Heparan sulfate proteoglycans mediate internalization and degradation of β-VLDL and promote cholesterol accumulation by pigeon macrophages

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Abstract Pigeon and rabbit β-migrating very low density lipoprotein (β-VLDL) are similar in size and composition, yet rabbit β-VLDL consistently stimulates greater cholesteryl ester accumulation in pigeon peritoneal macrophages than does pigeon β-VLDL. The purpose of this study was to determine the mechanism of this difference. Pigeon β-VLDL bound to both a high and low affinity site while rabbit β-VLDL bound primarily to a low affinity site. The high affinity site had the characteristics of the LDL receptor. Most rabbit β-VLDL and some pigeon β-VLDL bound to the low affinity site that was not down-regulated by cholesterol loading. β-VLDL binding to the low affinity site and subsequent internalization and degradation were mediated by cell surface heparan sulfate proteoglycans (HSPG). Evidence for this includes inhibition of binding and uptake by chlorate, which prevents sulfation of proteoglycans, and by treatment with heparinase but not chondroitinase ABC. β-VLDL uptake was stimulated by lipoprotein lipase (LpL) and apolipoprotein E (apoE), both known to bind HSPGs. Uptake and degradation of β-VLDL were not mediated by the LDL receptor or the α_2MR/LRP. Thus, binding of β-VLDL to low affinity, high capacity HSPG binding sites on pigeon macrophages appears to directly promote internalization and degradation and is largely responsible for the greater ability of rabbit β-VLDL to stimulate cholesterol accumulation.—**Seo, T., and R. W. St. Clair.** Heparan sulfate proteoglycans mediate internalization and degradation of β-VLDL and promote cholesterol accumulation by pigeon macrophages. *J. Lipid Res.* 1997. **38:** 765–779.

Supplementary key words proteoglycans • sodium chlorate • α_2MR/LRP • lipoprotein lipase • apolipoprotein E • White Carneau pigeon • macrophage • lipoprotein • β-VLDL • LDL receptor

White Carneau (WC) pigeons develop atherosclerosis naturally and at an accelerated rate with cholesterol feeding (1, 2). Pigeons fed a diet containing 0.5% cholesterol have plasma cholesterol concentrations that average about 1500 mg/dl. Under these conditions, beta-migrating very low density lipoprotein (β-VLDL) is the major lipoprotein responsible for transport of cholesterol in the blood (3). Pigeon β-VLDL is similar in size and composition to β-VLDL from other cholesterol-fed animals such as rabbits, but unlike mammalian β-VLDL, and like all birds, is devoid of apoE (3, 4). Atherosclerotic lesions of pigeons contain abundant macrophage foam cells that are enriched in cholesteryl esters and can be seen in the aorta of WC pigeons after only a few weeks of cholesterol feeding (5, 6). As macrophages are present in varying amounts throughout all of the stages of atherosclerotic plaque development, and macrophage foam cells play a central role in the pathogenesis of atherosclerosis in pigeons as in other animal models and humans, understanding the variety of mechanisms responsible for the uptake and metabolism of lipoproteins by these cells is essential to our understanding of a critical stage in the pathogenesis of atherosclerosis.

In previous studies, we compared the binding and uptake of pigeon β-VLDL in WC pigeon peritoneal macrophages with rabbit β-VLDL as a control. Although these lipoproteins have similar cholesterol/protein ratios, rabbit β-VLDL consistently caused a 2- to 3-fold greater

Abbreviations: α_2MR/LRP, α_2-macroglobulin receptor/low density lipoprotein receptor-related protein; α_2M, α_2-macroglobulin; α_2M^+, methylamine-treated α_2M; WC, White Carneau pigeon; β-VLDL, β-very low density lipoprotein; BSA, bovine serum albumin; MEM, minimal essential medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonylfluoride; PPACK, D-phenylalanyl-L-propyl-L-arginine chloromethylketone; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TPA, tetramyristate phorbol acetate; GLC, gas–liquid chromatography; EDTA, ethylenediaminetetraacetic acid; PG, proteoglycan; HSPG, heparan sulfate proteoglycan; LpL, lipoprotein lipase; apoE, apolipoprotein E; $NaClO_3$, sodium chlorate; TC, tyramine cellobiose.

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cholesteryl ester accumulation and esterification (4, 7). Pigeon β-VLDL was shown to be taken up primarily by LDL receptors, while rabbit β-VLDL appeared to bind to another site with low affinity and high capacity that is yet to be fully characterized (4). This difference in the ability of rabbit β-VLDL to load pigeon macrophages with cholesterol could be due to the presence of apoE on rabbit β-VLDL, which may direct rabbit β-VLDL to another site on macrophages that is distinct from the pigeon LDL receptor. One possible candidate for such a site is the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α_2MR/LRP). The α_2MR/LRP is a member of the LDL receptor superfamily and has been shown to bind lipoproteins that are enriched in apoE (8–10). Although we recently have shown that pigeon peritoneal macrophages express the α_2MR/LRP, this receptor did not recognize either pigeon or rabbit β-VLDL (7). Furthermore, addition of LpL to these β-VLDL, which has been shown to promote binding to the mammalian α_2MR/LRP (9), did not result in binding and metabolism of pigeon or rabbit β-VLDL by the α_2MR/LRP (7). These studies also showed that binding of rabbit β-VLDL and some pigeon β-VLDL could not be accounted for by binding to the LDL receptor, as binding occurred in the absence of calcium (presence of EDTA) and in cells where LDL receptors were down-regulated by cholesterol loading (4, 7). Previous studies also had shown that neither pigeon nor rabbit β-VLDL bound to the scavenger receptor (11).

A number of laboratories have suggested that proteoglycans (PG), specifically heparan sulfate proteoglycans (HSPG), play an important role in the initial binding of lipoproteins to certain cells, particularly hepatocytes (12–16). Binding to HSPGs could be mediated by positively charged heparin binding domains on apolipoprotein B-100 (apoB-100) or apoE (17), or via another protein such as lipoprotein lipase (LpL) that can form a complex with lipoproteins (18, 19). After binding, the HSPG-bound lipoproteins were shown to be transferred or "handed off" to a specific receptor such as the α_2MR/LRP or the LDL receptor for internalization and subsequent degradation (12, 16). Such a mechanism is unlikely to occur in pigeon macrophages, however, as the α_2MR/LRP on pigeon macrophages does not bind either pigeon or rabbit β-VLDL. Binding also could not be attributed to the LDL receptor as experiments were done under conditions where binding of β-VLDL to LDL receptors was inhibited or LDL receptors were down-regulated (7). As a result, in this study we considered an alternative mechanism for the uptake of rabbit and pigeon β-VLDL by pigeon macrophages that involved the initial binding to cell surface HSPGs and internalization without transfer to a lipoprotein receptor.

METHODS

Materials

Sodium ^{125}I-iodide (IMS 300, carrier-free, in NaOH solution, pH 7 to 11) and [1-^{14}C]oleic acid (CFA 243) were purchased from Amersham Corp., Arlington Heights, IL. [^{14}C]triolein (glycerol[1-^{14}C]trioleate) was purchased from DuPont NEM Research products, Boston, MA. Bovine serum albumin (BSA), bovine milk lipoprotein lipase (LpL), and heparinase I (H 2519) were purchased from Sigma Chemical Company, St. Louis, MO. Lipoprotein lipase was dialyzed first against deionized water containing 1 mg/ml EDTA and 1.2 M NaCl to remove ammonium sulfate, then against the 0.5 M NaCl solution and was stored in 2-ml vials at −70°C. Eagles's minimum essential medium (MEM) was purchased from JRH Biosciences, Lenexa, KS, and RPMI was purchased from Mediatech, Washington, DC. Fetal bovine serum (FBS) was from ICN/Flow, Costa Mesa, CA. The FBS was heat-inactivated before use by incubation at 56°C for 1 h. Aprotinin and phenylmethylsulfonylfluoride (PMSF) were purchased from Boehringer Mannhein Corp., Indianapolis, IN. D-Phenylalanyl-L-propyl-L-arginine chloromethylketone (PPACK) was purchased from Calbiochem Corp., San Diego, CA. Chondroitinase ABC (code No. 100330) was from Seikagaku Co., Tokyo, Japan. Sodium chlorate (40,301–6) was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Recombinant human apoE (E3 isoform) was produced by baculovirus gene transfer and was a gift from Dr. John S. Parks of the Department of Comparative Medicine, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC.

Isolation of β-VLDL from hypercholesterolemic pigeons and rabbits

Pigeon β-VLDL was isolated from the plasma of WC pigeons fed a diet of commercial pigeon pellets containing 0.5% cholesterol and 10% lard for at least 1 month. The rabbit β-VLDL was obtained from the plasma of New Zealand White rabbits (Robinson Services, Inc., Winston-Salem, NC) fed a commercial chow diet containing 0.5% cholesterol and 5% corn oil for at least 30 days. The β-VLDL was isolated by ultracentrifugation at d < 1.006 g/ml for 20 h as described previously (20). The isolated β-VLDL was labeled with ^{125}I using the iodine monochloride method of MacFarlane

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as modified by Bilheimer (21) or coupled with ^{125}I-labeled tyramine cellobiose (^{125}I-TC). ^{125}I-TC was prepared by using 1,3,4,6,-tetrachloro-3α,6α-diphenylglycouril (Iodogen) (0.06 to 0.1 mCi/nmol TC) (22, 23), and the ^{125}I-TC was covalently linked to pigeon and rabbit β-VLDL (5 to 10 nmol TC/mg β-VLDL protein) after activating the iodinated TC with cyanuric chloride as described previously (24, 25). The β-VLDLs, labeled with either ^{125}I or ^{125}I-TC, were dialyzed extensively against saline containing 1 mg/ml EDTA, sterilized by filtration through a Millipore filter (0.45 μm), and stored at 4°C for use within 2 weeks.

