High density lipoprotein$_3$ binding sites are related to DNA biosynthesis in the adenocarcinoma cell line A549

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Abstract The effect of high density (apoE-depleted HDL_3) on cell growth of a human tumor cell line (A549) was studied and related to its binding on the plasma membrane. HDL_3 were shown to stimulate the incorporation of [^{3}H]thymidine into DNA and cell proliferation; these effects were dose-dependent. As HDL_3, apoA-I and apoA-I-liposomes complexes (but not apoA-II) were able to stimulate DNA synthesis in serum-free conditions. This effect was maximum for 15-30 μg HDL_3 protein/ml concentration. Binding of HDL_3 on whole cells occurred by two mechanisms: the first was specific for HDL_3; the second, of lower affinity, was phospholipid-dependent and was inhibited by low density lipoprotein or by phospholipid particles. Internalization and degradation of bound HDL_3 were not observed. The specific sites (27.9 $\pm$ 2.2 ng HDL_3 protein/ng cell protein) accounted for only 2.5% of total (specific + phospholipid) binding sites and they bound HDL_3 with a dissociation constant (K_D) of 2.47 $\pm$ 0.46 μg HDL_3 protein/ml (2.6 $\pm$ 0.5 $\times$ 10^{-8} M). The apparent K_D value of total binding sites (specific + phospholipid) was eightfold higher (20.4 $\pm$ 6.1 μg HDL_3 protein/ml). Analysis of the membrane specific binding sites by ligand blotting with ^{125}I-labeled HDL_3 showed a single protein with an apparent molecular mass of 110 kDa. When HDL_3 binding on phospholipid sites was inhibited by rigid phospholipid particles, the stimulation of [^{3}H]thymidine incorporation related to HDL_3 concentration did not show a maximum peak as previously observed but reached a plateau at a concentration as low as 5 μg HDL_3 protein/ml. This low concentration also nearly saturated the specific binding sites with HDL_3. When binding on specific protein sites was suppressed by tetranitromethane, DNA synthesis was not stimulated but, in contrast, inhibited. The stimulating effect of HDL_3 on DNA biosynthesis is therefore likely dependent on HDL_3 occupying specific binding sites.—**Favre, G., K. A. Tazi, F. Le Gaillard, F. Bennis, H. Hachem, and G. Soula.** High density lipoprotein$_3$ binding sites are related to DNA biosynthesis in the adenocarcinoma cell line A549. *J. Lipid Res.* 1993. **34:** 1093-1106.

Supplementary key words high density lipoprotein subclasses • lipoprotein receptors • tumor cell lines • membrane phospholipid binding • tetranitromethane • DNA synthesis • cell proliferation

Blood plasma lipoproteins allow the transport of cholesterol between various tissues in the body. Cholesterol is needed for cell membrane synthesis; it is furnished by lipoproteins or synthesized by a highly regulated metabolic pathway. Low density lipoproteins (LDL) are the main source of cholesterol that enters the cell by endocytosis of the lipoprotein bound to its specific apoB/E receptor. High density lipoproteins (HDL) allow reverse cholesterol transport from peripheral tissues to the liver (1) but the mechanism is unclear and may be specific (receptor-mediated) (2) or nonspecific (possible substitution of phospholipid vesicles for HDL) (3). In addition to reverse transport, there is growing evidence for a mitogenic role of HDL on various cell types in culture such as normal mammary epithelial cells (4), endothelial cells (5), lymphocytes (6), cancer cell lines (7-9), and in vivo:rat thymus and spleen (10); a correlation has even been suggested between plasma HDL concentration and human breast cancer (9).

The mechanism by which HDL promote cell proliferation is unknown. It has been postulated that they induce phosphorylation of a 27 kDa protein, mediated by protein kinase C in cultured vascular endothelial cells (11). A cytosolic enhancement of Ca^{2+} has been recently shown in fibroblasts (12). A dose-dependent induction of 3-hydroxy -3-methylglutaryl-coenzyme A (HMG-CoA) reductase has been demonstrated in various cell types (13, 14). This rate-limiting enzyme is not only essential for the regulation of cholesterol biosynthesis needed for cell membranes, but also for the synthesis of mevalonate and

Abbreviations: BVSM, "Bio Vecteur Supra Moléculaire"; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DMPC, dimyristoylphosphatidylcholine; FCS, fetal calf serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HDL, high density lipoprotein; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; TNM-HDL_3, HDL_3 modified by tetranitromethane.

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isoprenoid derivatives (intermediary metabolites) that promote cell growth and transformation:stimulation of DNA synthesis by isopentenyl adenine (15), isopentenyl $_t$RNA (16), and transformation by activation of the RAS oncogene (17). The requirement of HMG-CoA reductase activity for cell growth has also been recently confirmed by inhibition of cell proliferation with mevinolin or lovastatin, competitive inhibitors of the enzyme (18).

Regulation of HMG-CoA reductase takes place in two ways: modulation of the gene transcription and protein product phosphorylation by an AMP-dependent protein kinase. Gene expression is regulated by a negative feedback mechanism in which cholesterol suppresses the transcription (19); this mechanism can explain the up-regulation of gene expression by HDL-dependent cholesterol efflux.

The interactions between cell and HDL are still unclear. According to several authors, binding of HDL to the plasma membrane does not require a specific protein receptor (20–22). It has been also postulated that specific and nonspecific binding may occur on the same cell type: cholesterol efflux is receptor-dependent at low HDL concentration range and receptor-independent at high concentration range when the specific binding is saturated (23). The existence of a specific HDL binding site was first demonstrated in hepatocytes (24) and there is now growing evidence in various cell types. This receptor has been identified as a 75–110 kDa protein that is able to bind apolipoproteins A-I and A-II (apoA-I and A-II) (25–28) but has also been suggested to be of lipid nature in endothelial cells (29).

A water-soluble apoA-I–protein A hybrid obtained by a recombinant DNA technique reproduces the binding characteristics of the whole HDL particle (30). ApoA-IV and C, but not apoE have been shown to compete on HDL binding sites (31). The apparent equal binding of apoA-I and A-II explains why both density subclasses HDL_2 (apoA-I-rich particles) and HDL_3 (apoA-I:apoA-II-rich particles) have similar affinities for the receptor (32). However, according to others, apoA-II is unable to bind the apoA-I sites (33).

On the other hand, cell growth seems to be promoted by apoA-I, apoC-III (34) and apoC-I (35). ApoA-I also seems to be responsible for the cholesterol efflux in contrast to apoA-II, which behaves as an antagonist (36).

High density lipoproteins, therefore, seem to be implicated in both cholesterol homeostasis and mitogenic effects but a relation between cell binding and mitogenic effects has never been studied. The present study was undertaken in a cancer cell line because regulation of cholesterol metabolism related to growth is particularly crucial in these cells. Indeed, *i)* LDL receptor activity is often enhanced (37); *ii)* biosynthesis of intracellular cholesterol and nonsterol metabolites has been shown to be essential for cell growth and transformation (17, 38); and *iii)* protein kinase C, which is involved in cholesterol efflux and stimulated by HDL_3 (39, 40), also plays a critical role in growth control and can play a role in the origin of certain human cancers (41). The human adenocarcinoma cell line A549 was chosen as it presents important metabolic properties already well known: LDL receptor activity and DNA synthesis modulated by autocrine growth factors (42), and proliferation modulated by protein kinase C activity (43, 44).

In the present study, it is shown for the first time that the mitogenic effect of HDL_3 is apoA-I-dependent and related to the specific cellular binding of the lipoprotein on a protein receptor of 110 kDa. This HDL_3 receptor was distinguished from nonspecific lower affinity binding sites of phospholipid nature that were able to counteract the protein receptor effect upon binding HDL_3.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, fetal calf serum (FCS) and phosphate-buffered saline (PBS) were obtained from Seromed. Cultured flasks (25 cm^2 and 75 cm^2) and multiwell plates were obtained from Nunc. All the chemicals and solvents used were of analytical grade. Human apolipoproteins A-I and A-II and human glycophorin were purchased from Sigma. [^{3}H]thymidine (29 Ci/mmol) was from Amersham.

