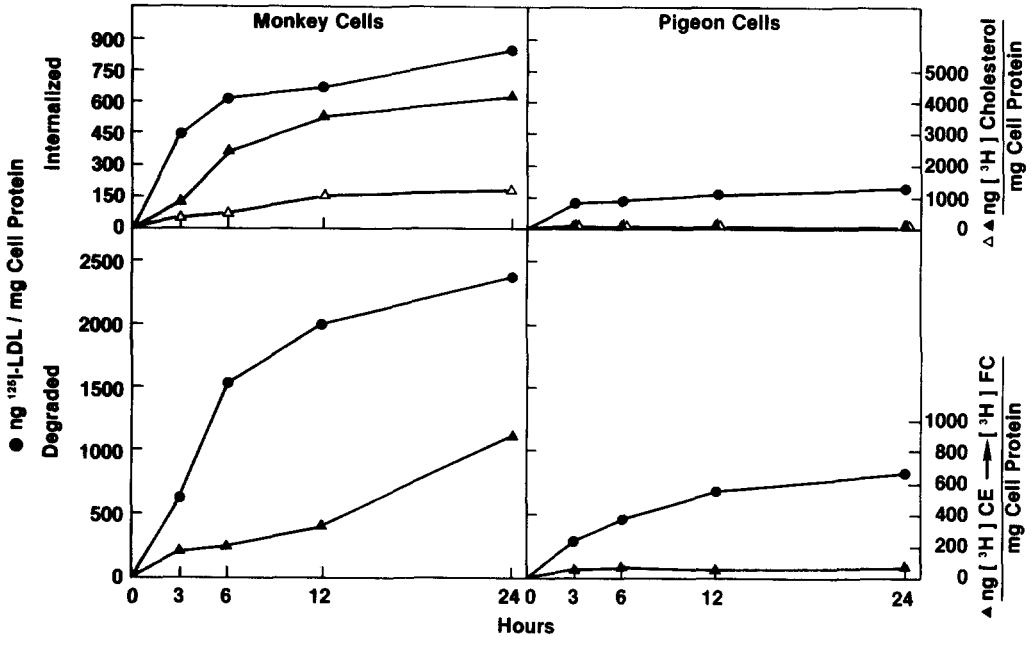

Fig. 6. Internalization and degradation of [^{3}H]CE/[^{125}I] monkey LDL by monkey and WC pigeon smooth muscle cells. Cells were grown to confluence in medium containing serum. Monkey cells were incubated for an additional 24 hr with LPDS in order to increase the number of LDL receptors. Fresh medium containing FBS (for pigeon cells) or LPDS (for monkey cells) was added along with the [^{3}H]CE/[^{125}I] monkey LDL at 20 μg protein/ml medium. At the indicated times, surface-bound LDL was removed with trypsin and the amount of [^{125}I] (●), [^{3}H]free cholesterol (▲), and [^{3}H]cholesteryl ester (△) internalized was measured. The ^{125}I-labeled LDL degradation was measured in the culture medium as described in Methods and the amount of [^{3}H]free cholesterol in the medium was determined as a measure of hydrolysis of the [^{3}H]cholesteryl ester of the LDL and its efflux from the cell as [^{3}H]free cholesterol. The original [^{3}H]CE/[^{125}I]LDL had greater than 99% of the [^{3}H] as [^{3}H]cholesteryl ester. In all of these studies control values (from dishes with no cells) were subtracted in order to correct for cholesteryl ester hydrolysis and protein degradation in the medium alone. Results represent total internalized and degraded LDL and were not corrected for nonspecific uptake. All values are the average of duplicate dishes.

TABLE 5. Effect of chloroquine on internalization and degradation of ^{3}H-CE/^{125}I-LDL by pigeon and monkey smooth muscle cells

	Cell-Associated			Degraded	
	^{125}I	^{3}H-FC	^{3}H-CE	^{125}I	^{3}H-FC
	ng/mg cell protein				
Monkey cells					
Control	841	4,168	1,181	2,350	981
Chloroquine	5,518	1,475	11,186	1,000	0
Pigeon cells					
Control	204	116	38	731	55
Chloroquine	220	97	21	791	60

Cells were grown and preincubated as described in the legend of Table 3. Cells then received medium containing FBS (pigeon cells) or LPDS (monkey cells) plus ^{3}H-CE/^{125}I-LDL at a protein concentration of 20 μg/ml and were incubated for 24 hr at 37°C with and without 50 μM chloroquine. Cell-associated radioactivity was determined after treatment of the cells with trypsin. Degradation and hydrolysis of ^{3}H-CE/^{125}I-LDL were determined as described under Methods. Results are the average of duplicate dishes.

(chloroquine) nor the specific protease inhibitor (leupeptin) were able to affect LDL degradation by pigeon cells. In contrast, chloroquine and leupeptin inhibited LDL degradation by monkey cells, consistent with the results of others using bovine arterial smooth muscle cells (34). These results, together with the results shown in Table 5, indicate that the lysosomes per se were not involved in the degradation of LDL by pigeon cells.