Cell culture

WC pigeon peritoneal macrophages were elicited using thioglycolate and maintained in culture in Eagle's MEM containing 10% heat-inactivated FBS, vitamins, glucose, L-glutamine and antibiotics as described previously (4). This will be referred to as MEM medium. To load pigeon macrophages with cholesterol in order to down-regulate the LDL receptor (4), rabbit β-VLDL (150 μg/ml) in MEM medium was incubated with cells for 24 h at 37°C. This resulted in the accumulation of 200–400 μg esterified cholesterol per mg cell protein. The cells were incubated for an additional 24 h in the absence of rabbit β-VLDL to allow internalization of surface bound β-VLDL (7).

The THP-1 human monocyte/macrophage cell line (26), the J774 murine macrophage cell line, and LDL receptor negative GM2000 human skin fibroblasts (27) were used for comparison with pigeon macrophages. THP-1 cells, incubated in the presence of phorbol ester (TPA) to convert them to the macrophage phenotype, and the J774 mouse macrophage cell line were grown in culture in RPMI medium containing 10% FBS as described previously (7). LDL receptor-negative GM2000 fibroblasts were incubated in MEM medium containing 10% FBS (7).

Results of cell culture studies represent the mean ± SEM of triplicate dishes at each point. All experiments were repeated at least once with comparable results.

Determination of the binding of ^{125}I-labeled β-VLDL to pigeon macrophages

Cells were incubated with ^{125}I labeled β-VLDL at the indicated concentrations for 3–6 h at 4°C. Chlorate ($NaClO_3$), an inhibitor of sulfate adenyltransferase, when incubated with cells reduces sulfation of PGs without affecting the secretion of the PG backbone (28). Cells were incubated with 25–50 mM $NaClO_3$ in MEM medium for 24–36 h prior to the binding experiments. Preliminary studies showed that incorporation of ^{35}S into cellular PGs was reduced by up to 80% with 50 mM $NaClO_3$. Using trypan blue dye exclusion and cell protein per dish, preliminary studies showed that concentrations of $NaClO_3$ of up to 100 mM were not toxic to pigeon macrophages when incubated at 37°C for 48 h. To distinguish the role of specific PGs, cells were incubated with heparinase and chondrotinase ABC prior to lipoprotein binding. Heparinase degrades the heparan sulfate glycosaminoglycans, while chondroitinase ABC digests chondroitin-6-sulfate, chondroitin-4-sulfate, and dermatan sulfate. Heparinase and chondroitinase ABC were incubated in HEPES-MEM with cells at concentrations of 3I U (heparinase) or 1.5I U (chondroitinase ABC) per ml of culture medium for 2 h at 37°C before incubation with lipoproteins. To eliminate the binding of β-VLDL to the LDL receptor on pigeon macrophages, binding experiments were done in the presence of EDTA, as binding of β-VLDL to the LDL receptor on pigeon macrophages, like mammalian macrophages, was shown to be strictly Ca^{2+}-dependent (4, 11). The concentration of EDTA in the medium was carefully calculated so that there was always an excess (0.5 mM) of EDTA over Ca^{2+}. Under the same conditions, the binding of methylamine-activated α_2M (α_2M^+) to the pigeon α_2MR/LRP, which also requires Ca^{2+}, was nearly completely abolished (7). At the end of the incubation with ^{125}I-labeled β-VLDL, the culture medium was poured off and the cell layer was washed five times with PBS containing 2 mg/ml BSA, then three times with PBS alone. Cells were digested with 1 N NaOH for a minimum of 3 h and an aliquot was counted in a Beckman gamma counter (model 5500B) to determine cell-associated ^{125}I radioactivity. A second aliquot of NaOH was taken from each dish for protein determination using the method of Lowry et al. (29). The results are expressed per mg of cell protein. The specific binding of β-VLDL was defined as the difference between the amount of ^{125}I-labeled-β-VLDL bound in the presence and absence of a 30- to 50-fold excess of unlabeled homologous β-VLDL. Specific binding was evaluated by Scatchard analysis as described by McPherson (30) using the EBDA and LIGAND computer programs (BIOSOFT, Cambridge, UK). Binding affinity (K_d) and capacity (B_{max}) were based on the least squares best fit of either a one-site or two-site model.

Determination of metabolism of conventionally radiolabeled β-VLDL (^{125}I-β-VLDL) and ^{125}I-TC-labeled β-VLDL

Pigeon macrophages were incubated with radiolabeled pigeon or rabbit β-VLDL in the presence or absence of LpL or apoE for 3 h at 37°C at the concentrations indicated. To eliminate the metabolism of β-VLDL mediated by LDL receptors on pigeon macrophages,

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studies were carried out in medium containing 0.5 mM excess EDTA. A 3-h incubation period was used for metabolism studies as longer incubations in the presence of EDTA caused the detachment of cells from the culture dish. To inhibit the metabolism of β-VLDL by PGs, cells were pretreated for 36 h with $NaClO_3$ as described above, and an equal amount of $NaClO_3$ was also incubated with cells during the 3-h metabolism studies to inhibit recovery of sulfate incorporation into newly synthesized PGs. The cells then were washed as described above and solubilized in 1 N NaOH to determine cell-associated ^{125}I-TC-β-VLDL and ^{125}I-β-VLDL. Aliquots of culture medium were taken from the dishes incubated with ^{125}I-β-VLDL to determine degradation by measurement of 10% TCA soluble noniodide ^{125}I as described in detail previously (4, 11).

Measurement of cellular cholesterol mass

To determine cholesterol accumulation, cells were incubated with MEM medium containing 20 mM HEPES, 2 mg/ml BSA, and 25 μg/ml pigeon or rabbit β-VLDL in the presence or absence of 50 nM LpL at 37°C for the times indicated. Cells were washed three times with PBS and the cellular lipids were extracted with isopropanol containing 10 μg/ml of stigmasterol as an internal standard. Free and esterified cholesterol mass were determined by gas–liquid chromatography (GLC) of the isopropanol extract, and results were corrected for recovery of the stigmasterol internal standard. Cell protein was measured by the method of Lowry (29) from the cells remaining on the dish after extraction of lipids and solubilization in 1 N NaOH. These methods have been described in detail elsewhere (31).

Lipoprotein lipase assay

Pigeon macrophages, J-774 and THP-1 macrophages were incubated with MEM medium for 48 h at 37°C as described. On day 3, fresh Eagle's MEM medium containing 2 mg/ml BSA, vitamins, glucose, L-glutamine, and antibiotics was added and cells were incubated for 12 h at 37°C. The culture medium was assayed for lipoprotein lipase as described by Iverius and Östlund-Lindqvist (32) in which heat inactivated human serum was used as a source of apoC-II as an activator for LpL. Unpasteurized bovine milk obtained fresh from a local farm was centrifuged at 4°C to remove the cream and used as a standard for the LpL assay. Results are expressed as nmol of free fatty acid liberated from [^{14}C]-triolein per mg cell protein. GM2000 human skin fibroblasts that do not secrete LpL were used as a negative control.

RESULTS

Our previous studies have shown that rabbit and pigeon β-VLDL bind specifically to pigeon peritoneal macrophages (4). The 4°C binding characteristics of pigeon and rabbit β-VLDL to pigeon macrophages are shown in **Fig. 1.** Binding of pigeon β-VLDL to pigeon macrophages was saturable and specific. In cells loaded with cholesterol the ascending limb (low concentrations) of the specific binding curve was reduced. As indicated by the curvilinear Scatchard plot, pigeon β-VLDL bound to both high and the low affinity sites, and the marked reduction in the high affinity sites upon loading cells with cholesterol is consistent with the interpretation that the high affinity binding of pigeon β-VLDL is mediated by an LDL receptor-like binding site. Binding of pigeon β-VLDL to the low affinity site was altered only slightly by cholesterol loading suggesting that this binding site was not regulated by cholesterol. Although rabbit β-VLDL also exhibited a curvilinear binding pattern upon Scatchard analysis, the binding characteristics were distinct from that of pigeon β-VLDL. Rabbit β-VLDL bound predominantly to a low affinity site that had a K_d of 122 μg/ml, while smaller amounts bound to a high affinity site (K_d 5.13 μg/ml). The observation that rabbit β-VLDL was bound primarily to the low affinity, high capacity site is consistent with the specific binding curves (Fig. 1), showing that rabbit β-VLDL binding did not reach saturation even at the highest concentration used (100 μg/ml). Cholesterol loading of cells had little effect on overall binding of rabbit β-VLDL. These results also are consistent with our previous data showing that rabbit β-VLDL was a poor ligand for the LDL receptor on pigeon macrophages (4, 7).

When identical studies were carried out in the presence of LpL (**Fig. 2**), different binding properties of pigeon and rabbit β-VLDL were observed. The filled squares show the Scatchard plots without LpL (these are the same data shown in Fig. 1), while open circles show the binding of β-VLDL in the presence of 50 nM LpL. In the presence of LpL the Scatchard analysis indicated one dominant binding component for pigeon β-VLDL with a much greater capacity than in the absence of LpL (Fig. 2A). Cholesterol loading had little effect on this differential in binding (Fig. 2B). $NaClO_3$ treatment virtually eliminated binding of pigeon β-VLDL in the presence of LpL (Fig. 2C) consistent with the conclusion that most binding was to cell surface PGs. Binding of rabbit β-VLDL also was resolved into one binding component in the presence of LpL (Fig. 2D), and there was little effect of cholesterol loading on binding kinetics of rabbit β-VLDL (Fig. 2E). Most rabbit β-VLDL