A549 cells were obtained from the American Type Culture Collection (Rockville, MD). The cell line, which possesses epithelial-like morphology, was initiated through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male.

The "Bio Vecteur Supra Moléculaire" (BVSM), a new drug delivery system elaborated by "A et S Biovecteurs" Society (Theogone, Parc Technologique du Canal, F-31520 Ramonville), consists of synthetic biomimetic particles that duplicate the natural and most efficient transport vehicles in living organisms, the lipoproteins. BVSM have the characteristics of lipoproteins in terms of the multilayered structure. The core, made up of lipid substances such as triglycerides and cholesterol in lipoproteins is, in BVSM, an internal hydrophilic solid cross-linked polysaccharide that is acylated on the surface with fatty acids. This externally lipophilic core is surrounded with a phospholipid layer. The size of the BVSMs can be engineered, through a specific process, to that of lipoproteins, in particular LDL (20 nm diameter) (Samain et al: World Patent PCT/FR89/00229 – Priority date May 27, 1988).

Cell culture

The A549 cells were maintained in RPMI 1640 medium containing 3.5 g/l of glucose and 2 g/l of $NaHCO_3$ supplemented with 10% fetal calf serum, and

kept in a humidified incubator with 5% CO_2. Under these conditions, the doubling time of the cell culture was 24 h. The cells were routinely subcultured once a week.

Cell growth measurements

To remove serum components from cell membranes, cells were incubated 24 h in RPMI 1640 alone. The cells were seeded at an initial density of 25,000 cells/ml in 6-well multidishes. Cells were then washed once and exposed to RPMI 1640 medium with or without 5% LPDS and supplemented with various concentrations of HDL_3. At appropriate times, duplicate dishes were trypsinized and counted with a hematocytometer.

Lipoproteins, lipoprotein-deficient serum, and HDL_3 labeling

Lipoproteins were prepared from fresh human plasma of healthy donors by ultracentrifugal flotation at 105,000 *g* in a KBr density gradient using a vertical ultracentrifugation rotor (Kontron). The following fractions were obtained: VLDL ($d \leq 1.020$ g/ml), LDL (d 1.030–1.050 g/ml), and total HDL (d 1.063–1.210 g/ml).

HDL_3 were isolated from total HDL by refrigerated differential ultracentrifugation; the fraction between densities 1.125 and 1.210 g/ml was recovered (45). They were further purified by chromatography on heparin-Sepharose (Pharmacia) to remove apolipoprotein E (apoE)-containing fractions (46).

LDL and apoE-depleted HDL_3 were dialyzed extensively against 10 mM Tris buffer, pH 7.4, containing 150 mM NaCl and 0.01% disodium EDTA. They were then sterilized by filtration through a membrane filter (Millipore, 0.45 μm pore size) and stored at 4°C (up to 3 weeks). Lipoprotein purity was assessed by 1% agarose electrophoresis and sodium dodecylsulfate (SDS) (10% polyacrylamide gel) electrophoresis as described by Laemmli (47). No obvious contamination by plasma proteins was observed.

Lipoprotein-deficient serum of d 1.25 g/ml was prepared by ultracentrifugal flotation (45). After extensive dialysis against Krebs-Ringer solution (0.25 g/l $CaCl_2$, 0.42 g/l KCl, 9 g/l NaCl), lipoprotein-deficient serum was heat-inactivated at 56°C for 30 min, sterilized by filtration through a membrane filter (Millipore, 0.45 μm pore size), and stored frozen until used (up to 4 months).

Protein concentrations were determined by the method of Bradford using Bio-Rad protein assay reagent and bovine serum albumin as a standard. Hereafter the concentrations of lipoproteins refer to their protein content.

ApoE-depleted HDL_3 were iodinated by the McFarlane monochloride procedure as modified for lipoproteins (48). Specific activity ranged from 120 to 180 cpm/ng of protein.

The measure of the cellular degradation of lipoproteins was performed according to Brown and Goldstein (49).

Methylation of LDL was carried out according to Weisgraber, Innerarity, and Mahley (50).

Modification of HDL_3 by tetranitromethane

Modification of HDL_3 with tetranitromethane (TNM) was performed as described by Brinton et al. (51). Briefly, 20 μl freshly prepared solution of 0.6 M TNM in absolute ethanol was added to 2 ml HDL_3 (2 mg/ml in 0.9% NaCl, final TNM concentration 6 mM) and the reaction was allowed to proceed in the dark at room temperature for 60 min. The sample was chilled on the ice and subsequently desalted on a Sephadex PD-10 column (Pharmacia).

Preparation of protein-containing liposomes

Dimyristoylphosphatidylcholine-cholesterol (DMPC-cholesterol) liposomes were obtained after adding 1 ml of solution containing 150 mM NaCl–1 mM EDTA–10 mM Tris-HCl buffer (pH 7.6) to 10 mg DMPC supplemented with 3.3 mg cholesterol that had been dissolved in chloroform and dried by lyophilization. The solution was sonicated for 10 min at 30°C using a Branson sonifier at a setting of 50 W. The sonicated preparation of DMPC-cholesterol liposomes was added to a solution of apoA-I, apoA-II, or glycophorin at a protein concentration 1 mg/ml in the same buffer as above and at a lipid-to-protein ratio 3.75:1 (w/w). The mixture was blended and treated at room temperature. The complexes of liposomes and reconstituted proteins were isolated by a centrifugation as described by Brissette, Roach, and Noll (52) and the isolated fraction was dialyzed against 150 mM NaCl–1 mM EDTA–10 mM Tris-HCl buffer (pH 7.6).

[^{3}H]thymidine incorporation studies

Tritiated thymidine incorporation studies were performed on A549 cells in 6-well multidishes. At day 0, cells were seeded with 25,000 cells per well and allowed to grow in RPMI 1640 supplemented with 10% fetal calf serum. At day 4, they were preincubated in RPMI 1640 alone for 24 h in order to remove lipoproteins and growth factors of fetal calf serum from cell membranes (this time of preincubation is not sufficient to synchronize cells). At day 5, cells were washed successively: twice with ice-cold 10 mg/ml bovine serum albumin in PBS buffer and twice with ice-cold PBS buffer alone. They were then incubated, in duplicate, with increasing concentrations of apoE-depleted HDL_3 for 5 h at 37°C in culture medium (RPMI 1640 with or without 5% lipoprotein-deficient serum). [^{3}H]thymidine (1 μCi/ml) was added simultaneously to HDL_3. After incubation, the dishes were rinsed twice with PBS buffer and DNA was precipitated by adding 1 ml of 10% trichloracetic acid per well for 15 h at 4°C. Cells were digested with 1 ml of 1 M NaOH per well at ambient temperature. Aliquots were assayed for [^{3}H]thymidine radioactivity and protein content.

The [^{3}H]thymidine incorporation index (I) was defined as follows: the results for cells incubated in RPMI 1640 medium with or without LPDS were subtracted from corresponding results for cells incubated in the presence of HDL_3 or FCS; the index was expressed as the ratio of the HDL_3 value/FCS value. The ratio is representative of the efficiency of DNA synthesis in the presence of HDL_3 compared to synthesis in the presence of FCS (I = 1 if HDL_3 were more efficient than FCS) and the one in the presence of RPMI 1640 with or without lipoprotein-deficient serum (I = 0 if HDL_3 stimulate DNA synthesis).

Binding of ^{125}I-labeled apoE-depleted HDL_3 to cells

As in [^{3}H]thymidine incorporation studies (see above), experiments were carried out in 6-well multidishes and cells were seeded, at day 0, with 25,000 cells per well. They were allowed to grow in RPMI 1640 medium supplemented with 10% fetal calf serum. At day 4, they were preincubated in RPMI 1640 alone for 24 h. Occasionally, cells were loaded with cholesterol, according to Oram (53), during this preincubation. For this purpose, cholesterol was added either as ethanolic solution (final concentration 50 μg/ml in the presence of 2 mg/ml bovine serum albumin) or as LDL (final concentration of 50 μg protein LDL/ml in the presence of 5% lipoprotein-deficient serum).