The pattern of fluorescence of cells incubated with DiI-labeled LDL is shown in **Fig. 7.** Fluorescence was compared in pigeon and monkey smooth muscle cells and in skin fibroblasts from a human patient with the LDL receptor-negative form of familial hypercholesterolemia. In contrast to cells that lack functional LDL receptors (FH cells), pigeon cells exhibited fluorescence that was more abundant and more evenly distributed over the cell surface. Under the same conditions, monkey cells had much more fluorescence, with greatest intensity in the perinuclear region of the cells. There was no such perinuclear concentration of fluorescence in pigeon cells. These results are consistent with the binding and

TABLE 6. Effect of chloroquine and leupeptin on the degradation of LDL by monkey and pigeon smooth muscle cells

Cells	Inhibitor	ng ^{125}I-labeled LDL Degraded/ mg Cell Protein
Pigeon	None	572
	Leupeptin	548
	Chloroquine	560
Monkey	None	1309
	Leupeptin	906
	Chloroquine	601

Pigeon and monkey smooth muscle cells were grown to confluence in medium containing serum. Monkey cells were preincubated an additional 24 hr in medium containing LPDS. Fresh medium containing FBS (pigeon cells) or LPDS (monkey cells) plus 20 μg ^{125}I-labeled monkey LDL protein/ml and leupeptin (20 μg/ml) or chloroquine (50 μM) were added and incubation was continued for 5 hr at 37°C. Aliquots of medium were taken for determination of ^{125}I-labeled LDL degradation. Average cellular protein concentration for dishes of pigeon and monkey cells were 147 and 226 μg protein/dish, respectively. Values are the average of duplicate dishes.

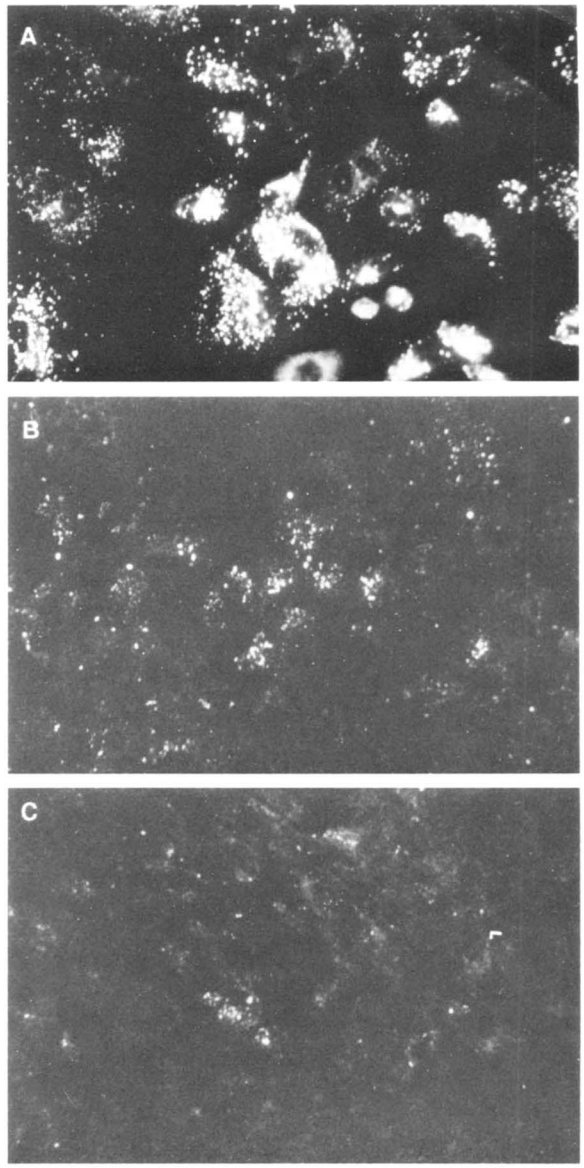

Fig. 7. Binding and uptake of DiI-monkey LDL by monkey (A) and WC pigeon (B) smooth muscle cells and skin fibroblasts from a patient with the homozygous LDL-receptor negative form of FH (C). Cells were grown to confluence on glass cover slips in medium containing FBS and monkey and FH cells were incubated for an additional 24 hr with medium containing LPDS. Cells were incubated with fresh FBS-containing medium (for pigeon cells) or LPDS-containing medium (for monkey and FH cells) plus DiI-labeled monkey LDL (20 μg of protein/ml) for 3 hr at 37°C. Cells were then washed extensively, fixed with neutral buffered formalin, and observed by fluorescence microscopy (80×).