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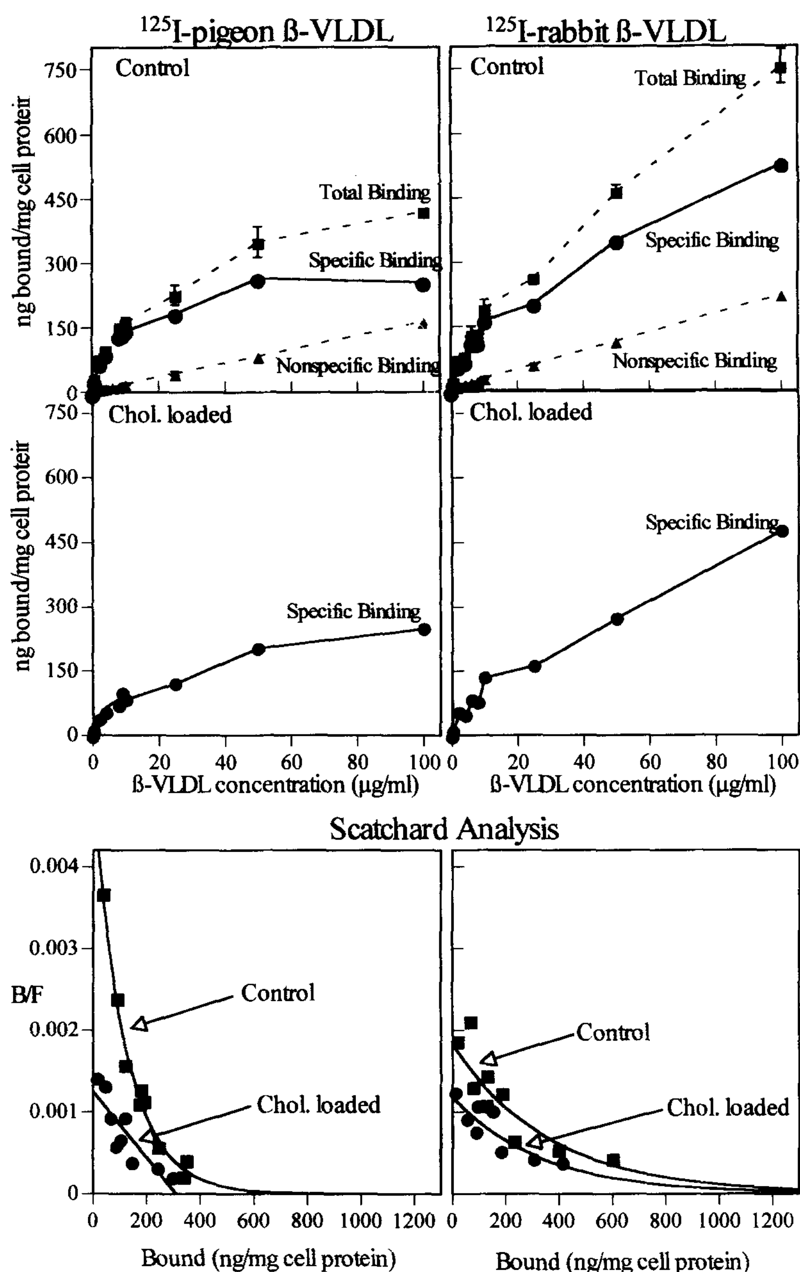

Fig. 1. Concentration-dependent binding of pigeon and rabbit β-VLDL to pigeon macrophages at 4°C. Cells were incubated with HEPES-MEM containing 2% FBS and the indicated amount of ^{125}I-labeled pigeon or rabbit β-VLDL in the presence or absence of a 30-fold excess of homologous unlabeled β-VLDL. Specific binding was determined after a 5-h incubation at 4°C by subtracting nonspecific binding from total binding. Two days before the experiment, another set of dishes (cholesterol-loaded) was incubated with 150 µg/ml of rabbit β-VLDL for 24 h. This was followed by incubation in HEPES-MEM with 10% FBS for an additional 24 h to allow the bound β-VLDL to be internalized and cellular cholesterol to equilibrate. Specific binding of ^{125}I-labeled pigeon and rabbit β-VLDL was determined as described above. Results are the mean of total, nonspecific, and specific binding of triplicate dishes at each point. Scatchard plots with the calculated affinity (K_d) and capacity (B_{max}) were generated from the specific binding data using the EBDA, LIGAND computer programs as described in Methods. A straight line indicates a best fit for a single site model while a curved line indicates a two-site model is the best fit.

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binding was lost when cells were treated with $NaClO_3$ (Fig. 2F). These studies demonstrated that binding of pigeon and rabbit β-VLDL was mediated by at least two components. Pigeon β-VLDL bound both to high affinity LDL receptors and to low affinity high capacity sites that were most likely cell surface PG. The binding of rabbit β-VLDL, on the other hand, was primarily mediated by the low affinity, high capacity PG sites. Addition of LpL to both rabbit and pigeon β-VLDL primarily increased binding to the low affinity, high capacity PG sites.

To confirm that binding was mediated by cell surface PGs, cells were pretreated for 24 h at 37°C with 25 mM $NaClO_3$ to inhibit sulfation of PGs prior to the binding of ^{125}I-β-VLDL at 4°C (**Fig. 3**). The incubation of cells with $NaClO_3$ did not change the amount of cell protein per dish or the binding of another ligand, ^{125}I-labeled α_2M^+ to the α_2MR/LRP (4°C binding for 5 h to pigeon macrophages was 114 ± 2.2 and 129 ± 19.8 ng/mg cell protein (n = 3), respectively, for control cells and cells preincubated with $NaClO_3$ for 24 h at 37°C). When pigeon macrophages were pretreated with $NaClO_3$, the

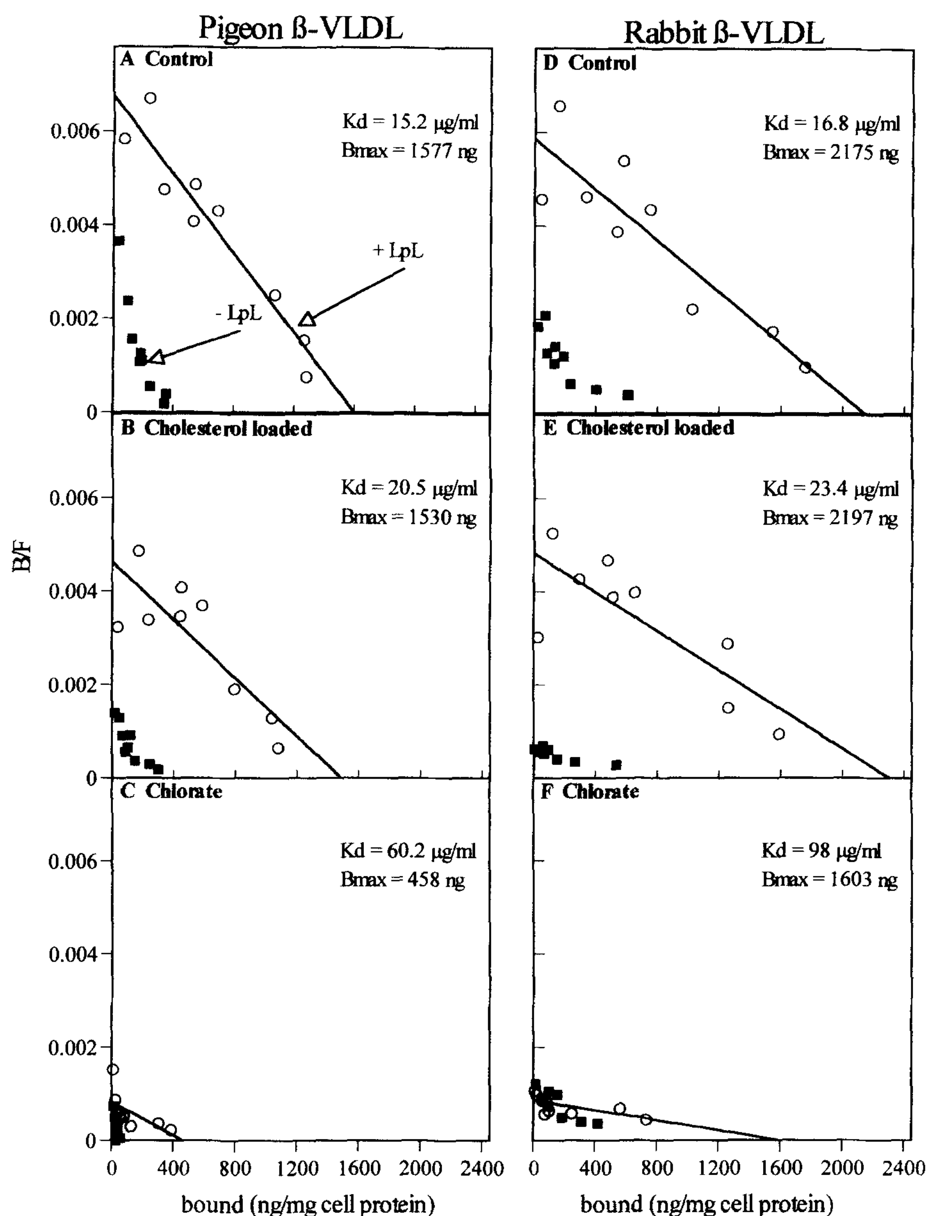

Fig. 2. Concentration-dependent binding of pigeon and rabbit β-VLDL incubated with lipoprotein lipase at 4°C. Cells were incubated with HEPES-MEM containing 2% FBS and the indicated amount of ^{125}I-labeled pigeon or rabbit β-VLDL in the presence (+LpL, open circles) of 50 mM bovine LpL. For comparison, the Scatchard plots for β-VLDL in the absence of LpL (−LpL) from Fig. 1 have been included as the closed squares. Nonspecific binding was obtained by incubation of labeled β-VLDL with a 30-fold excess of homologous unlabeled β-VLDL. The $NaClO_3$-treated cells were incubated with 25 mM $NaClO_3$ in HEPES-MEM with 10% FBS for 24 h prior to incubation with medium containing labeled β-VLDL to measure binding. All other conditions and procedures are identical to those described in Fig. 1.

binding of both pigeon and rabbit β-VLDL was reduced by 30–50% (Fig. 3). As pigeon β-VLDL binds primarily to LDL receptors, this suggests that PGs also may play a cooperative role in binding of lipoproteins to the pigeon LDL receptor. Addition of LpL caused a 500–700% increase in binding of both β-VLDLs, and this enhanced binding was nearly eliminated by $NaClO_3$ treatment, suggesting that binding was mediated primarily by cell surface PGs. As the formation of soluble lipoprotein PG complexes does not require calcium (33), binding studies were carried out in the absence and presence of EDTA in order to distinguish between LDL receptor-mediated binding, which requires calcium (4, 7, 11). At the concentrations used (50 μg/ml), EDTA