Saturation and competition studies were performed in RPMI 1640 medium supplemented with fatty acid-free bovine serum albumin (2 mg/ml). A549 cells were incubated for 1 h at 37°C or 3 h at 4°C in 6-well multidishes with increasing concentrations, in duplicate, of ^{125}I-labeled apoE-depleted HDL_3; unlabeled apoE-depleted HDL_3 or other competitors were added at indicated concentrations in a final volume of 1 ml. After incubation, dishes were washed rapidly three times with ice-cold bovine serum albumin in PBS buffer (10 mg/ml) and twice with ice-cold PBS buffer alone. Cells were then digested in 1 ml 0.5 M NaOH per well. Aliquots were assayed for radioactivity and protein content. A saturation control experiment in wells without cells showed insignificant radioactivity binding.

High affinity binding was calculated by subtracting the amount of bound ^{125}I-labeled HDL_3 obtained in the presence of a 20-fold excess of unlabeled HDL_3 (nonspecific binding) from the one obtained in absence of unlabeled HDL_3 (total binding). Binding data were analyzed by the ligand computer program Enzfitter (Elsevier-Biosoft); total and high affinity binding curves were determined by a nonlinear curve-fitting program that uses the enhanced algorithm of Marquart; low affinity binding was determined by linear regression.

Preparation of cell membranes

Cell membranes were prepared according to Basu, Goldstein, and Brown (54). Briefly, cell monolayers were washed three times with PBS containing 0.2% BSA, dislodged from dishes with a nylon policeman into 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl (TBS buffer), 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride dissolved in dimethylsulfoxide. Cells from six flasks (75 cm^2 each) were combined, pelleted by centrifugation at 200 *g* for 5 min at 4°C, and resuspended in the same buffer. They were lysed by successive freezing at −70°C and warming at 37°C (3 times), and homogenized by 10-sec pulses of a polytron homogenizer (Virtis); the mixture was then centrifuged at 800 *g* for 10 min at 4°C. The 800-*g* supernatant was centrifuged at 100,000 *g* for 60 min at 4°C, and the pellet was solubilized by aspiration in TBS buffer containing the detergent CHAPS (15 mM) and incubation for 15–20 min at 0°C. The mixture was then centrifuged at 800 *g* for 10 min at 4°C and the supernatant was centrifuged at 100,000 *g* for 60 min at 4°C. The pellet was stored frozen for use in ligand blotting studies.

SDS-polyacrylamide gel electrophoresis and ligand blotting

Membrane proteins were isolated by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (47) using 7.5% polyacrylamide, 3-mm-thick slab gels. Membrane pellets were solubilized by aspiration into 1% SDS electrophoresis buffer and boiled for 3 min in the presence of 5% β-mercaptoethanol (v/v); samples containing equal amounts of protein (500 μg) were applied to separate lanes. Isolated proteins were transferred to nitrocellulose membranes (0.45 μm) by semi-dry electrophoresis. Gels were calibrated using prestained molecular weight standards (from Sigma). For protein binding assay, nitrocellulose membranes were first incubated for 2 h with blocking buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2% bovine serum albumin, 2% non-fat dried milk, 0.01% antifoam A, 50 μg/ml LDL) and then incubated for 3 h at room temperature in the same buffer containing 5 μg/ml ^{125}I-labeled HDL_3 with or without a 100-fold excess of unlabeled HDL_3. Nitrocellulose membranes were washed at room temperature once rapidly and then five times for 15 min with LDL-free blocking buffer. Radioactive protein bands were visualized by autoradiography.

RESULTS

ApoE-depleted HDL_3 or apolipoprotein A-I were mitogenic for A549 cells

When A549 cells were incubated in the presence of increasing amounts of apoE-depleted HDL_3, a maximum stimulation of [^{3}H]thymidine incorporation was obtained for low concentrations (less than 50 μg HDL_3 protein/ml). The stimulation was always higher than the one observed

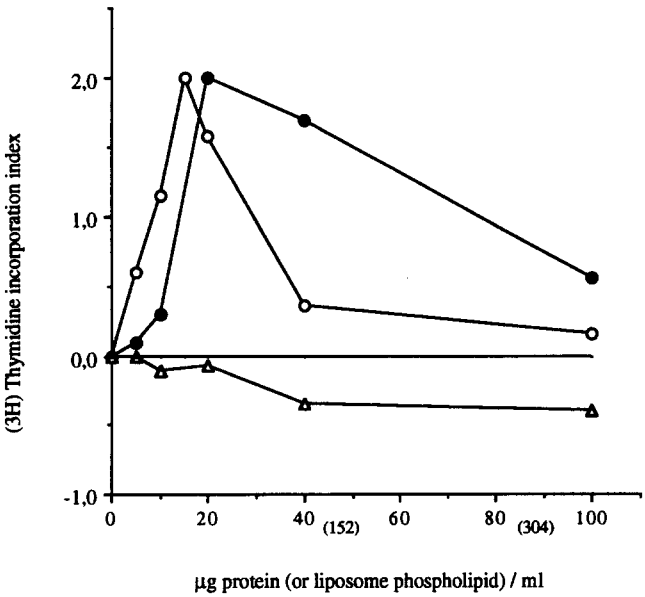

Fig. 1. Effects of increasing concentrations of apoE-depleted HDL_3 or apoA-I-liposome complexes on [^{3}H]thymidine incorporation into DNA of A549 cells. Cells were preincubated for 24 h in RPMI 1640 medium and washed successively with ice-cold bovine serum albumin (10 mg/ml) in PBS buffer and ice-cold PBS buffer alone as described in Materials and Methods. Cells were then incubated for 5 h with increasing concentrations of protein (HDL_3, O-O; apoA-I-liposome complexes, ●-●) and the respective liposome phospholipid concentrations (△-△) in the presence of [^{3}H]thymidine (1 µCi/ml) in RPMI 1640 medium. A control was carried out in the same medium containing 10% fetal calf serum (FCS). After washing with ice-cold PBS buffer, DNA was precipitated with 10% trichloracetic acid and resolubilized in 1 M NaOH for radioactivity counting and protein determination. Results are expressed as dpm/mg cell protein and are means of duplicate culture dishes. The [^{3}H]thymidine incorporation index was calculated as described in Materials and Methods.

with 10% fetal calf serum (FCS), as the index value, as defined previously in Materials and Methods, was higher than 1 (**Fig. 1**); the effect was obtained whatever the incubation medium (RPMI 1640 medium with or without 5% lipoprotein-deficient serum (LPDS)) (**Table 1**). The weak stimulation observed with LDL was lower than the one obtained with 10% FCS; methylated LDL were without effects in the same conditions (**Table 2**).

To test the possibility that the stimulation of DNA synthesis was accompanied by an enhancement of proliferation, cells were exposed to RPMI 1640 medium supplemented or not with 5% LPDS in the presence of increasing concentrations of apoE-depleted HDL_3. As shown in Table 1, addition of HDL_3 at concentrations ranging from 20 to 100 µg protein/ml markedly enhanced cell proliferation. Only part of the cells maintained in RPMI 1640 medium alone (but not in RPMI 1640 medium with 5% LPDS) survived 5 or 6 days which explains the very low number of cells in the experiment without HDL_3 ($50 \pm 10 \times 10^3$); that experiment was also carried out in the presence of LPDS. The observed stimulation of DNA synthesis was therefore accompanied by a enhancement of cell proliferation.