TABLE 7. Metabolism of ^{125}I-labeled LDL in confluent and nonconfluent pigeon smooth muscle cells

	Protein/Dish	Bound[a]	Cell-Associated[a]	Degraded[a]
	μg	ng ^{125}I-labeled LDL/mg cell protein		
Confluent	500 ± 11	28 ± 6	118 ± 5	335 ± 24
Nonconfluent	222 ± 12	38 ± 3	135 ± 4	445 ± 24

White Carneau pigeon smooth muscle cells (BB-1) were plated on day 0 at 0.75 × 10^6 or 1.5 × 10^6 cells/60-mm dish in medium containing 10% fetal bovine serum. On day 2, the medium was replaced with fresh medium. On day 3, 15 μg of ^{125}I-labeled LDL/ml, isolated from cholesterol-fed pigeons, was added and incubated with the cells for 5 hr at 37°C. After washing the cells as described in Methods, the surface-bound LDL was removed with trypsin, and cell-associated and degraded LDL were determined as described. Degradation was corrected for the amount of TCA-soluble noniodide ^{125}I in no-cell control dishes.

[a] Mean ± SEM; N = 4.

internalization of LDL by monkey cells and the concentration of the fluorescent label in the lysosomes (25), whereas in pigeon cells the pattern of fluorescence is consistent with surface binding, but a lack of internalization.

Cultured bovine endothelial cells have been reported to bind LDL to surface receptors, but to internalize the LDL only when actively proliferating (35). Since our studies were done with confluent cells, it was possible that, like bovine endothelial cells, LDL was internalized only while cells were nonconfluent. To test this possibility we incubated pigeon smooth muscle cells with ^{125}I-labeled LDL under conditions where the cells were either confluent or nonconfluent (**Table 7**). The nonconfluent cells were 60–70% confluent as judged by phase contrast microscopy. This was consistent with the difference in cell protein per dish. Although slightly more LDL was bound, internalized, and degraded in the nonconfluent group there was no evidence to suggest that this was the result of a functional internalization component that was absent in the confluent cells. This conclusion is supported by the observation that there was little change in the proportion of cell-associated and degraded ^{125}I-labeled LDL relative to the amount bound in the confluent and nonconfluent cells. As a result, under the conditions of this experiment, pigeon smooth muscle cells appear to lack a functional LDL receptor pathway while actively proliferating as well as while quiescent. Since only one stage of cell growth was studied, we cannot exclude the possibility that during some short period of the cell cycle a functional LDL receptor pathway is expressed. This seems to us unlikely, however.

To test whether LDL binding to pigeon cells could be regulated, pigeon smooth muscle cells were preincubated with LPDS, LDL, cholesterol dissolved in ethanol, or 25-OH cholesterol dissolved in ethanol for up to 24 hr, and the extent of specific binding of ^{125}I-labeled LDL was determined. As can be seen from **Fig. 8**, neither LDL nor cholesterol had any effect on LDL binding, while LPDS reduced LDL binding at the 24-hr time period. Incubation with 25-OH cholesterol, in contrast, resulted in a marked increase in LDL binding. The failure to alter LDL binding by incubation with LDL or cholesterol occurred in spite of an approximate doubling in cellular free cholesterol content (**Table 8**). Incubation with LPDS for 24 hr did not change the cholesterol content of the cells but, nevertheless, reduced LDL binding by 40%. The response of LDL binding in

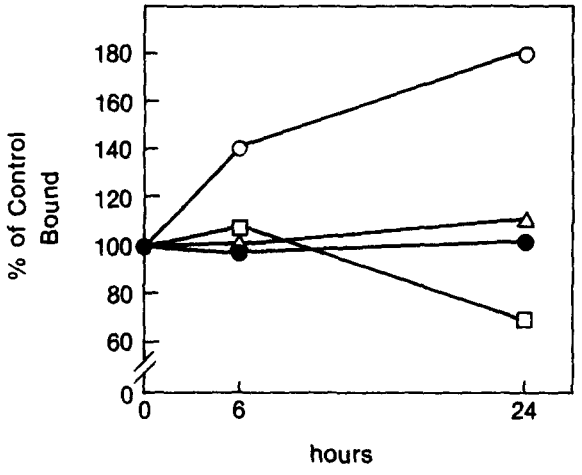

Fig. 8. Effect of LDL, LPDS, 25-OH cholesterol; and cholesterol added in ethanol on binding of ^{125}I-labeled LDL by WC pigeon smooth muscle cells. Cells were grown to confluence in medium containing FBS. At zero time, cells received medium containing LPDS (□), medium containing FBS and 50 μg of monkey LDL protein/ml (●), medium containing FBS plus cholesterol (△) (sufficient to give a final concentration of 100 μg/ml) in 20 μl ethanol, or medium containing FBS plus 25-OH cholesterol (○) (giving a final concentration of 10 μg/ml). Control dishes containing LDL and LPDS also received 20 μl of ethanol. At the indicated times, cells were washed and incubated with 20 μg of ^{125}I-labeled monkey LDL/ml for an additional 5 hr at 37°C in the presence or absence of a 20-fold excess of unlabeled LDL. Binding was determined by releasing surface ^{125}I with trypsin. Results represent specific binding only. Zero time specific binding was 260 ng of ^{125}I-labeled LDL protein/mg cell protein. Results are the average of duplicate cultures.