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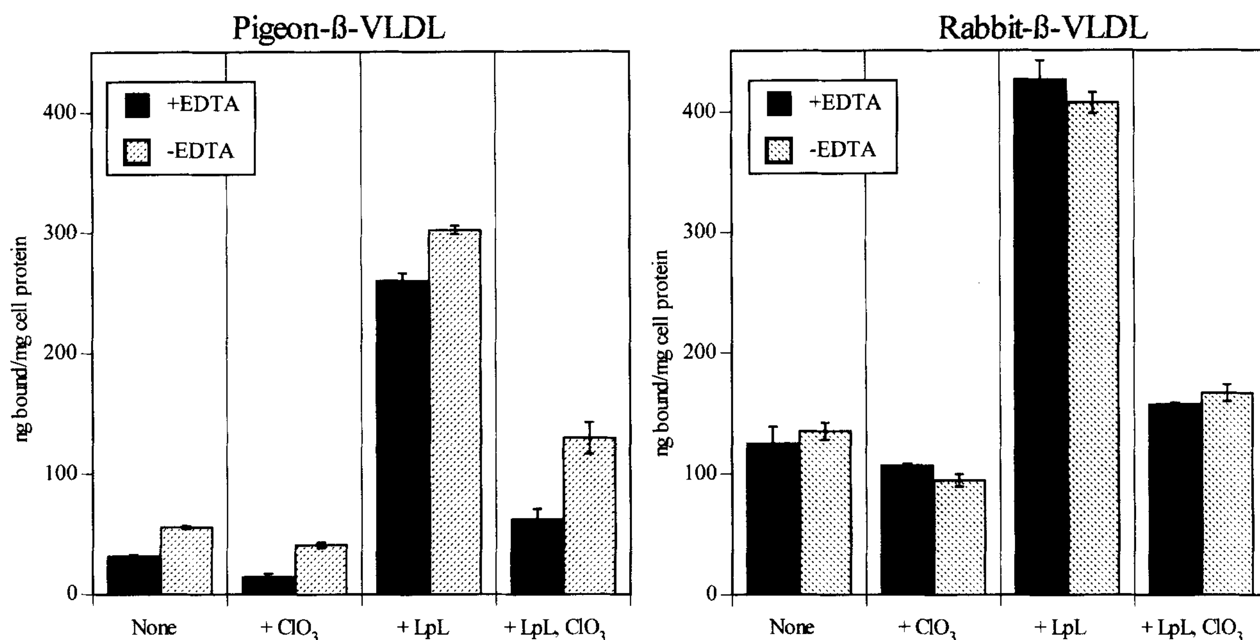

Fig. 3. Effect of lipoprotein lipase and $NaClO_3$ on binding of ^{125}I-labeled pigeon and rabbit β-VLDL. Cells were incubated for 24 h in HEPES-MEM containing 10% FBS. On the second day the medium was replaced with fresh HEPES-MEM (10% FBS) with or without 25 mM $NaClO_3$ and incubated for an additional 24 h with the cells. Cells were chilled to 4°C and washed three times with cold PBS. Cells were then incubated with HEPES-MEM containing 25 μg/ml ^{125}I-labeled pigeon or rabbit β-VLDL in the presence or absence of 50 nM bovine LpL for 5 h at 4°C. The EDTA-containing medium (+EDTA) had 0.5 mM excess EDTA over Ca^{2+}, while the EDTA-free (−EDTA) medium had 2.5 mM free Ca^{2+}. Nonspecific binding was obtained by incubation with a 50-fold excess of homologous unlabeled β-VLDL. The height of the bars indicates the mean of specific binding of triplicate dishes ±SEM.

reduced the binding of pigeon β-VLDL by 20–50%, while having no effect on binding of rabbit β-VLDL. This is consistent with the binding of some pigeon β-VLDL to the LDL receptor, while almost no rabbit β-VLDL binds to the LDL receptor (4, 7). As the enhanced binding of both β-VLDLs with LpL was largely independent of calcium, it suggests that the greater binding of β-VLDL in the presence of LpL was not mediated by the LDL receptor on pigeon macrophages.

To determine which family of PGs mediated the binding of pigeon and rabbit β-VLDL to pigeon macrophages, cells were treated with either heparinase or chondroitinase ABC prior to binding in order to distinguish between binding to HSPGs and other PGs such as chondroitin sulfate and dermatan sulfate. Cells were treated with these enzymes for 2 h at 37°C followed by incubation with ^{125}I-labeled β-VLDL for 3 h at 4°C. As a control, a set of the same dishes was treated with 50 mM $NaClO_3$ for 36 h prior to the 4°C binding experiment. Results are shown in **Fig. 4.** Consistent with previous experiments, binding of pigeon and rabbit β-VLDL in the presence of 50 mM LpL (Fig. 4A) was significantly enhanced. As before, this enhancement was abolished when cells were treated with $NaClO_3$. The treatment of cells with heparinase was as effective as $NaClO_3$ in reducing β-VLDL binding, while chondroitinase ABC had no effect. When identical experiments were performed in the absence of LpL (Fig. 4B), similar results were seen although the magnitude of binding was less. Thus, binding of both pigeon and rabbit β-VLDL at 4°C was mediated in part by cell surface HSPGs. HSPGs also mediated the enhanced binding of β-VLDL in the presence of LpL.

Although binding of β-VLDL to PGs is enhanced in the presence of LpL, the actual internalization and ultimate degradation of β-VLDL have been shown to be mediated largely by other receptors such as the α_2MR/LRP or the LDL receptor (10, 13, 16). If the binding of β-VLDL to PGs on the surface of pigeon macrophages results in internalization of β-VLDL, the mechanism must be different from mammalian cells as neither the α_2MR/LRP nor the LDL receptor mediates binding under the conditions of these experiments (7). Alternatively, it is possible that HSPGs on the cell surface directly mediate the internalization and subsequent degradation of β-VLDL. To test this possibility, cells were incubated with ^{125}I-TC-labeled β-VLDLs in the presence or absence of LpL for 3 h at 37°C and total metabolism was measured. To eliminate any metabolism that might be mediated by lipoprotein receptors, the incubations were carried out in the presence or absence of 0.5 mM excess EDTA. Incubations were limited

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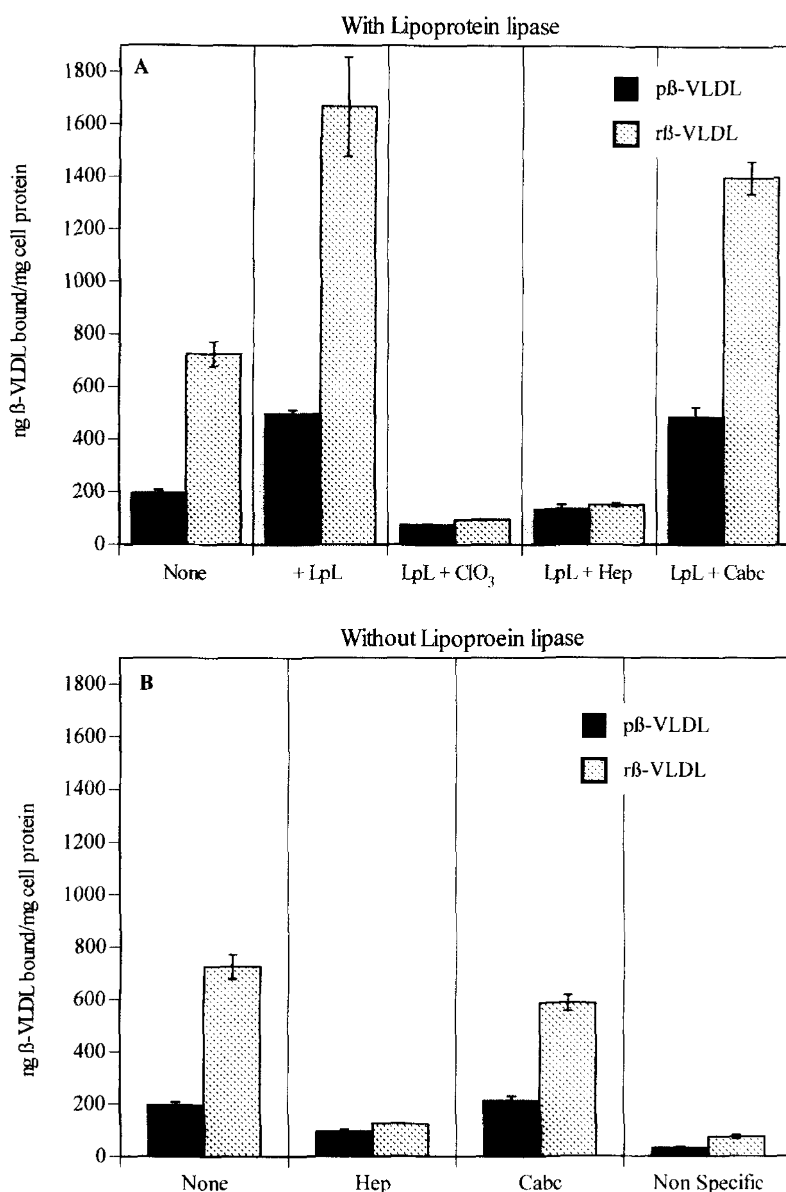

Fig. 4. Effect of heparinase and chondroitinase ABC on pigeon and rabbit β-VLDL binding at 4°C. Cells were incubated in HEPES-MEM medium containing 10% FBS with or without 50 mM $NaClO_3$ for 36 h. Another set of dishes was incubated with 3 unit/ml of heparinase (hep) or 1 unit/ml of chondroitinase ABC (Cabc) for 2 h at 37°C prior to the binding experiments. Dishes were washed with cold PBS and incubated with MEM containing 50 μg/ml of ^{125}I-TC-labeled pigeon or rabbit β-VLDL in the presence or absence of 50 nM bovine LpL for 5 h at 4°C to measure cell surface binding. Nonspecific binding was determined by incubation with a 30-fold excess of unlabeled homologous β-VLDL. Results are the mean of triplicate dishes ± SEM. Pigeon β-VLDL (pβ-VLDL); rabbit β-VLDL (rβ-VLDL).

to 3 h to prevent the detachment of cells from culture dishes in the presence of EDTA. Results are shown in **Fig. 5.** These studies were done with β-VLDL labeled with ^{125}I-TC. The TC is residualized in lysosomes, thus total ^{125}I count in the cells represents all of the β-VLDL internalized over the 3-h period. Approximately 700 ng of pigeon β-VLDL and 1000 ng of rabbit β-VLDL were internalized without addition of LpL, and both were reduced with $NaClO_3$. When LpL was added, internalization of pigeon and rabbit β-VLDL was increased by 70% and 150%, respectively. The enhanced internalization of both β-VLDLs in the presence of LpL was abolished when cells were treated with $NaClO_3$. Similar results were seen in the presence and absence of EDTA.