When apolipoprotein A-I (apoA-I) was substituted for HDL_3, a stimulation was also obtained but it occurred to a lesser extent and for higher protein concentrations (Table 2). Curiously, an unexplained inhibitory effect was observed for lower concentrations. Since both phospholipid and cholesterol are required for the complete reorganization of secondary and tertiary structure of apoA-I HDL (55, 56), it was incorporated into liposomes as described in Materials and Methods. As shown in Fig. 1 and Table 2,

TABLE 1. Effects of HDL_3 on A549 cell proliferation and [^{3}H]thymidine incorporation into DNA

HDL_3	10% FCS		RPMI 1640		5% LPDS	
	Cells/Dish ($\times 10^{-3}$)	dpm/mg ($\times 10^{-2}$)	Cells/Dish ($\times 10^{-3}$)	dpm/mg ($\times 10^{-2}$)	Cells/Dish ($\times 10^{-3}$)	dpm/mg ($\times 10^{-2}$)
µg/ml						
0	698 ± 20	2081 ± 43	50 ± 10	1649 ± 26	504 ± 14	1707 ± 49
20	nd	nd	202 ± 20[a]	2520 ± 86[a]	590 ± 10[b]	2404 ± 36[a]
50	nd	nd	761 ± 80[a]	2287 ± 24[a]	860 ± 40[a]	2120 ± 62[a]
100	nd	nd	896 ± 25[a]	1658 ± 12[c]	796 ± 60[a]	1732 ± 19[c]

A549 cells were preincubated for 24 h in RPMI 1640 medium and seeded at initial density of 25,000 cells/ml in 6-well multidishes in the presence of RPMI 1640 medium alone or supplemented with 5% LPDS or 10% FCS with HDL_3 added at indicated concentrations. Dishes were counted on day 5 as described under Materials and Methods. Results are means ± SE of duplicate cultures and given as cells/dish ($\times 10^{-3}$). For [^{3}H]thymidine incorporation cells were preincubated and washed as described in Fig. 1. They were then incubated for 5 h with increasing concentrations of HDL_3 in the presence of RPMI 1640 medium alone or supplemented with 5% LPDS or 10% FCS. After washing with ice-cold PBS buffer, DNA was precipitated with 10% trichloracetic acid and resolubilized in 1 M NaOH for radioactivity counting and protein determination. Results are expressed as dpm/mg cell protein ($\times 10^{-2}$) and are means ± SE of duplicate cultures.

[a] $P < 0.001$; [b] $P < 0.05$; [c] no significant difference compared to the same experiment without HDL_3.

TABLE 2. [^{3}H]thymidine incorporation in the presence of apolipoprotein, non-apolipoprotein-liposome complexes, LDL, or methylated LDL

Addition	[^{3}H]Thymidine Incorporation					
	ApoA-I	ApoA-I/Lipo	ApoA-II/Lipo	Glyco/Lipo	LDL	met LDL
μg/ml	*dpm/mg cell protein (× 10^{-2})*					
0	1557 ± 21	1617 ± 21	1432 ± 30	1441 ± 21	1301 ± 23	1605 ± 22
5	nd	1575 ± 41[c]	1511 ± 40[c]	1488 ± 27[c]	1562 ± 13[c]	1671 ± 11[c]
10	553 ± 59[a]	1721 ± 36[c]	1435 ± 9[c]	1494 ± 10[c]	1341 ± 27[c]	1492 ± 39[c]
20	642 ± 44[a]	2670 ± 18[a]	1405 ± 20[c]	1300 ± 46[c]	1674 ± 33[b]	1721 ± 72[c]
50	2142 ± 72[a]	2481 ± 72[a]	1341 ± 18[c]	1389 ± 33[c]	1710 ± 49[b]	1573 ± 19[c]
200	2204 ± 80[a]	1884 ± 84[c]	1344 ± 29[c]	1277 ± 48[c]	1636 ± 17[b]	1314 ± 42[b]
Control						
10% FCS	2017 ± 92	2136 ± 48	1874 ± 63	1841 ± 15	1879 ± 81	1920 ± 49

A549 cells were preincubated and washed as described in Fig. 1. They were incubated for 5 h with increasing concentrations (μg/ml) of apolipoprotein A-I (apoA-I), apolipoprotein-liposome complexes (apoA-I/lipo or apoA-II/lipo), glycophorin-liposome complexes (glyco/lipo), LDL, or methylated LDL (met LDL). The values of positive controls (10% FCS) without other addition are shown for each experiment. Results are expressed as dpm/mg cell protein (× 10^{-2}) and are means ± SE of duplicate culture dishes.

[a] $P < 0.001$; [b] $P < 0.009$; [c] no significant difference compared to the same experiment without HDL_3.

an inhibitory effect did not occur and apoA-I-containing liposomes were able to mimic the effect of whole HDL_3 on the stimulation of [^{3}H]thymidine incorporation. This stimulation was obtained at concentrations less than 50 μg protein/ml as for whole HDL_3. As liposomes alone had an insignificant effect at low concentration and low inhibitory effect at high concentration (Fig. 1), the observed stimulation of DNA synthesis seems to be due to apoA-I. To ascertain that the mitogenic effect was not due to a nonspecific interaction between a lipid-binding protein and the cell, two proteins other than apoA-I were tested: apoA-II, the other major protein component of HDL_3, and glycophorin, a protein of erythrocyte plasma membranes. Both proteins were complexed with liposomes as was apoA-I but, in contrast to apoA-I, they were unable to stimulate [^{3}H]thymidine incorporation into DNA (Table 2). For the purpose of specifying what kind of interaction occurred between HDL_3 and cells, binding studies were carried out with ^{125}I-labeled apoE-depleted HDL_3.

ApoE-depleted ^{125}I-labeled HDL_3 bound on cells with a high affinity

Fig. 2 shows the binding of increasing amounts of apoE-depleted ^{125}I-labeled HDL_3 on whole cells at 37°C in the presence and absence of an excess of unlabeled HDL_3. Binding that could be prevented by excess unlabeled HDL_3 (high affinity binding) saturated at a concentration of 50 μg HDL protein/ml or more. At these concentrations, total binding and low affinity binding increased linearly with the same slope; this slope was subtracted from total ^{125}I-labeled HDL_3 binding curve to obtain the

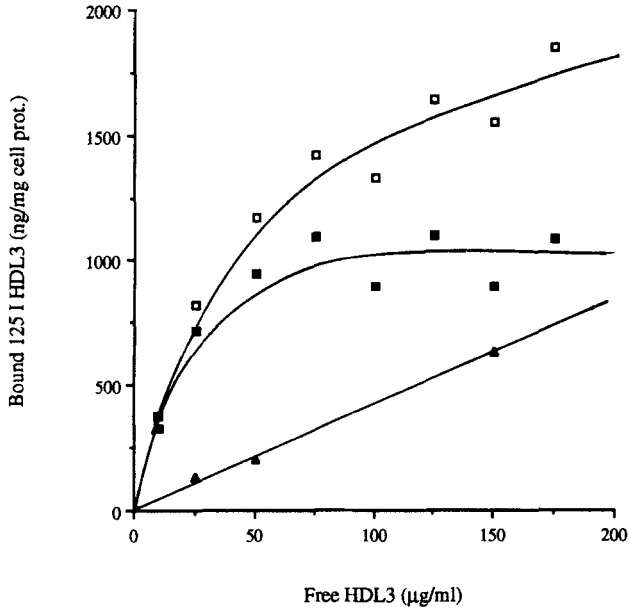

Fig. 2. ApoE-depleted ^{125}I-labeled HDL_3 binding to A549 cells. Cells were preincubated and washed as described in Fig. 1. They were then incubated for 1 h at 37°C with increasing concentrations of apoE-depleted ^{125}I-labeled HDL_3 in the presence of 2 mg/ml fatty acid-free bovine serum albumin. Total binding (□-□) was measured on 0.5 M NaOH digests after washing unbound lipoproteins and precipitating cell proteins with trichloracetic acid as described in Materials and Methods. Results are means of duplicate culture dishes. The high affinity binding curve (■-■) was calculated by subtracting values for binding in the presence of excess unlabeled HDL (low affinity) (▲-▲) from the values for total binding. The straight line for low affinity binding was obtained by linear regression and the curves for total and high affinity binding were obtained by a nonlinear regression computer program that uses the enhanced algorithm of MARQUART (Enzfitter program, Elsevier Biosoft).

high affinity curve. Calculated dissociation constant and capacity of binding sites for the high affinity curve were, respectively, 20.4 ± 6.1 μg HDL_3 protein/ml and 1195 ± 97 ng HDL_3 protein/mg cell protein. Similar results were obtained whatever the calculation method: nonlinear regression determined by computer analysis or Scatchard analysis of high affinity binding. Scatchard plot of the ascending part of the high affinity curve (not shown) was linear. Internalization and degradation of ^{125}I-labeled HDL_3 were not observed whatever its concentration.