TABLE 8. Effect of LPDS, LDL, and cholesterol on cholesterol mass in pigeon smooth muscle cells

Treatment	FC	CE
	μg/mg cell protein	
FBS control	15.4	0.6
LPDS control	16.0	0.4
LDL	25.5	0.9
Cholesterol	33.6	0.7

Cells were grown to confluence in medium containing FBS. The various treatment groups were prepared exactly as described for Fig. 8. After 24 hr of incubation at 37°C, free cholesterol (FC) and cholesteryl ester mass (CE) were determined as described. Results are the mean of duplicate cultures.

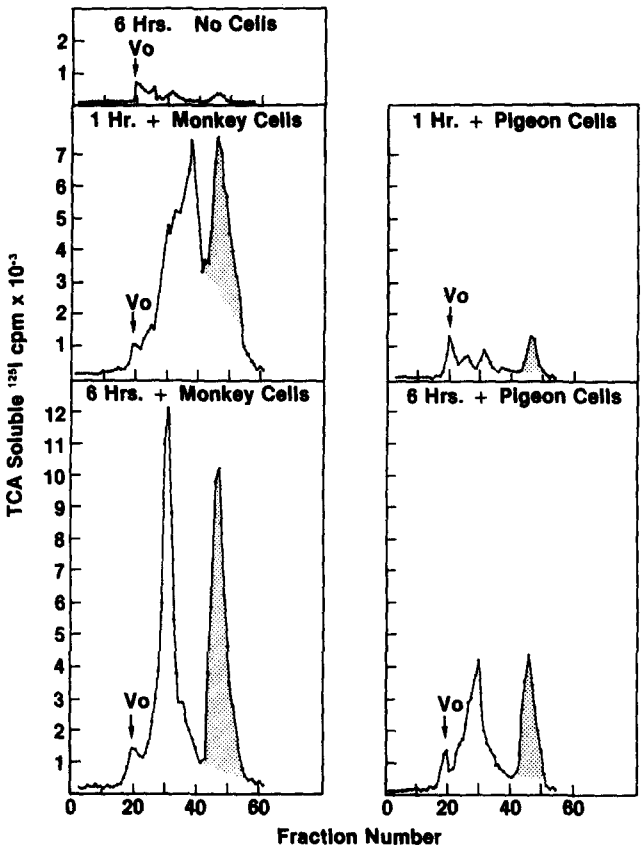

Fig. 9. Sephadex G-10 elution profile of ^{125}I-labeled TCA-soluble LDL degradation products from monkey and WC pigeon smooth muscle cells. Prior to the experiment, pigeon and monkey cells were preincubated with medium containing FBS or LPDS, respectively, for 48 hr in order to maximize LDL binding and degradation. At the beginning of the experiment, the medium in each dish was replaced with 2 ml of medium containing LPDS and 10 μg of ^{125}I-labeled pigeon LDL/ml. One group of ten dishes with no cells was incubated for 6 hr at 37°C. Other groups of cells of ten dishes each were incubated for 1 or 6 hr at 37°C. After the appropriate incubation period, medium from each group of ten dishes was collected, pooled, and prepared for chromatography as described under Methods. Retained peaks eluting at exactly the same volume are indicated by the stippling. Recovery of radioactivity after chromatography was approximately 77%. The void volume of the column was determined using blue dextran. The average cell protein for dishes from which the monkey or pigeon medium was obtained was 362 μg and 212 μg/dish, respectively.

cells incubated with LPDS or 25-OH cholesterol was opposite to that demonstrated in mammalian cells treated in a similar fashion (36).

Even though bound LDL was not internalized by pigeon cells, there was considerable LDL degradation that could not be accounted for by "nonspecific" processes (Fig. 3). Although small amounts of proteolytic activity against ^{125}I-labeled LDL could be detected in the cell-free culture medium that had been incubated with pigeon cells for greater than 24 hr, little proteolytic activity could be detected in culture medium exposed to cells for less than 24 hr. Consequently, the degradation that is measured after 5 hr of incubation (Tables 3, 6, and Fig. 4) appears to be entirely the result of cell-associated (presumably cell surface) proteolytic activity.