To be certain that internalization of β-VLDL resulted in lysosomal degradation, a similar 3-h, 37°C incubation experiment in the presence of EDTA was carried out using conventionally ^{125}I-labeled β-VLDL, and degradation products as well as cell-associated ^{125}I were measured (**Fig. 6**). Results indicate that the enhancement of β-VLDL internalization with LpL and the reduction in internalization with $NaClO_3$ were associated with parallel changes in the degradation of β-VLDL. Thus internalization of β-VLDL by HSPGs results in the delivery of a proportional amount of β-VLDL for degradation.

The greater binding of rabbit β-VLDL to pigeon macrophages results in greater internalization and degradation culminating in greater cholesterol accumulation

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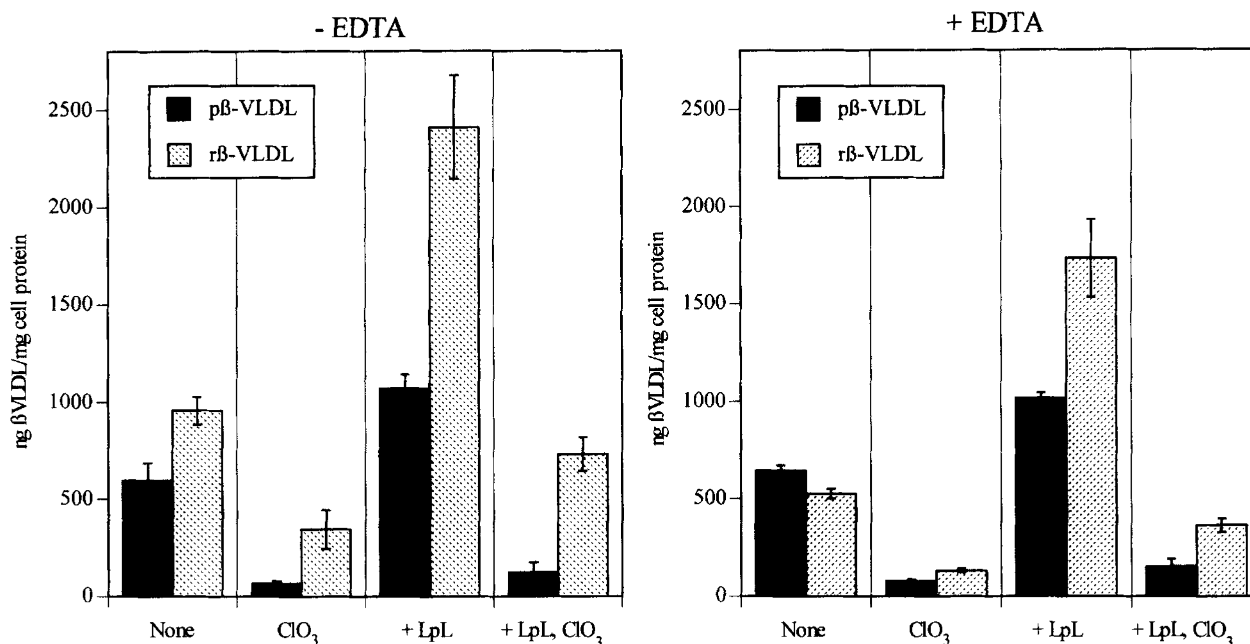

Fig. 5. Effect of EDTA on metabolism of pigeon and rabbit β-VLDL incubated with lipoprotein lipase and sodium chlorate at 37°C for 3 h. Cells were incubated with 50 μg/ml of ^{125}I-TC-labeled pigeon or rabbit β-VLDL in the presence or absence of 100 nM bovine LpL for 3 h at 37°C. The EDTA-containing medium (+EDTA) had 0.5 mM excess EDTA over Ca^{2+}, while the EDTA-free medium (−EDTA) had 2.5 mM excess Ca^{2+}. Where indicated dishes were pretreated with 50 mM $NaClO_3$ for 36 h prior to the experiments, and an equal amount of $NaClO_3$ was added to the medium during the 3 h incubation with ^{125}I-TC-β-VLDL to inhibit the sulfation of newly synthesized proteoglycans. After 3 h at 37°C, cells were washed and assayed for cell-associated radioactivity which represents the sum of surface bound and internalized β-VLDL. Nonspecific binding was obtained by incubation with a 30-fold excess of unlabeled homologous β-VLDL. Data represent specific binding of pigeon (pβ-VLDL) and rabbit (rβ-VLDL) β-VLDL. Results are the mean of triplicate dishes ±SEM.

than with pigeon β-VLDL (4, 7). As described earlier, a major difference between pigeon and rabbit β-VLDL is the presence of apoE on rabbit β-VLDL. ApoE is known to bind HSPGs with high affinity (17). Thus we investigated whether addition of apoE to pigeon β-VLDL would increase its uptake by pigeon macrophages. To eliminate the binding of pigeon β-VLDL to pigeon LDL receptors, the experiment was carried out in the presence of 0.5 mM EDTA. When cells were incubated for 3 h at 37°C with ^{125}I-TC-labeled pigeon β-VLDL to which exogenous recombinant human apoE had been added at a mass ratio of 1 μg apoE to 10 μg β-VLDL protein, there was a 3-fold increase in the internalization of pigeon β-VLDL (**Fig. 7**). This was similar to the metabolism of pigeon β-VLDL in the presence of 100 nM LpL. When both apoE and LpL were added to pigeon β-VLDL, its internalization was further enhanced. $NaClO_3$ treatment markedly reduced the enhanced binding due to apoE, LpL, and apoE + LpL. As the study was performed in a calcium-free medium, the effect was independent of LDL receptors and the α_2MR/LRP. Despite the fact that native rabbit β-VLDL already had significant amounts of apoE, further enrichment with apoE induced even greater binding of rabbit β-VLDL to pigeon macrophages and the effect of apoE was eliminated by $NaClO_3$ treatment.

To determine whether PG-mediated β-VLDL metabolism induced cholesteryl ester accumulation in pigeon macrophages, normal or $NaClO_3$-treated cells were incubated with or without LpL for up to 18 h and cholesterol mass in the cells was measured. As a control, cells also were incubated without β-VLDL for the same length of time. The results of this study are shown in **Fig. 8.** Cholesterol content of cells incubated without β-VLDL remained unchanged throughout the course of the experiment. When cells were incubated with pigeon or rabbit β-VLDL in the presence of LpL, there was nearly a doubling in cholesterol accumulation compared with cells incubated with β-VLDL alone. $NaClO_3$ treatment markedly reduced cholesterol accumulation in cells incubated with pigeon or rabbit β-VLDL in the presence or absence of LpL. These studies are consistent with the conclusion that metabolism of β-VLDL by a PG-mediated process may play a significant role in cholesterol accumulation by pigeon macrophages.

Although LpL clearly stimulated β-VLDL uptake by a PG-mediated uptake process, it was not clear whether this process could have any physiological importance

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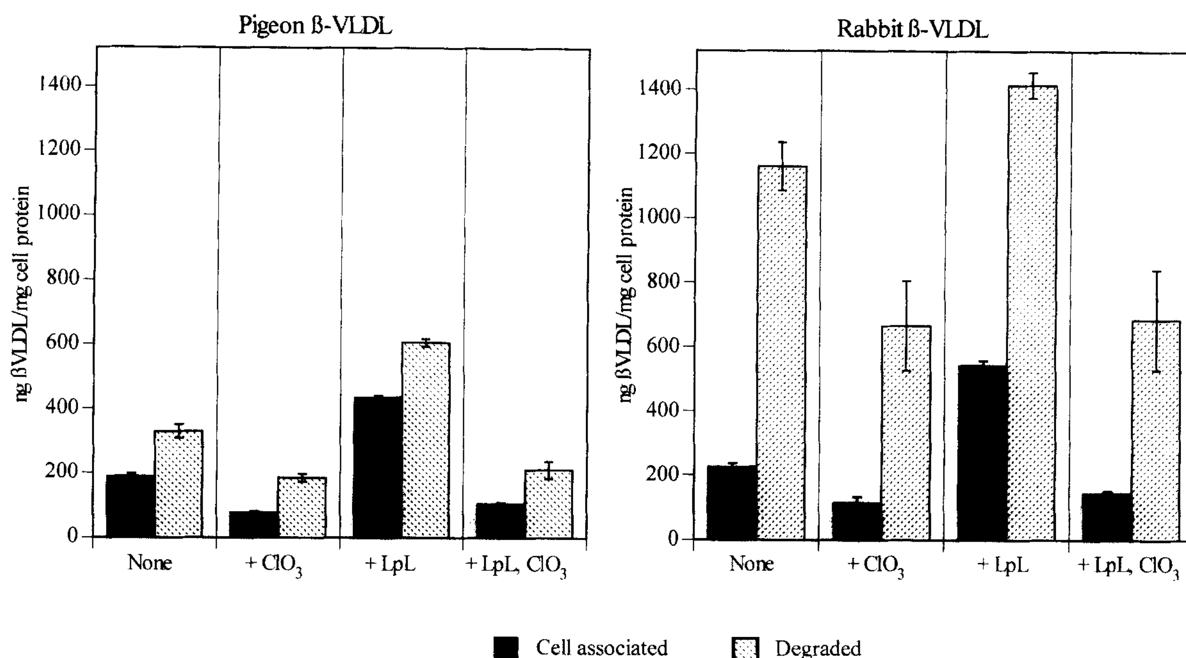