Competition between lipoprotein subfractions and phospholipid particles suggested the presence of two classes of HDL_3 binding sites

To assess specificity of the HDL binding sites, A549 cells were incubated with apoE-depleted ^{125}I-labeled HDL_3, with or without increasing concentrations of unlabeled lipoprotein subfractions (**Fig. 3**). Experiments were carried out at 4°C to prevent internalization of LDL. ApoE-depleted HDL_3 competed well for apoE-depleted ^{125}I-labeled HDL_3 as expected, but LDL and methylated LDL also competed. With a twofold excess of competitors over ^{125}I-labeled HDL_3, the labeled residual binding percentages were, respectively, 48%, 43%, and 32% for methylated LDL, LDL, and HDL_3. Although the residual binding values after competition by LDL and methylated LDL were significantly higher than the value obtained after competition by HDL_3, the slight difference does not support specificity of the high affinity binding sites previously described. Moreover, as it is well known that methylated LDL are not able to bind LDL receptors (50), the competition by LDL cannot be explained by ^{125}I-labeled HDL_3 bound to LDL receptors.

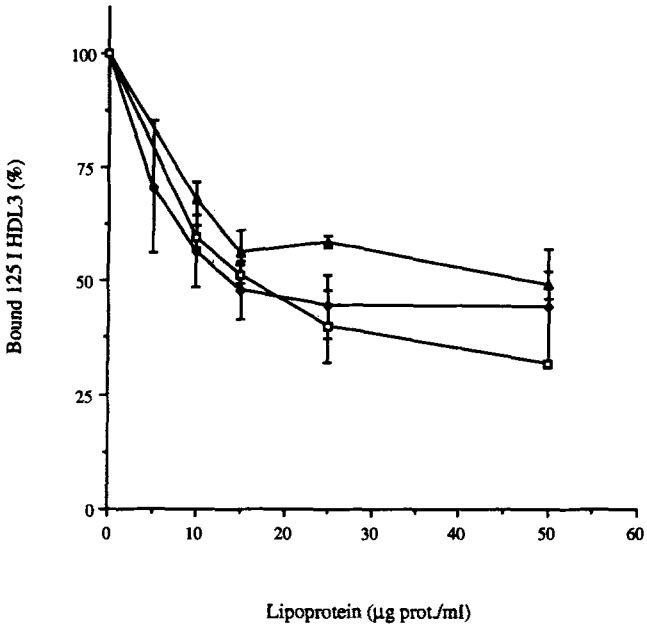

Fig. 3. Competitive inhibition of apoE-depleted ^{125}I-labeled HDL_3 binding to A549 cells by lipoprotein subfractions. After preincubation and washing as in Fig. 1, A549 cells were incubated at 4°C for 3 h with 20 μg protein/ml apoE-depleted ^{125}I-labeled HDL_3 in the presence of increasing concentrations of unlabeled apoE-depleted HDL_3 (□-□), LDL (♦-♦), or methylated LDL (▲-▲). Bound labeled HDL_3 values were measured as described in Fig. 2 and are means ± 2 SE of duplicate culture dishes.

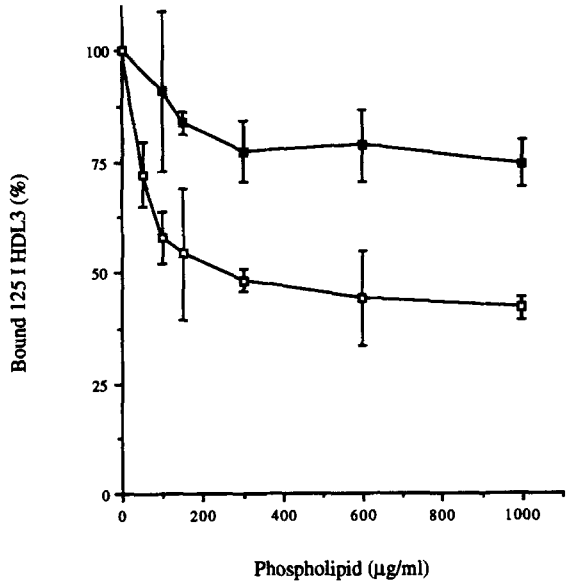

Fig. 4. Competitive inhibition of apoE-depleted ^{125}I-labeled HDL_3 binding to A549 cells by phospholipids. After preincubation and washing as described in Fig. 1, A549 cells were incubated at 4°C for 3 h with 10 μg protein/ml apoE-depleted ^{125}I-labeled HDL_3 in the presence of increasing concentrations of dimirystoyl-phosphatidylcholine (■-■) or of phospholipid particles BVSM (□-□). Bound labeled HDL_3 values were measured as described in Fig. 2 and are means ± 2 SE of duplicate culture dishes.

The question then arises as to the nature of the apoE-depleted ^{125}I-labeled HDL_3 high affinity binding. Specific lipoprotein high affinity protein binding sites (LDL receptors, HDL receptors) did not seem to be implicated. Therefore, we investigated a possibly nonspecific phospholipid interaction between cell membrane and lipoproteins. For that purpose, A549 cells were incubated with apoE-depleted ^{125}I-labeled HDL_3, with or without increasing concentrations of phospholipids, either as dimyristoylphosphatidylcholine (DMPC) or as phospholipid particles ("bio vecteur supra moléculaire": BVSM) which may be considered as a rigid model of "lipoprotein without apolipoprotein." A rapid decrease of bound labeled HDL_3 occurred for low concentrations of phospholipid (below 150 μg/ml) particularly with BVSM (**Fig. 4**). The first part of the curve reflects high affinity binding; in the second part of the curve (above 150 μg/ml), bound labeled HDL_3 decreased more slowly, which reflects a low affinity binding and a high number of binding sites.

The ^{125}I-labeled HDL_3 binding sites described in Fig. 2, therefore, implicate, at least partially, nonspecific inter-

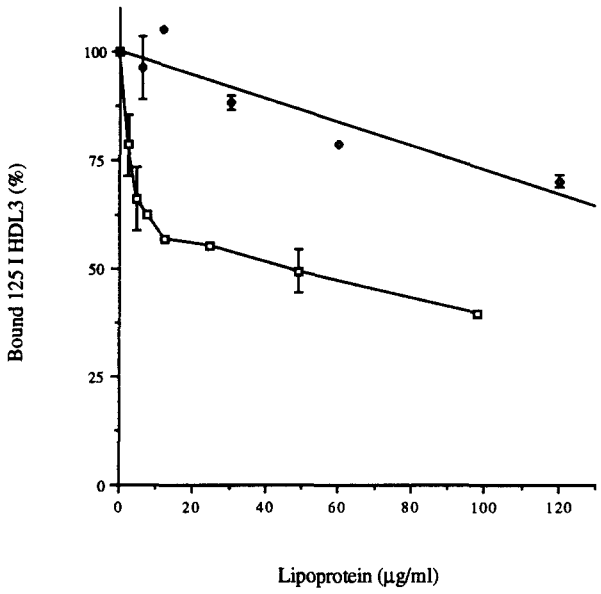

Fig. 5. Competitive inhibition of apoE-depleted ^{125}I-labeled HDL_3 binding to A549 cells by lipoprotein subfractions in the presence of BVSM. The conditions were essentially the same as in Fig. 3 except that incubations were carried out with 10 µg protein/ml apoE-depleted ^{125}I-labeled HDL_3 and 150 µg BVSM phospholipid/ml in the presence of increasing concentrations of unlabeled apoE-depleted HDL_3 (□-□) and LDL (◇-◇). Results are means ± 2 SE of duplicate culture dishes.

actions with phospholipids; but the presence of specific HDL binding sites cannot be excluded. In order to test this possibility, cells were incubated at 4°C with apoE-depleted ^{125}I-labeled HDL_3 in the presence of 150 µg BVSM phospholipid/ml, with or without increasing concentrations of unlabeled apoE-depleted HDL_3 or LDL (**Fig. 5**). HDL_3 competed with a rapid decrease of bound radioactivity, whereas LDL competed to a much smaller extent. The finding that LDL competed slightly for HDL binding may be explained by low affinity binding sites discussed previously (Fig. 4) and by the presence of apoA-I in the LDL density fraction that accounted for approximately 2% of total protein content.