The characterization of the TCA-soluble degradation products of ^{125}I-labeled pigeon LDL incubated with pigeon and monkey smooth muscle cells is shown in **Figs. 9** and **10.** Separation of degradation products by chromatography on Sephadex G-10 indicated two major peaks for both pigeon and monkey cells, with greater than 90% of the TCA-soluble material from the 6-hr incubation eluting in the inclusion volume of the column

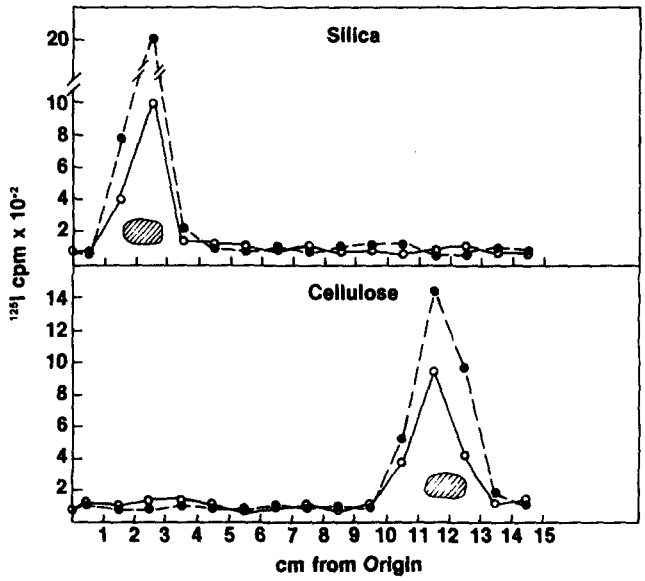

Fig. 10. Thin-layer chromatography profile of ^{125}I radioactivity from the most retained fraction (stippled area) from Sephadex G-10 column chromatography shown in Fig. 9. The ^{125}I-labeled TCA-soluble degradation products from the stippled peak from Fig. 9 (6 hr incubation) were pooled and separated by TLC on plastic sheets coated with silica gel or cellulose. An authentic L-tyrosine standard was spotted on sheets with the test material and co-chromatographed. After chromatography (as described under Methods), the sheets were dried, sprayed with ninhydrin, and developed at 90°C for 5 min to localize the L-tryrosine. Thin-layer sheets were cut into 1-cm strips and counted directly for ^{125}I. The cross-hatched spot indicates the location of the L-tryrosine standard. Open symbols (○) represent material from pigeon cells and closed symbols (●) represent material from monkey cells.

(Fig. 9). The material in the peak indicated by the stippling was further characterized by TLC and found to be identical with ^{125}I-labeled tyrosine (Fig. 10). The other major peak eluted earlier than tyrosine and probably represents a mixture of polypeptide degradation products. Thus, greater than 90% of the TCA-soluble ^{125}I-labeled LDL degradation products from monkey and pigeon cells were amino acids or short polypeptides.

DISCUSSION

Results of this study show that LDL binds to pigeon smooth muscle cells by a saturable and high affinity process ($T_{1/2}$ approximately 15 μg protein/ml), and that a significant proportion of the bound LDL can be released by heparin. These are characteristics of LDL binding to the classical mammalian LDL receptor (1). On the other hand, there are some obvious differences in the characteristics of LDL binding to pigeon cells that distinguish it from the classical LDL receptor pathway. Scatchard analysis showed a complex pattern of binding suggestive of both positive cooperativity and at least two classes of binding sites. When the same pigeon LDL was incubated with monkey cells, Scatchard analysis indicated binding to only a single class of receptors as has been shown previously with monkey LDL (16). As a result, these differences are clearly a property of the cells themselves and are not due to differences in the pigeon LDL.

Low density lipoprotein binding to the classical mammalian LDL receptor has an absolute requirement for calcium (26), is diminished by pretreatment of cells with proteolytic enzymes such as pronase (26), and is highly specific as seen by the failure of certain other lipoproteins, unrelated proteins and chemically modified lipoproteins such as acetyl LDL, to compete for binding (1, 37). In contrast, LDL binding to pigeon smooth muscle cells did not require calcium, was not inhibited by treatment with pronase, and was competed for by acetyl LDL and to a lesser extent by HDL. Competition with HDL could not be explained by the presence of apoE since no apoprotein of the same size as mammalian apoE was seen on PAGE gels. Taken together, these data suggest that LDL binding to pigeon cells is not mediated by the classical LDL receptor. Instead it has many of the properties of a less specific binding site that has been described for lipoprotein binding to hepatic and adrenal cell membranes. Binding of LDL to this site does not require calcium, is not influenced by pronase treatment, and is competed for by a variety of lipoproteins such as LDL, apoE-free HDL, acetyl LDL, and methyl LDL (38–43). A further characteristic of the multiple lipoprotein binding site of liver and adrenal is that LDL, although bound, is not degraded, presumably because it is not internalized (39). Again, this is similar to pigeon cells in which no LDL internalization was demonstrated. It is also possible that the binding of LDL to pigeon cells does not represent binding to a true receptor but rather may be the result of lipid-lipid interactions between lipid domains of the LDL and the plasma membrane, as has been suggested by Fogelman et al. (44) for the uptake of methyl LDL by human monocyte macrophages.