Fig. 6. Determination of cell-associated and degraded β-VLDL in pigeon macrophages in Ca^{2+}-free medium. Cells were incubated with 50 μg/ml of conventionally labeled ^{125}I-labeled pigeon or rabbit β-VLDL in the presence or absence of 100 nM bovine LpL for 3 h at 37°C. The medium contained 0.5 mM excess EDTA over Ca^{2+}. Some dishes were pretreated with 50 mM $NaClO_3$ for 36 h prior to the experiments, and an equal amount of $NaClO_3$ was added to the medium during the 3 h incubation with ^{125}I-labeled β-VLDL in order to inhibit the sulfation of newly synthesized proteoglycans. After 3 h, the culture medium was removed and assayed for TCA-soluble noniodide ^{125}I degradation products. Cells were washed and assayed for the cell-associated ^{125}I count as described in Methods. Results are the mean of triplicate determinations ±SEM.

without knowing whether pigeon macrophages could secrete LpL, as do mammalian macrophages. To determine whether pigeon macrophages secrete LpL, cells were incubated with MEM containing BSA without serum for 24 h, and the culture medium was analyzed for LpL activity by measuring the hydrolysis of radiolabeled triglyceride emulsion particles. The J774 murine and THP-1 human macrophage cell lines were used for comparison. LDL receptor negative GM2000 human skin fibroblasts, which do not secrete LpL, were used as a negative control. Both normal and cholesterol-loaded pigeon peritoneal macrophages were studied. As shown in **Table 1,** pigeon macrophages secreted an amount of LpL activity similar to that of THP-1 cells. LpL activity was similar for normal and cholesterol-loaded cells. J774 cells had the highest activity of the cells tested, while GM2000 fibroblasts had no detectable lipolytic activity.

DISCUSSION

The present study shows that pigeon and rabbit β-VLDL are taken up by pigeon peritoneal macrophages by two processes: a high affinity LDL receptor-like mechanism and a low affinity, high capacity process that involves binding to cell surface HSPGs. Pigeon β-VLDL is taken up by both processes while rabbit β-VLDL is taken up primarily by the low affinity HSPG mechanism.

Several pieces of evidence support the presence of these two mechanisms for the uptake of β-VLDL. Kinetic analysis of 4°C binding of ^{125}I-labeled β-VLDL shows the presence of at least two classes of binding sites. The high affinity binding site was down-regulated by loading the cells with cholesterol, while the low affinity site was unaffected. In contrast, binding to the low affinity site was inhibited by $NaClO_3$ which prevents sulfation of PGs. LpL stimulated binding to the low affinity site. This enhanced binding with LpL was completely inhibited by $NaClO_3$ but unaffected by cholesterol loading. In the presence of LpL there was an increase in the number of β-VLDL particles bound to the cell surface. WC pigeon peritoneal macrophages have been shown to synthesize a variety of classes of PGs (34). Chondroitin sulfate PGs (CSPG) are largely secreted from the cells and can be found in the culture medium, while HSPGs are localized primarily on the cell surface (35). Based on the inhibition of binding of pigeon and rabbit β-VLDL by heparinase, but not by chondroitinase

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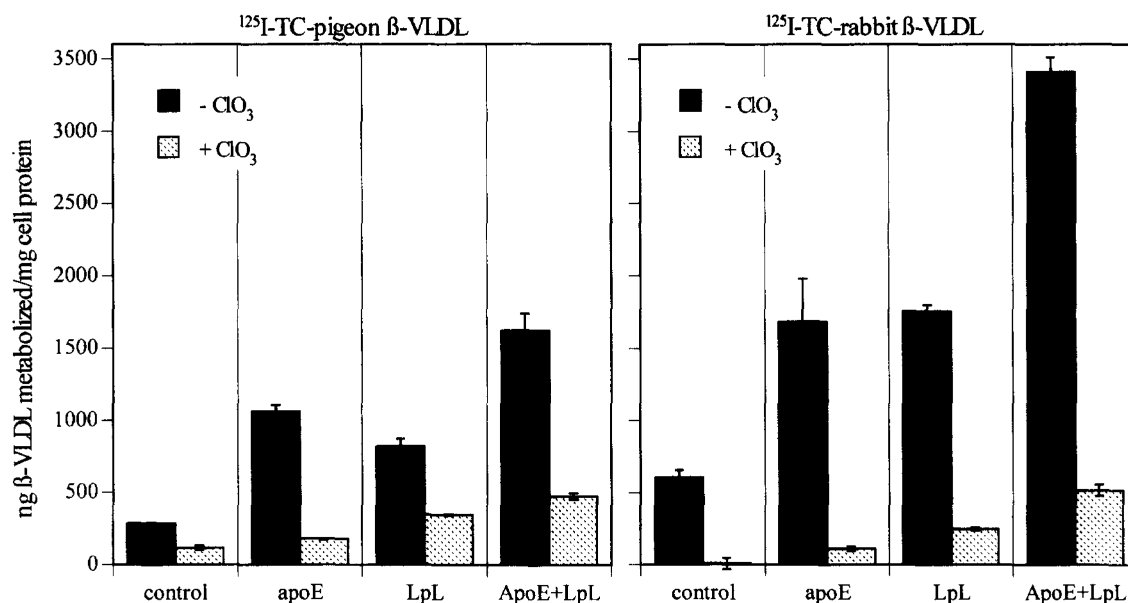

Fig. 7. Effect of apolipoprotein E and lipoprotein lipase on pigeon and rabbit β-VLDL metabolism by pigeon macrophages in Ca^{2+}-free medium. Cells were incubated with 50 μg/ml of ^{125}I-TC-labeled pigeon or rabbit β-VLDL in the presence or absence of recombinant human apoE (12.5 μg/ml) and/or bovine LpL (100 nM) for 3 h at 37°C in Ca^{2+}-free medium. The amount of apoE added to the β-VLDL is approximately 3.6 mol apoE per mol β-VLDL using molecular weights of 34,000 and 500,000 for apoE and β-VLDL protein, respectively. As the TC is not degraded and is trapped in lysosomes, its accumulation in cells provides an accurate measure of total β-VLDL bound and internalized. The medium contained 0.5 mM excess EDTA over Ca^{2+} to inhibit Ca^{2+}-dependent pathways. The indicated sets of dishes were treated with 50 mM $NaClO_3$ for 36 h to inhibit the sulfation of proteoglycans prior to addition of ^{125}I-TC-β-VLDL, and an equal amount of $NaClO_3$ was added to the medium during the 3 h incubation with ^{125}I-TC-β-VLDL to inhibit the sulfation of newly synthesized proteoglycans. After 3 h at 37°C, cells were washed and assayed for cell-associated radioactivity. Nonspecific binding was obtained by incubation with a 30-fold excess of unlabeled homologous β-VLDL. Data represent specific binding. Results are the mean of the triplicate dishes ±SEM.

ABC, we conclude that the low affinity binding of pigeon and rabbit β-VLDL is mediated largely by cell surface HSPGs and not CSPGs. Interestingly, treatment of cells with $NaClO_3$ also reduced the binding capacity of pigeon β-VLDL to the high affinity LDL receptor by about 80%, while having no effect on affinity (data not shown). It is possible to speculate that initial binding to PGs could facilitate the binding of pigeon β-VLDL to the LDL receptor by putting β-VLDL in a favorable orientation for optimal binding or by concentrating β-VLDL in localized areas where abundant LDL receptors are found. It is difficult to know whether a similar mechanism operates for rabbit β-VLDL due to the small amount of high affinity binding of rabbit β-VLDL to pigeon macrophages.

Using a variety of cell types including skin fibroblasts, hepatocytes, and macrophages, others have reported that several lipoproteins (LDL, VLDL, β-VLDL, chylomicron remnants) bind to HSPGs (12–17). In most cases, however, this did not result in direct internalization of these lipoproteins (16). Instead, the lipoproteins were transferred or "handed off" to the LDL receptor or α_2MR/LRP for internalization (12, 16, 17). Recent observations in human skin fibroblasts, however, have shown that LDL-LpL complexes can bind with low affinity and high capacity to a receptor-independent site, while LDL in the absence of LpL is taken up by the conventional LDL receptor pathway (36). The authors concluded that this pathway was not the α_2MR/LRP, as LDL-LpL complexes were localized differently from α_2M^+ on cells, but rather that the complexes were binding to cell surface PGs. This observation agrees with our conclusion that β-VLDL are taken up after binding with low affinity and high capacity by cell surface HSPGs. In the presence of LpL, such a mechanism predominates in mediating the binding and metabolism of β-VLDL (Fig. 2). Obunike et al. (37) showed that THP-1 macrophages metabolized significant amounts of LpL-LDL complexes by direct uptake via cell surface PGs in addition to internalization by the α_2MR/LRP.