A possible misinterpretation of data could be due to an eventual transfer of radioactive material (from ^{125}I-labeled HDL_3 to BVSM) or to the formation of phospholipid–^{125}I-labeled apolipoprotein discs (57). These possibilities would explain the apparent poor competitive effect of DMPC (Fig. 4) but are unlikely to explain the results with BVSM. Indeed, ^{125}I-labeled HDL_3 were incubated for 3 h at 4°C with BVSM and without cells using concentrations similar to those in competitive experiments. After gel exclusion chromatography of the mixture, the radioactivity was eluted with HDL_3 the BVSM fraction did not contain any radioactive material. Moreover, radioactive particles of size other than HDL_3 and BVSM were not observed (**Fig. 6**).

At least two components must therefore be distinguished in the apoE-depleted ^{125}I-labeled HDL_3 high affinity binding of Fig. 2: one specific for HDL_3 and a nonspecific site that also bound vesicular phospholipids such as LDL or BVSM.

A specific protein binding site for apoE-depleted ^{125}I-labeled HDL_3

Fig. 7 shows the binding of increasing amounts of apoE-depleted ^{125}I-labeled HDL_3 on whole cells in the presence of BVSM. A 20-fold excess of unlabeled apoE-depleted HDL_3 was added in some experiments to deter-

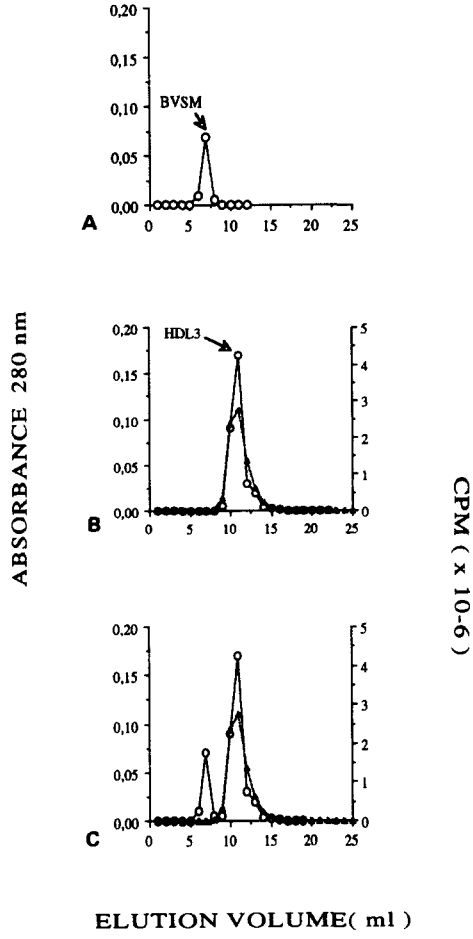

Fig. 6. Elution profiles of BVSM (A), ^{125}I-labeled HDL_3 (B), and BVSM + ^{125}I-labeled HDL_3 incubation mixture (C). Incubation of 10 µg/ml ^{125}I-labeled HDL_3 with 200 µg phospholipid/ml was performed in cell-free multiwell plates at 4°C for 3 h in RPMI 1640 medium supplemented with 2 mg/ml bovine serum albumin (final volume of 1 ml). Aliquots of 200 µl from BVSM alone (A), ^{125}I-labeled HDL_3 alone (B), and BVSM + ^{125}I-labeled HDL_3 incubation mixture (C) were fractionated through a Superose 6 exclusion chromatography column (8 × 300 mm) using a fast protein liquid chromatography system (FPLC Pharmacia). Elution was performed with phosphate buffer saline, pH 7.4. Fractions (1 ml) were collected and assayed for absorbance at 280 nm (○-○) and radioactivity (▲-▲). The elution profiles were obtained from one experiment but were representative of duplicate incubations and chromatographies. Similar results were obtained when incubation was carried out at 37°C for 1 h.

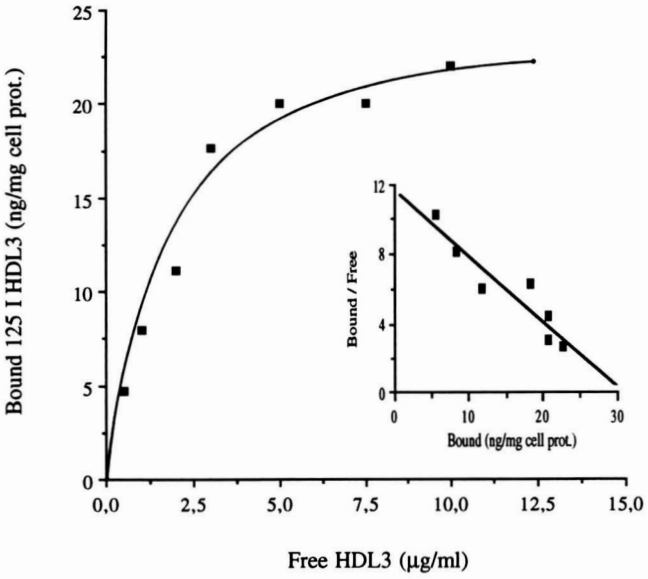

Fig. 7. ApoE-depleted ^{125}I-labeled HDL_3 specific binding to A549 cells in the presence of BVSM. The conditions were essentially the same as in Fig. 2 except that all the experiments were carried out with 150 µg BVSM phospholipid/ml. The specific high affinity binding curve was measured as described in Fig. 2 by subtracting low affinity binding from total binding curve (not shown). The specific curve was transformed by Scatchard analysis (inset) with a ligand computer program (Enzfitter, Elsevier-Biosoft).

mine nonspecific low affinity binding. Nonlinear regression and Scatchard analysis of the high affinity curve (Fig. 7) indicated a dissociation constant K_D of 2.47 ± 0.46 µg HDL_3 protein/ml ($2.6 \pm 0.5 \times 10^{-8}$ M assuming that HDL_3 have a 55% protein content and a molecular mass of 175 kDa) and a capacity of binding sites of 27.9 ± 2.2 ng HDL_3 protein/mg cell protein at 4°C. Values obtained at 37°C were not significantly different; exchange of radioactive material between ^{125}I-labeled HDL_3 and BVSM at this temperature is unlikely as the same elution profile as in Fig. 6 was obtained when ^{125}I-labeled HDL_3 (10 µg/ml) were incubated for 1 h at 37°C with BVSM (200 µg phospholipid/ml) without cells.

The specific HDL binding capacity seemed independent of growth state as a significant difference was not observed between cells in log phase and cells at confluence. Moreover, in contrast to fibroblasts (27), it was also independent of cholesterol pre-loading of cells.

These binding experiments were also carried out after substituting LDL or methylated LDL (100 µg/ml) for BVSM. Whatever the incubation temperature (4°C with LDL or 37°C with methylated LDL), the K_d value was not significantly different from the one determined in the presence of BVSM. Because transfer of radioactive material from HDL to LDL is unlikely, this result further sustains previous results and their interpretation in the presence of BVSM. The capacity of specific binding sites was slightly lower (18.3 ± 0.7 and 20.0 ± 2.8 ng HDL_3 protein/mg cell protein, respectively, at 4°C and 37°C). This difference may be due to occupancy of some HDL specific binding sites by an apoA-I contaminant of LDL.

Membrane proteins from A549 cells were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and incubated with ^{125}I-labeled HDL_3 in the presence of LDL. Autoradiographs revealed that ^{125}I-labeled HDL_3 bound to a single membrane protein that had an apparent molecular mass of 110 kDa; a 100-fold excess of unlabeled HDL_3 completely blocked binding of ^{125}I-labeled HDL_3 (**Fig. 8**).