Pigeon cells also differ from mammalian cells in their inability to down-regulate the surface binding of LDL even under conditions in which the cellular cholesterol content is nearly doubled. The mechanism of free cholesterol enrichment in pigeon cells incubated with LDL presumably involves surface transfer of free cholesterol from LDL to the plasma membranes of the cells. A similar enrichment of cellular free cholesterol content can also be observed in FH cells incubated with LDL (24). In normal mammalian cells and in FH cells, the addition of cholesterol in ethanol results in an increase in cellular cholesterol content and the down-regulation of LDL receptor activity on the cell surface (29). This did not occur in pigeon cells, however, suggesting that the site on pigeon cells to which LDL binds is not down-regulated by an increase in cellular cholesterol content similar in magnitude to that shown to down-regulate the LDL receptor on mammalian cells.

LDL binding to pigeon cells can be modulated, however, by LPDS and 25-OH cholesterol. LPDS caused a decrease in LDL binding while 25-OH cholesterol caused an increase. These changes are opposite to those observed in mammalian cells under similar conditions and suggest that LDL binding to pigeon cells does not function to deliver cholesterol to cells. Rather it might be speculated that the site on pigeon cells that binds LDL mediates cellular cholesterol efflux in response to enhancement of the concentration of certain critical intracellular cholesterol pools. Such an hypothesis is consistent with the report by Biesbroek et al. (43) indicating the presence of an HDL receptor on mammalian skin fibroblasts having many of the properties of LDL binding to pigeon cells and whose activity is up-regulated by increases in cholesterol content. Although enrichment of the cells with cholesterol did not alter LDL binding to pigeon cells, 25-OH cholesterol did increase LDL binding in a manner similar to its enhancement of HDL binding to endothelial cells (45) and to fibroblasts and smooth muscle cells (46). It should be emphasized, however, that we have yet to demonstrate a physiological function of lipoprotein binding to pigeon cells.

In spite of the failure of pigeon cells to internalize LDL, there was considerable degradation of LDL. Sev-

eral lines of evidence support the conclusion that this degradation occurred on the cell surface and not within the lysosomes of the cells. Degradation was not susceptible to inhibition by the general lysosomal enzyme inhibitor chloroquine, or the lysosomal cathepsin B inhibitor, leupeptin. Degradation of LDL could not be attributed to secretion of soluble proteases into the culture medium since there was little proteolytic activity detectable in the culture medium in incubations of less than 24 hr. Taken together, these observations lead to the conclusion that degradation occurred on the cell surface and not within the lysosomes of the cells.

Although there was no evidence of lysosomal degradation of LDL in pigeon cells, the products of proteolytic degradation of LDL were remarkably similar to those seen in monkey cells. Similar results have been reported for the LDL degradation products from normal and receptor-negative FH skin fibroblasts (26). This observation, plus the demonstration that chloroquine has a small, but measurable, inhibitory effect on the degradation of LDL by FH cells, has been interpreted by Goldstein and Brown (26) as evidence that the presumed "nonspecific" degradation of LDL occurs in the lysosomes. Since chloroquine had little effect on LDL degradation, it does not appear that a similar "nonspecific" pathway could be responsible for the degradation of LDL by pigeon cells.

A major question raised by these studies is the physiological role in pigeon cells of LDL binding in the absence of internalization. In mammalian cells LDL binds to LDL receptors, is internalized, and ultimately provides cholesterol to the cell for a variety of cellular needs. Low density lipoprotein does not appear to serve this function in pigeon cells, suggesting that this function is served either by another lipoprotein or by endogenous cholesterol synthesis. Previous studies from this laboratory have shown that even whole pigeon serum is ineffective in stimulating cholesteryl ester accumulation and cholesterol esterification in pigeon cells (15). This suggests that the other lipoproteins present in whole serum are also ineffective in promoting the influx of extracellular cholesterol. As a result, cellular cholesterol homeostasis in pigeon cells must be regulated at the level of cholesterol synthesis and/or cholesterol efflux.

Since the present studies utilized only cells from the atherosclerosis-susceptible WC pigeon, it is not known whether the atherosclerosis-resistant Show Racer (SR) pigeon also lacks a functional LDL receptor pathway. In a previous study, however, both WC and SR pigeon smooth muscle cells failed to accumulate cholesteryl esters or to have cholesterol esterification stimulated when exposed to whole serum (15). This suggests that a similar pattern of LDL metabolism occurs in cells from both WC and SR pigeons. Consequently, it remains to be seen whether the lack of LDL internalization plays a direct role in the susceptibility or resistance of the two breeds of pigeons to atherosclerosis. Nevertheless, the absence of a functional LDL receptor pathway and the marked susceptibility to the development of atherosclerosis gives the WC pigeon a high degree of similarity to humans with familial hypercholesterolemia (1) and to Watanabe rabbits (47).

We acknowledge the excellent technical assistance of Ms. Molly Leight, Ms. Grayce Green, Mr. Ramesh Shah, and Mrs. Patricia Hester, and the assistance of Mrs. Barbara Lindsay and Mrs. Brenda Warner in the preparation of this manuscript. This work was supported by SCOR Grant HL-14164 from the National Heart, Lung, and Blood Institute. Dr. Randolph was supported by National Research Service Award Institutional Grant HL-07115 and by a Special Fellowship from the R. J. Reynolds Co.