Although pigeon peritoneal macrophages have both LDL receptors (4, 11) and α_2MR/LRP (7), several pieces of evidence indicate that neither is required for internalization and ultimate degradation of β-VLDL. Most of our binding, internalization, and degradation studies were done in the presence of excess EDTA. As binding to both the LDL receptor and α_2MR/LRP requires calcium (4, 7, 18), while binding to HSPGs does not (33), this is strong evidence that a component of binding and internalization of β-VLDL is not mediated

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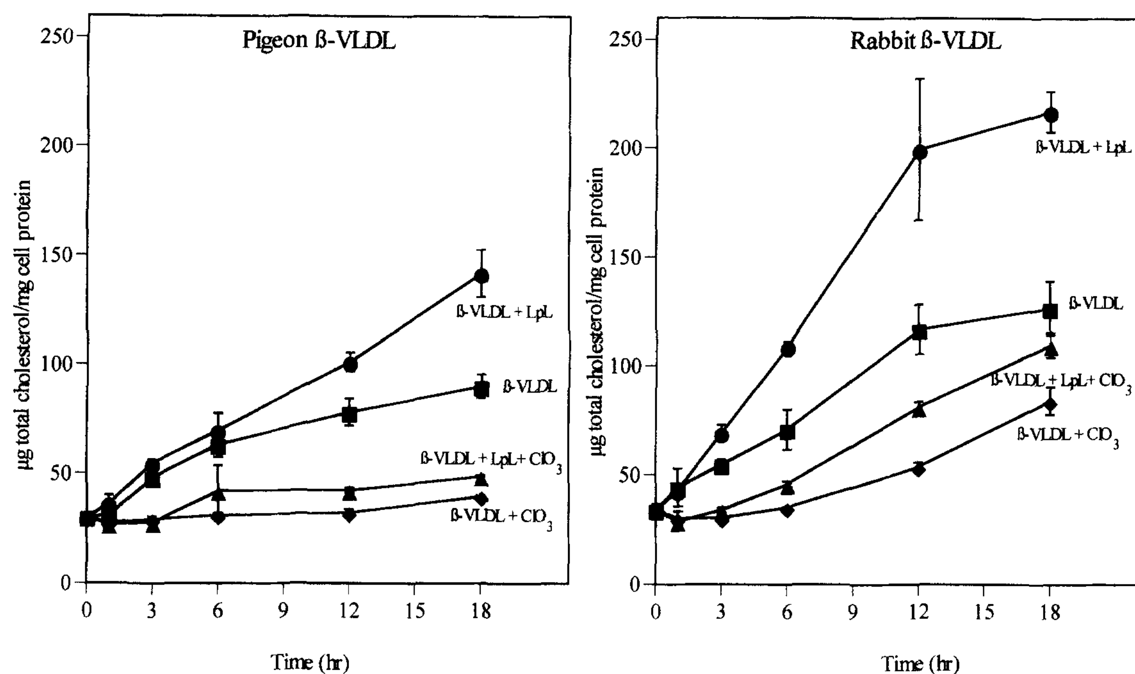

Fig. 8. Cholesterol accumulation in pigeon peritoneal macrophages incubated with pigeon or rabbit β-VLDL in the presence or absence of lipoprotein lipase and/or $NaClO_3$. Cells were incubated for 24 h with HEPES-MEM containing 10% FBS with or without 50 mM $NaClO_3$. Cells were washed with PBS, then incubated with HEPES-MEM containing 25 µg/ml of pigeon or rabbit β-VLDL with or without 50 nM bovine LpL for the indicated periods of time. These B-VLDL also were labeled with ^{125}I-TC, but the ^{125}I data were not used for the purpose of this experiment. The cells that were preincubated with $NaClO_3$ continued to have the same concentration of $NaClO_3$ during incubation with β-VLDL to inhibit the sulfation of newly synthesized proteoglycans. Cell cholesterol content was measured by gas–liquid chromatography. Results are the mean of triplicate dishes at each point ±SEM.

by either of these receptors. Another possible candidate for mediating the uptake of rabbit β-VLDL is the recently characterized VLDL receptor (38). Although some role for this receptor cannot be completely eliminated, as discussed previously (7) in another avian species (chicken) VLDL receptors appear to be restricted to oocytes and there is no evidence that chicken macrophages express VLDL receptors. Direct binding studies also indicated that neither rabbit nor pigeon β-VLDL bound to the α_2MR/LRP (7). The stimulation of binding of β-VLDL by LpL was unaltered in cells preloaded with cholesterol in order to down-regulate LDL receptors. Although 4°C binding to HSPGs was enhanced somewhat more by LpL (up to 7-fold) compared with

TABLE 1. Measurement of lipoprotein lipase activity in medium incubated with pigeon, J-774, or THP-1 macrophages, and GM2000 human skin fibroblasts

Cell types	LpL activity
	nmol FFA released/min/mg cell protein
Pigeon macrophages	32.75 ± 3.52
Pigeon macrophages (cholesterol loaded)	26.07 ± 1.40
J774 macrophages	93.80 ± 21.25
THP-1 macrophages	22.40 ± 9.35
GM2000 fibroblasts	−5.59 ± 9.19

Each cell line was incubated as described in Methods for 3 days at 37°C. In order to load pigeon peritoneal macrophages with cholesterol they were incubated in HEPES-MEM containing 2% FBS and 150 µg/ml of rabbit β-VLDL for 24 h at 37°C. These dishes were washed and incubated with fresh medium containing 2% FBS for an additional 24 h at 37°C to allow internalization of surface-bound rabbit β-VLDL. On the day of the experiments, each cell line was incubated with HEPES-MEM containing 2 mg/ml of BSA for 12 h at 37°C. The cell-conditioned medium was then analyzed for lipoprotein lipase activity as described by Iverius et al. (32) using a triglyceride emulsion containing [^{14}C]triolein. After removal of the medium, cells were washed with PBS and solubilized in 1 N NaOH and assayed for protein. Results have been normalized to equivalent amounts of cell protein per dish. Results are mean of triplicate dishes ± SEM.

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internalization at 37°C (up to 2-fold), there was a clear increase in the internalization and ultimate degradation of β-VLDL when binding to HSPGs was stimulated. Others have also shown that binding of ^{125}I-labeled LDL to PGs was stimulated more than internalization (39).

Binding to HSPGs was stimulated by LpL and apoE. Both of these proteins have been shown by others to bind HSPGs (8, 9, 18). Added apoE stimulated binding and metabolism of both pigeon and rabbit β-VLDL. As a result, the greater binding of native rabbit β-VLDL to pigeon peritoneal macrophages, and its consequent greater ability to stimulate cholesterol accumulation, may be the result of this endogenous apoE. It should be pointed out, however, that we did not test this possibility directly by trying to block the effect of endogenous apoE on rabbit β-VLDL. This will be necessary before a final conclusion on this point can be drawn. The effect of apoE was additive over the already enhanced binding of β-VLDL by LpL. This suggests either that the capacity for LpL enhancement of β-VLDL binding had not been achieved at the concentration of LpL used (50–100 nM), or LpL and apoE bind to different sites on the HSPGs. The latter is the most likely possibility as different binding domains for LpL and apoE on HSPGs have been described (40).

Most importantly, internalization of β-VLDL via the HSPG pathway in pigeon peritoneal macrophages results in the delivery of large amounts of cholesterol to these cells and their storage as cholesteryl esters. If binding to PGs is blocked by $NaClO_3$, cholesteryl ester accumulation is reduced. This suggests that under appropriate conditions this pathway may play an important role in cholesterol delivery to macrophages. Nevertheless, the mechanisms by which β-VLDL that is bound to HSPGs on the cell surface of macrophages is internalized are unclear. In most cells the rate of turnover of cell surface PGs is 5–20 h depending on cell type (41) and is considerably slower than that of cell surface receptors such as the LDL receptor (15–20 min) (42). In macrophages, however, the turnover of the plasma membrane occurs in a matter of minutes (43), thus, it is possible that by simply binding to cell surface HSPGs, some lipoproteins are internalized and directed to lysosomes for degradation. Despite the slower turnover of cell surface PGs, the large quantities of PGs on the surface of cells and the fact that a single glycosaminoglycan chain on HSPGs binds multiple lipoproteins (44) could result in significant amounts of cholesteryl esters delivered to cells via a PG-mediated pathway.

A popular current concept of the pathogenesis of atherosclerosis suggests that macrophage foam cells form as the result of uptake of abnormal lipoproteins by scavenger receptors (45). Oxidized lipoproteins are one of the abnormal forms that have been suggested. Although there seems little doubt that lipoprotein oxidation is one process that can result in cholesteryl ester accumulation in macrophages, it does not exclude a role for other mechanisms as well. One such potential mechanism is the direct uptake of lipoproteins by HSPGs on macrophages. Being low affinity but high capacity, such a process would be most efficient in situations of high concentrations of lipoproteins and in the presence of agents like LpL or apoE to enhance binding to HSPGs. The atherosclerotic plaque would appear to be an ideal environment for this to occur. The concentration of plasma lipoproteins has been shown to be quite high in the extracellular environment of the plaque (25, 46). Macrophages have been shown both in vitro (47–50) and in vivo (51–53) to synthesize and secrete LpL and apoE. Our data show that LpL secretion from pigeon macrophages continues at the same rate even after cholesterol loading; in mammalian macrophages, cholesterol loading increases apoE secretion (50, 54, 55). Thus, the conditions of the atherosclerotic plaque seem ideal for promoting binding of lipoproteins to cell surface HSPGs, suggesting that such a mechanism may play an important role in the development of macrophage foam cells of the atherosclerotic plaque.

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REFERENCES

1. Clarkson, T. B., and H. B. Lofland, Jr. 1961. Effect of cholesterol-fat diets on pigeons susceptible and resistant to atherosclerosis. *Circ. Res.* **9:** 106–109.
2. Prichard, R. W., T. B. Clarkson, H. O. Goodman, and H. B. Lofland, Jr. 1966. Coronary atherosclerosis. A study of coronary disease occurring naturally in White Carneau pigeons. *Arch. Pathol.* **81:** 292–301.
3. Barakat, H. A., and R. W. St. Clair. 1985. Characterization of plasma lipoproteins of grain-and cholesterol-fed White Carneau and Show Racer pigeons. *J. Lipid Res.* **26:** 1252–1268.
4. Adelman, S., and R. W. St. Clair. 1989. β-VLDL metabolism by pigeon macrophages: evidence for two binding sites with different potentials for promoting cholesterol accumulation. *Arteriosclerosis.* **9:** 673–683.
5. Lewis, J. C., R. G. Taylor, and W. G. Jerome. 1985. Foam cell characteristics in coronary arteries and aortas of White Carneau pigeons with moderate hypercholesterolemia. *Ann. NY Acad. Sci.* **454:** 91–100.
6. St. Clair, R. W., H. B. Lofland, and T. B. Clarkson. 1970.