Stimulation of [^{3}H]thymidine incorporation into DNA of A549 cells by apoE-depleted HDL_3 is related to specific binding site occupancy

The question then arises as to the relation between both components of HDL_3 binding sites and the observed stimulation peak of DNA synthesis. In order to prevent binding of HDL_3 on phospholipid binding sites, A549 cells were incubated with increasing amounts of HDL_3 in the presence of BVSM. These phospholipid particles alone were shown to have no effects on DNA synthesis in our experimental conditions; however, two responses resulted from the presence of these phospholipid particles: the quenching of stimulation at high concentrations of HDL_3 was almost suppressed and there was a shift of the maximum stimulation to lower concentration of HDL_3 as a plateau was reached as early as a concentration of 5 µg HDL_3 protein/ml (**Fig. 9**). As this low concentration is also the one that allowed a nearly complete saturation of specific binding sites (Fig. 7), the 110 kDa HDL_3 binding

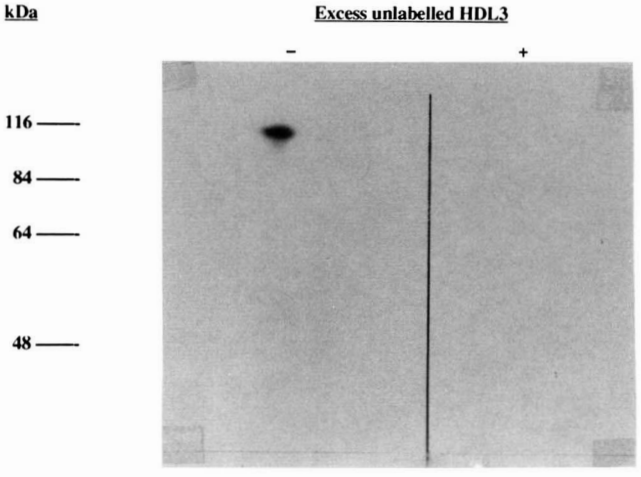

Fig. 8. Ligand blotting of membrane proteins isolated from A549 cells. Solubilized cell membranes (500 µg of protein per lane) were subjected to 7.5% SDS-polyacrylamide electrophoresis and proteins were transferred to nitrocellulose by semi-dry electrophoresis. After incubation of nitrocellulose with blocking buffer as described in Materials and Methods, nitrocellulose strips were incubated with 5 µg/ml apoE-free ^{125}I-labeled HDL_3 plus either no or excess unlabeled HDL_3 (500 µg/ml) and washed extensively. Membrane proteins that bind ^{125}I-labeled HDL_3 were visualized by autoradiography. Molecular masses of protein standards are indicated.

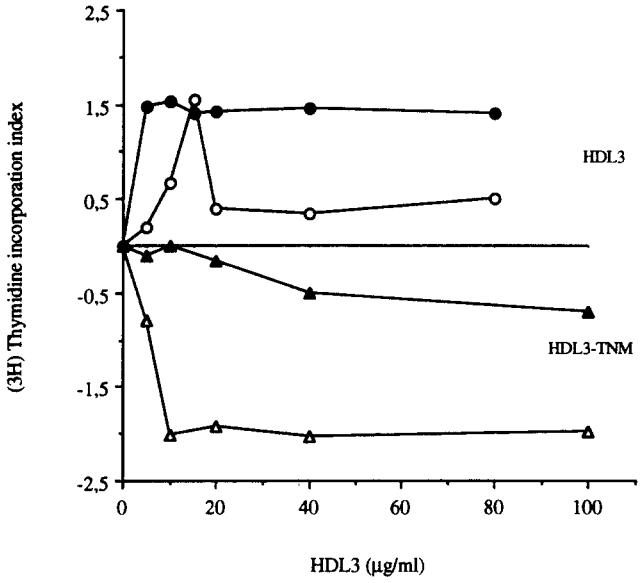

Fig. 9. Modulation of DNA synthesis in A549 cells by native HDL_3 and TNM-HDL_3 in the presence or absence of BVSM. Cells were preincubated and washed as described in Fig. 1. They were incubated for 5 h with increasing concentrations of HDL_3 (circles) or TNM-HDL_3 (triangles) in the presence of [^{3}H]thymidine (1 μCi/ml) in RPMI 1640 medium alone (open symbols) or supplemented with 200 μg BVSM phospholipid/ml (solid symbols). The [^{3}H]thymidine incorporation index was calculated as in Materials and Methods. All results are means of duplicate culture dishes.

protein could be implicated in the stimulation of DNA synthesis. In contrast, the quenching of stimulation for high concentrations of HDL_3 in the absence of BVSM seems related to binding of HDL_3 to phospholipid binding sites.

In contrast to BVSM, LDL or methylated LDL (50 μg/ml) were unable to prevent the inhibitory effects of high concentrations of HDL_3 although the concentration was shown to be sufficient for a maximum competition on phospholipid sites with HDL_3 (Fig. 3). This inability might be explained by a different membrane effect of these lipoproteins during the 5-h incubation with cells at 37°C (see Discussion below).

Modification of tyrosine and cysteine residues by tetranitromethane has been shown to be efficient in inactivating various proteins, including HDL_3 apolipoproteins (3). When HDL_3 were modified by tetranitromethane (HDL_3-TNM), they not only lost their ability to stimulate DNA synthesis but, in contrast, had an inhibitory effect (negative [^{3}H]thymidine incorporation index) as shown in Fig. 9. This effect was almost suppressed in the presence of BVSM (Fig. 9): it appeared then only for higher concentrations of HDL_3 which might indicate a competition on lipid sites between HDL_3-TNM and BVSM.

HDL_3 were, therefore, herein shown to have a biphasic effect on DNA synthesis: a stimulatory one that was receptor-dependent and an inhibitory one that appeared to be dependent on nonspecific binding on membrane lipids.

DISCUSSION

High density lipoproteins are mitogenic in various cell types (4–8, 12) which might imply a synergistic action between these lipoproteins and growth factors (8). Growth factors have been shown to be released in culture medium of the human lung adenocarcinoma cell line A549 (58). They have been identified from culture medium; among them, Transforming Growth Factor α and Insulin-like Growth Factor 1 have been purified (58). Transforming Growth Factor β (TGF β) is released in culture medium in higher amounts by A549 cells than by normal bronchial epithelial cells, but it seems to be unable to bind its receptors although the same receptor level is present. It is unclear whether this defect is due to the nonprocessing of the latent form of TGF β or to unability of the activated form to bind its receptors (59). These autocrine growth factors have been shown to stimulate concomitantly LDL receptor activity, cholesterol and DNA synthesis (42).

As in some other normal and tumor cell lines, a mitogenic effect of HDL_3 was demonstrated in the human lung adenocarcinoma cells A549. A mitogenic effect was also observed with apoA-I (alone or complexed with liposomes) but not with apoA-II nor with another lipid-binding protein that is not an apolipoprotein (glycophorin). The mitogenic effect seems, therefore, to be specifically apoA-I-dependent; such a specificity for apoA-I has been suggested for cholesterol efflux induced by HDL_3 (36). The stimulation of DNA synthesis was only observed for low concentrations of HDL_3. It was accompanied by a stimulation of cell proliferation which was not quenched by high concentrations of HDL_3; this difference might be explained by the long time of incubation with HDL_3 required for cell counting experiments (5 days versus 5 h for DNA synthesis determinations) and then might reflect a consumption of HDL_3 by cells.

With the object of explaining the quenching of the stimulation of DNA synthesis at high HDL_3 concentrations, we studied the relationship between these effects and the binding mechanism of HDL_3 on cells. Binding of HDL on cells by nonspecific lipid interactions has been suggested by several authors (20–22) who deny existence of a specific protein binding site. In contrast, a specific protein binding site for HDL has been demonstrated by others (25–28) and an HDL binding protein has even been cloned recently (60). These controversial results have been observed sometimes on the same cell type, e.g., fibroblasts (20, 21, 27), and might be explained if both types of binding sites are present.

The specific binding sites for HDL in A549 cells accounted for only 2.5% of total high affinity binding sites (30 and 1200 ng HDL_3 protein/mg cell protein for, respec-

tively, specific and phospholipid + specific binding sites). This low ratio explains why the population of specific binding sites was not detected in the absence of phospholipid particles or LDL and might explain some controversial results on other cell types. Recently, two classes of binding sites were also described on rat liver plasma membranes (61) and the higher affinity sites accounted for a low percentage (5%) of total high affinity sites; this is similar to what was determined in A549 cells (**Table 3**).