Manuscript received 22 February 1984.

REFERENCES

1. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46:** 897–930.
2. Guertler, L. S., and R. W. St. Clair. 1980. Low density lipoprotein receptor activity on skin fibroblasts from rhesus monkeys with diet-induced or spontaneous hypercholesterolemia. *J. Biol. Chem.* **255:** 92–99.
3. Shimado, Y., K. Tanzawa, M. Kurada, Y. Tsujita, M. Arai, and Y. Watanabe. 1981. Biochemical characterization of skin fibroblasts derived from WHHL-rabbit: a notable animal model for familial hypercholesterolemia. *Eur. J. Biochem.* **118:** 557–564.
4. Weinstein, D. B., T. E. Carew, and D. Steinberg. 1976. Uptake and degradation of low density lipoprotein by swine arterial smooth muscle cells with inhibition of cholesterol biosynthesis. *Biochim. Biophys. Acta.* **424:** 404–421.
5. Assmann, G., G. B. Brown, and R. W. Mahley. 1975. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured swine aortic smooth muscle cells by plasma lipoproteins. *Biochemistry.* **14:** 3996–4002.
6. Davies, P. F., and C. Kerr. 1982. Modification of low density lipoprotein metabolism by growth factors in cultured vascular cells and human skin fibroblasts. Dependence upon duration of exposure. *Biochim. Biophys. Acta.* **712:** 26–32.
7. Bierman, E. L., O. Stein, and Y. Stein. 1974. Lipoprotein uptake and metabolism by rat aortic smooth muscle cells in tissue culture. *Circ. Res.* **35:** 136–150.
8. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1980. Disparities in the interaction of rat and human lipoproteins with cultured rat fibroblasts and smooth muscle cells. Requirements for homology for receptor binding activity. *J. Biol. Chem.* **255:** 11163–11172.
9. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1974.

Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 789–796.
10. Brown, M. S., J. R. Faust, and J. L. Goldstein. 1975. Role of the low density lipoprotein receptor in regulating the content of free and esterified cholesterol in human fibroblasts. *J. Clin. Invest.* **55:** 783–793.
11. Brown, M. S., and J. L. Goldstein. 1974. Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Proc. Natl. Acad. Sci. USA.* **71:** 788–792.
12. Mahley, R. W., and T. L. Innerarity. 1978. Properties of lipoproteins responsible for high affinity binding to cell surface receptors of fibroblasts and smooth muscle cells. *Adv. Exp. Med. Biol.* **109:** 99–127.
13. Brown, M. S., and J. L. Goldstein. 1974. Suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in inhibition of growth of human fibroblasts by 7-ketocholesterol. *J. Biol. Chem.* **249:** 7306–7314.
14. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1975. Cholesterol ester formation in cultured human fibroblasts. *J. Biol. Chem.* **250:** 4025–4027.
15. Smith, B. P., R. W. St. Clair, and J. C. Lewis. 1979. Cholesterol esterification and cholesteryl ester accumulation in cultured pigeon and monkey arterial smooth muscle cells. *Exp. Mol. Pathol.* **30:** 190–208.
16. St. Clair, R. W., J. J. Mitschelen, and M. A. Leight. 1980. Metabolism by cells in culture of LDL of abnormal composition from nonhuman primates with diet-induced hypercholesterolemia. *Biochim. Biophys. Acta.* **618:** 63–79.
17. St. Clair, R. W., B. P. Smith, and L. L. Wood. 1977. Stimulation of cholesterol esterification in rhesus monkey arterial smooth muscle cells. *Circ. Res.* **40:** 166–173.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randal. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
20. Ishikawa, T. T., J. MacGee, J. A. Morrison, and C. J. Glueck. 1974. Quantitative analysis of cholesterol in 5 to 20 μl of plasma. *J. Lipid Res.* **15:** 286–291.
21. St. Clair, R. W., and M. A. Leight. 1978. Differential effects of isolated lipoproteins from normal and hypercholesterolemic rhesus monkeys on cholesterol esterification and accumulation in arterial smooth muscle cells in culture. *Biochim. Biophys. Acta.* **530:** 279–291.
22. Rudel, L. L., L. L. Pitts II, and C. A. Nelson. 1977. Characterization of plasma low density lipoproteins of nonhuman primates fed dietary cholesterol. *J. Lipid Res.* **18:** 211–222.
23. Parks, J. S., and L. L. Rudel. 1979. Isolation and characterization of high density lipoprotein apoproteins in the nonhuman primate (vervet). *J. Biol. Chem.* **254:** 6716–6723.
24. St. Clair, R. W., P. Greenspan, and M. Leight. 1983. Enhanced cholesterol delivery to cells in culture by low density lipoproteins from hypercholesterolemic monkeys: correlation of cellular cholesterol accumulation with low density lipoprotein molecular weight. *Arteriosclerosis.* **3:** 77–86.
25. Pitas, R. E., T. L. Innerarity, J. N. Weinstein, and R. W. Mahley. 1981. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. *Arteriosclerosis.* **1:** 177–185.
26. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. A comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
27. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its surface receptor by sulfated glycosaminoglycans. *Cell.* **7:** 85–95.
28. Rudel, L. L., D. G. Greene, and R. Shah. 1977. Separation and characterization of plasma lipoproteins of rhesus monkeys (*Macaca mulatta*). *J. Lipid Res.* **18:** 734–744.
29. Brown, M. S., and J. L. Goldstein. 1976. Analysis of a mutant strain of human fibroblasts with a defect in the internalization of receptor-bound low density lipoprotein. *Cell.* **9:** 663–674.
30. Drevon, C. A., A. D. Attie, S. H. Pangburn, and D. Steinberg. 1981. Metablism of homologous and heterologous lipoproteins by cultured rat and human skin fibroblasts. *J. Lipid Res.* **22:** 37–46.
31. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51:** 660–672.
32. Goldstein, J. L., G. Y. Brunschede, and M. S. Brown. 1975. Inhibition of the proteolytic degradation of low density lipoprotein in human fibroblasts by chloroquine, concanavalin A and Triton WR 1139. *J. Biol. Chem.* **250:** 7854–7862.
33. Krupp, M. N., and M. D. Lane. 1982. Evidence for different pathways for the degradation of insulin and insulin receptor in the chick liver cell. *J. Biol. Chem.* **257:** 1372–1377.
34. Van Der Westhuyzen, D. R., W. Gevers, and G. A. Coetzee. 1980. Cathepsin-D-dependent initiation of the hydrolysis by lysosomal enzymes of apoprotein B from low-density lipoproteins. *Eur. J. Biochem.* **112:** 153–160.
35. Fielding, P. E., I. Vlodavsky, D. Gospodarowicz, and C. J. Fielding. 1979. Effect of contact inhibition on the regulation of cholesterol metabolism in cultured vascular endothelial cells. *J. Biol. Chem.* **254:** 749–755.
36. Brown, M. S., and J. L. Goldstein. 1975. Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. *Cell.* **6:** 307–316.
37. Basu, S. K., J. L. Goldstein, G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
38. Basu, S. K., M. S. Brown, Y. K. Ho, and J. L. Goldstein. 1979. Degradation of low density lipoprotein: dextran sulfate complexes associated with deposition of cholesteryl esters in mouse macrophages. *J. Biol. Chem.* **254:** 7141–7146.
39. Bachorik, P. S., F. A. Franklin, D. G. Virgil, and P. O. Kwiterovich, Jr. 1982. High-affinity uptake and degradation of apolipoprotein E free high-density lipoprotein and low-density lipoprotein in cultured porcine hepatocytes. *Biochemistry.* **21:** 5675–5684.