ASBMB JOURNAL OF LIPID RESEARCH

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Influence of duration of cholesterol feeding on esterification of fatty acids by cell-free preparation of pigeon aorta. *Circ. Res.* **27:** 213–225.
7. Seo, T., H-C. Wang, S. R. Feldman, and R. W. St. Clair. 1997. Characterization of α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α_2MR/LRP) in White Carneau pigeon peritoneal macrophages: its role in lipoprotein metabolism. *Biochim. Biophys. Acta.* **1344:** 171–188.
8. Beisiegel, U., W. Weber, G. Ihrke, J. Herz, and K. K. Stanley. 1989. The LDL-receptor-related protein, LRP, is an apolipoprotein E binding protein. *Nature.* **341:** 162–164.
9. Beisiegel, U., W. Weber, and G. Bengtsson-Olivecrona. 1991. Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein. *Proc. Natl. Acad. Sci. USA.* **88:** 8342–8346.
10. Willnow, T. E., J. L. Goldstein, K. Orth, M. S. Brown, and J. Herz. 1992. Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator- inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *J. Biol. Chem.* **267:** 26172–26180.
11. Adelman, S., and R. W. St. Clair. 1988. Lipoprotein metabolism by macrophages from atherosclerosis-susceptible White Carneau and resistant Show Racer pigeons. *J. Lipid Res.* **29:** 643–656.
12. Ji, Z-S., W. J. Brecht, R. D. Miranda, M. M. Hussain, T. L. Innerarity, and R. W. Mahley. 1993. Role of heparan sulfate proteoglycans in the binding and uptake of apolipoprotein E-enriched remnant lipoproteins by cultured cells. *J. Biol. Chem.* **268:** 10160–10167.
13. Ji, Z-S., S. Fazio, Y-L. Lee, and R. W. Mahley. 1994. Secretion-capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **269:** 2764–2772.
14. Ji, Z-S., D. A. Sanan, and R. W. Mahley. 1995. Intravenous heparinase inhibits remnant lipoprotein clearance from the plasma and uptake by the liver: in vivo role of heparan sulfate proteoglycans. *J. Lipid. Res.* **36:** 583–592.
15. Mulder, M., P. Lombardi, H. Jansen, T. J. C. Van Berkel, R. R. Frants, and L. M. Havekes. 1992. Heparan sulfate proteoglycans are involved in the lipoprotein lipase-mediated enhancement of the cellular binding of very low density and low density lipoproteins. *Biochem. Biophys. Res. Commun.* **185:** 582–587.
16. Mulder, M., P. Lombardi, H. Jansen, T. J. C. Van Berkel, R. R. Frants, and L. M. Havekes. 1993. Low density lipoprotein receptor internalizes low density and very low density lipoproteins that are bound to heparan sulfate proteoglycans via lipoprotein lipase. *J. Biol. Chem.* **268:** 9369–9375.
17. Mahley, R. W., and M. M. Hussain. 1991. Chylomicron and chylomicron remnant catabolism. *Curr. Opin. Lipidol.* **2:** 170–176.
18. Nykjaer, A., G. Bengtsson-Olivecrona, A. Lookene, S. K. Moestrup, C. M. Petersen, W. Weber, U. Beisiegel, and J. Gliemann. 1993. The α_2-macroglobulin receptor/low density lipoprotein receptor-related protein binds lipoprotein lipase and β-migrating very low density lipoprotein associated with the lipase. *J. Biol. Chem.* **268:** 15048–15055.
19. Sivaram, P., S. Y. Choi, L. K. Curtiss, and I. J. Goldberg. 1994. An amino-terminal fragment of apolipoprotein B binds to lipoprotein lipase and may facilitate its binding to endothelial cells. *J. Biol. Chem.* **269:** 9409–9412.
20. St. Clair, R. W., R. K. Randolph, M. P. Jokinen, T. B. Clarkson, and H. A. Barakat. 1986. Relationship of plasma lipoproteins and the monocyte-macrophage system to atherosclerosis severity in cholesterol-fed pigeons. *Arteriosclerosis.* **6:** 614–626.
21. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
22. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80:** 849–857.
23. Pittman, R. C., T. E. Carew, C. K. Glass, S. R. Green, C. A. Taylor, Jr., and A. D. Attie. 1983. A radio iodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation in vivo. *Biochem. J.* **212:** 791–800.
24. Schwenke, D. C., and T. E. Carew. 1989. Initiation of atherosclerotic lesions in cholesterol-fed rabbits: I. Focal increases in arterial LDL concentration precede development of fatty streak lesions. *Arteriosclerosis.* **9:** 895–907.
25. Schwenke, D. C., and R. W. St. Clair. 1992. Accumulation of ^{125}I-tyramine cellobiose-labeled low density lipoprotein is greater in the atherosclerosis-susceptible region of White Carneau pigeon aorta and further enhanced once atherosclerotic lesions develop. *Arterioscler. Thromb.* **12:** 446–460.
26. Auwerx, J. 1991. The human leukemia cell line, THP-1: a multifaceted model for the study of monocyte-macrophage differentiation. *Experientia.* **47:** 22–31.
27. 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.
28. Greve, H., Z. Cully, P. Blumberg, and H. Kresse. 1988. Influence of chlorate on proteoglycan biosynthesis by cultured human fibroblasts. *J. Biol. Chem.* **263:** 12886–12892.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
30. McPherson, G. A. 1985. Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacol. Methods.* **14:** 213–218.
31. Yancey, P. G., and R. W. St. Clair. 1992. Cholesterol efflux is defective in macrophages from atherosclerosis-susceptible White Carneau pigeons relative to resistant Show Racer pigeons. *Arterioscler. Thromb.* **12:** 1291–1304.
32. Iverius, P-H., and A. M. Östlund-Lindqvist. 1986. Preparation, characterization, and measurement of lipoprotein lipase. *Methods Enzymol.* **129:** 691–704.
33. Fenske, D. B., and R. J. Cushley. 1988. Soluble complex formation between low-density lipoprotein and glycosaminoglycans. A^2H and ^{31}P-NMR, and quasi-elastic light scattering study. *Chem. Phys. Lipids* **49:** 15–29.
34. Owens, R. T., and W. D. Wagner. 1992. Chondroitine sulfate proteoglycan and heparan sulfate proteoglycan production by cultured pigeon peritoneal macrophages. *J. Leukocyte Biol.* **51:** 626–633.
35. Edwards, I. J., W. D. Wagner, and R. T. Owens. 1990. Macrophage secretory products selectively stimulate dermatan sulfate proteoglycan production in cultured arterial smooth muscle cells. *Am. J. Pathol.* **136:** 609–621.
36. Fernández-Borja, M., D. Bellido, E. Vilella, G. Olivecrona, and S. Vilaró. 1996. Lipoprotein lipase-mediated uptake of lipoprotein in human fibroblasts: evidence for an LDL

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receptor-independent internalization pathway. *J. Lipid Res.* **37:** 464–481.
37. Obunike, J. C., I. J. Edwards, S. C. Rumsey, L. K. Curtiss, W. D. Wagner, R. J. Deckelbaum, and I. J. Goldberg. 1994. Cellular differences in lipoprotein lipase-mediated uptake of low density lipoproteins. *J. Biol. Chem.* **269:** 13129–13135.
38. Takahashi, S., Y. Kawarabayasi, T. Nakai, J. Sakai, and T. Yamamoto. 1992. Rabbit very low density lipoprotein receptor—a low density lipoprotein receptor-like protein with distinct ligant specificity. *Proc. Natl. Acad. Sci. USA.* **89:** 9252–9256.
39. Hendricks, W. L., H. van der Boom, L. C. Van Vark, and L. M. Havekes. 1996. Lipoprotein lipase stimulates the binding and uptake of moderately oxidized low-density lipoprotein by J774 macrophages. *Biochem. J.* **314:** 563–568.
40. Saxena, U., E. Ferguson, and C. L. Bisgaier. 1993. Apolipoprotein E modulates low density lipoprotein retention by lipoprotein lipase anchored to the subendothelial matrix. *J. Biol. Chem.* **268:** 14812–14819.
41. Yanagishita, M., and V. C. Hascall. 1992. Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **267:** 9451–9454.
42. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
43. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. *J. Cell Biol.* **68:** 665–687.
44. Wagner, W. D., I. J. Edwards, R. W. St. Clair, and H. Barakat. 1989. Low density lipoprotein interaction with artery-derived proteoglycans: the influence of LDL particle size and the relationship to atherosclerosis susceptibility. *Atherosclerosis.* **75:** 49–59.
45. Krieger, M., and J. Herz. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63:** 601–637.
46. Schwenke, D. C., and R. W. St. Clair. 1993. Influx, efflux, and accumulation of LDL in normal arterial areas and atherosclerotic lesions of White Carneau pigeons with naturally occurring and cholesterol-aggravated aortic atherosclerosis. *Arterioscler. Thromb.* **13:** 1368–1381.
47. Khoo, J. C., E. M. Mahoney, and J. L. Witztum. 1981. Secretion of lipoprotein lipase by macrophages in culture. *J. Biol. Chem.* **256:** 7105–7108.
48. Chait, A., P.-H. Iverius, and J. D. Brunzell. 1982. Lipoprotein lipase secretion by human monocyte-derived macrophages. *J. Clin. Invest.* **69:** 490–493.
49. Basu, S. K., M. S. Brown, Y. K. Ho, R. J. Havel, and J. L. Goldstein. 1981. Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* **78:** 7545–7549.
50. Basu, S. K., J. L. Goldstein, and M. S. Brown. 1983. Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages. *Science.* **219:** 871–873.
51. Yla-Herttuala, S., B. A. Lipton, M. E. Rosenfeld, I. J. Goldberg, D. Steinberg, and J. L. Witztum. 1991. Macrophages and smooth muscle cells express lipoprotein lipase in human and rabbit atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **88:** 10143–10147.
52. O'Brien, K. D., D. Gordon, S. Deeb, M. Ferguson, and A. Chait. 1992. Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques. *J. Clin. Invest.* **89:** 1544–1550.
53. O'Brien, K. D., S. S. Deeb, M. Ferguson, T. O. McDonald, M. D. Allen, C. E. Alpers, and A. Chait. 1994. Apolipoprotein E localization in human coronary atherosclerotic plaques by in situ hybridization and immunohistochemistry and comparison with lipoprotein lipase. *Am. J. Pathol.* **144:** 538–548.
54. Mazzone, T., K. Basheeruddin, and C. Poulos. 1989. Regulation of macrophage apolipoprotein E gene expression by cholesterol. *J. Lipid Res.* **30:** 1055–1064.
55. Crespo, P., M. A. Ros, J. M Ordovas, and J. C. Rodriguez. 1992. Foam cells from aorta and spleen overexpress apolipoprotein E in the absence of hypercholesterolemia. *Biochem. Biophys. Res. Commun.* **183:** 514–523.

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