Phospholipid and/or apolipoprotein transfer between HDL and phospholipid vesicles and formation of apolipoprotein-phospholipid discs have been reported (57). However, such transfer or association was not observed between ^{125}I-labeled HDL_3 and BVSM under our conditions. This could be due to the rigid structure of BVSM (polysaccharide core) which would impede molecular transfer at the external phospholipid layer; indeed, flexibility has been suggested to be one of the mechanisms by which transfers are made easier in HDL (57).

Binding sites for phospholipids have been suggested on fibroblasts and other cell types to explain results of metabolic studies (20, 23). These sites are generally considered as nonspecific but they have also been claimed as specific for HDL_3 on endothelial cells (29). We showed, herein, experimental arguments for the presence of two classes of HDL_3 binding sites on A549 cells: nonspecific phospholipid sites and specific protein sites.

Different determinations of dissociation constants for HDL_3 specific binding on various cell types have shown similar results at either 4°C or 37°C, on either whole cells or isolated membranes, with either HDL_3 or apoA-I-protein A hybrid: the determined K_d values have been 1.5-10 $\times$ 10^{-8} M (23, 26, 30, 32). These values are in good agreement with that determined on A549 cells (2.6 $\times$ 10^{-8} M). HDL binding experiments on A549 cells were carried out on monolayer cells. Other results on such monolayer cells are from Slotte, Oram, and Bierman (2) and Biesbroeck et al. (62) with human fibroblasts; Biesbroeck et al. (62) showed a high affinity specific binding curve that saturated at approximately 70 ng HDL protein/mg cell protein. Other determinations on whole cells have been carried out on cell suspensions (23, 30). Some results are compiled in Table 3 and compared with ours. It should be noted that our results are close to the ones recently determined on rat liver plasma membranes by Morrison, McPherson, and Fidge (61).

Identification of the HDL_3 protein binding sites by ligand blotting showed an apparent molecular mass of 110 kDa which is the same as that previously determined in fibroblasts and some other cell types by Graham and Oram (27).

When compared to fibroblasts, HDL_3 specific binding on A549 cells showed essentially two significant differences: a lower binding capacity (Table 3) and an absence of up-regulation by cholesterol (27, 63). These differences might be characteristic of neoplastic or dividing cells; for example, both HDL_3 receptor number and cholesterol efflux have been shown to be enhanced in fibroblasts when cell proliferation is inhibited by gamma-interferon (64).

The stimulation of [^{3}H]thymidine incorporation into DNA of A549 cells was obtained with low concentrations of HDL_3 or apoA-I-containing liposomes, the same ones that were shown to be able to saturate the specific binding sites. These sites seem therefore implicated in the stimulatory effect of HDL_3, the mechanism being unknown but probably related to the intracellular events induced by HDL_3-bound receptors. It has been shown that interaction of HDL_3 with their receptors promotes membrane-associated protein kinase C activity. This, in turn, stimulates the translocation of cholesterol from intracellular pools to the plasma membrane in adipose cells (59) and in fibroblasts (60), and phosphorylates a 27 kDa protein related to cell proliferation in vascular endothelial cells (11). Protein kinase C activators (such as phorbol esters)

TABLE 3. HDL high affinity binding K_d and capacities in some cell types

	Whole Cells		Isolated Membranes	
Cells	K_d	Capacity	K_d	Capacity
	μg/ml	*ng/mg cell protein*	*μg/ml*	*ng/mg membrane protein*
Adipocytes (26)	18	160	10	2400
Fibroblasts	nd	70[a]	1.5[b]	1300[b]
Rat liver (61)				
Lower affinity	nd	nd	36	700
Higher affinity	nd	nd	0.94	36
A549 cells				
Phospholipid				
+ Specific	20	1195	nd	nd
− Specific	2.5	28	nd	nd

nd, not determined.
[a] From Biesbroeck et al. (62).
[b] From Mendel et al. (63).

have similar effects (11, 59, 60). However, A549 cells have their growth stimulated by HDL_3 but inhibited by phorbol esters; this inhibition occurs with a rapid transient increase in membrane-associated protein kinase C activity followed by a total disappearance of cellular protein kinase C activity (44). Such a growth inhibition effect of phorbol esters has also been observed in human breast cancer cells (65). All these results suggest that receptor-mediated actions of HDL_3, as well as phorbol ester effects, need protein kinase C activity but the mechanisms are different. We showed that the mitogenic effect of HDL_3 occurs in the absence of internalization; one possibility is that binding of HDL_3 to its receptor generates an intracellular signal that enhances the receptor activity of growth factors either by a direct effect on these receptors or indirectly on growth factor secretion. Indeed, receptors for a variety of growth factors, including IGF1, are themselves protein kinases. Moreover, the enzyme activity of these receptors appears to be regulated both by autophosphorylation and by phosphorylation catalyzed by protein kinase C (66).

In order to understand the molecular events in A549 cells after HDL_3 binding to their receptors, it will be necessary to determine protein kinase C activity and cholesterol metabolism modification (cholesterol efflux, LDL receptor, and HMG-CoA reductase activities). Accumulation of cholesterol in plasma membranes has been shown to be related to cell growth (67). A stimulation of HMG-CoA reductase activity by HDL_3 would also explain a mitogenic action as it promotes synthesis not only of cholesterol but also of isoprenoid compounds necessary to the activation of the protein $p21^{ras}$ (17). Moreover, the mitogenic action of $p21^{ras}$ needs a down-regulation of cytosolic protein kinase C and a parallel increase of nuclear-associated enzyme activity (68).

The inhibition of mitogen-induction DNA synthesis obtained with higher concentrations has been previously observed for the proliferation of lymphoblastoid cells (6). However, quenching of stimulation did not occur in A549 cells when phospholipid binding sites were occupied by phospholipid particles (BVSM). Binding of HDL_3 on phospholipid sites seems, therefore, to antagonize the effects induced by specific binding sites. Such a possibility was further sustained by the negative effect of TNM-HDL_3 on DNA synthesis. Because these modified HDL_3 are unable to bind their specific sites (2, 21), it is likely that the negative effect is due to TNM-HDL_3 binding on lipid sites. This phenomenon might be explained by the modulation of membrane function by cholesterol (69) and the relationship between increase in cholesterol content of plasma membrane and the high level of cell growth, two events that have been shown to be linked in fibroblasts (67). When HDL_3 are in low concentration, they almost totally bind to the specific protein receptor, which then might promote cholesterol transfer from intracellular pools to the plasma membrane; cells are then in mitogenic conditions. When receptors are saturated in the presence of high concentrations of HDL_3, excess HDL_3 bind to cells by phospholipidic interactions; they can act as cholesterol acceptors and then the cholesterol content of plasma membrane should decrease, which is related to a loss of cell division conditions. Such a mechanism would explain quenching of DNA synthesis stimulation in A549 cells for high concentration of HDL_3 and the negative effect of TNM-HDL_3. In contrast, when BVSM are added in medium culture, they impede HDL_3 binding by phospholipid interactions; cholesterol should remain anchored in plasma membrane due to inability of these particles to incorporate cholesterol. Indeed, BVSM have a rigid structure and do not contain apolipoproteins; the latter have been shown to facilitate desorption of cholesterol molecules by perturbing phospholipid-cholesterol interactions (70). The result is that mitogenic conditions are maintained with high concentrations of HDL_3 in the presence of BVSM.

The question of whether the plasma HDL have a similar effect in vivo and of its importance relative to other growth factors in the neoplastic process is unclear. Total HDL concentration in extravascular fluids and serum is likely to be 10 to 100 times higher (71) than the concentration of HDL_3 required to saturate specific sites on A549 cells. The other major density subclass of high density lipoproteins, HDL_2, is known to behave as an antagonist as it binds to the same cell sites as HDL_3 but is unable to promote cholesterol efflux (50). The relative proportion of HDL subclasses in the extracellular fluid must therefore be considered in vivo.

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