40. Traber, M. G., V. Defendi, and H. J. Kayden. 1981. Receptor activities for low-density lipoprotein and acetylated low-density lipoprotein in a mouse macrophage cell line (IC21) and in human monocyte-derived macrophages. *J. Exp. Med.* **154:** 1852–1857.
41. Kita, T., M. S. Brown, Y. Watanabe, and J. L. Goldstein. 1981. Deficiency of low density lipoprotein receptors in liver and adrenal gland of the WHHL rabbit, an animal model of familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **78:** 2268–2272.
42. Hui, D. Y., T. S. Innerarity, and R. W. Mahley. 1981. Lipoprotein binding to canine hepatic membranes: metabolically distinct apoE and apoB,E receptors. *J. Biol. Chem.* **256:** 5646–5655.
43. Biesbroek, R., J. F. Oram, J. J. Albers, and E. L. Bierman. 1983. Specific high-affinity binding of high density lipoproteins to cultured human skin fibroblasts and arterial smooth muscle cells. *J. Clin. Invest.* **71:** 525–539.
44. Fogelman, A. M., M. M. Hokom, M. E. Haberland, R. D. Tanaka, and P. A. Edwards. 1982. Lipoprotein regulation of cholesterol metabolism in macrophages derived from human monocytes. *J. Biol. Chem.* **257:** 14081–14086.
45. Tauber, J. P., D. Goldminz, and D. Gospodarowicz. 1981. Up-regulation in vascular endothelial cells of binding sites of high density lipoprotein induced by 25-hydroxycholesterol. *Eur. J. Biochem.* **119:** 327–339.
46. Oram, J. F., E. A. Brinton, and E. L. Bierman. 1983. Regulation of high density lipoprotein receptor activity in cultured human skin fibroblasts and human skin fibroblasts and human arterial smooth muscle cells. *J. Clin. Invest.* **72:** 1611–1621.
47. Buja, L. M., T. Kita, J. L. Goldstein, Y. Watanabe, and M. S. Brown. 1983. Cellular pathology of progressive atherosclerosis in the WHHL rabbit: an animal model of familial hypercholesterolemia. *Arteriosclerosis.* **3:** 87–101.