Uptake of type IV hypertriglyceridemic VLDL by cultured macrophages is enhanced by interferon-γ

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Abstract Hypertriglyceridemic (HTG) very low density lipoproteins (VLDL) from subjects with type IV hyperlipoproteinemia induce both cholesteryl ester (CE) and triglyceride (TG) accumulation in cultured J774 macrophages. We examined whether the cytokine interferon-γ (IFN-γ), which is expressed by lymphocytes in atherosclerotic lesions, would modulate macrophage uptake of HTG-VLDL. Incubation of cells with HTG-VLDL alone significantly increased cellular CE and TG mass 17- and 4.3-fold, respectively, while cellular free cholesterol (FC) was unaffected. Preincubation of cells with IFN-γ (50 U/ml) prior to incubation with HTG-VLDL caused a marked enhancement in cellular CE and TG 27- and 6-fold over no additions (controls), respectively, and a 1.5-fold increase in FC. IFN-γ increased low density lipoprotein (LDL)-induced cellular CE 2-fold compared to LDL alone. IFN-γ did not enhance the uptake of type III (apoE2/E2) HTG-VLDL or VLDL from apoE knock-out mice. Incubations in the presence of a lipoprotein lipase (LPL) inhibitor or an acylCoA:cholesterol acyltransferase (ACAT) inhibitor demonstrated that the IFN-γ-enhanced HTG-VLDL uptake was dependent on LPL and ACAT activities. IFN-γ significantly increased the binding and degradation of ^{125}I-labeled LDL. Binding studies with ^{125}I-labeled α2-macroglobulin, a known LDL receptor-related protein (LRP) ligand, and experiments with copper-oxidized LDL indicated that the IFN-γ-enhanced uptake was not due to increased expression of the LRP or scavenger receptors. Thus, IFN-γ may promote foam cell formation by accelerating macrophage uptake of native lipoproteins. IFN-γ-stimulated CE accumulation in the presence of HTG-VLDL occurs via a process that requires receptor binding-competent apoE and active LPL. IFN-γ-enhanced uptake of both HTG-VLDL and LDL is mediated by the LDL-receptor and requires ACAT-mediated cholesterol esterification.—Whitman, S. C., C. A. Argmann, C. G. Sawyez, D. B. Miller, R. A. Hegele, and M. W. Huff. **Uptake of type IV hypertriglyceridemic VLDL by cultured macrophages is enhanced by interferon-γ. *J. Lipid Res.* 1999.** 40: **1017–1028.**

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T lymphocytes have been identified in human atherosclerotic plaques at all stages of lesion development (1–4). The presence of these immune cells, many of them activated (1, 4), lends support to the idea that an immune response may play an active role in atherogenesis. As a result, investigations have begun to focus on examining whether the cytokine interferon-γ (IFN-γ), which is produced by certain T lymphocyte subsets (5, 6), may play an important role in modulating the development of atherosclerotic lesions. A potential role for IFN-γ in atherogenesis stems from the presence of this cytokine with T lymphocyte-rich regions of human plaques (4, 7). Furthermore, macrophages and smooth muscle cells (SMCs), both of which are capable of forming foam cells, express the specific IFN-γ receptor (8).

The role of T-cells and IFN-γ in atherogenesis is controversial. T-cell-deficient mice develop normal fatty streak lesions when fed atherogenic diets (9), and immune-deficient RAG-2 knockout mice crossed with apolipoprotein (apo) E knockout mice (EKO) develop lesions that are indistinguishable from the EKO controls (10). In vitro, IFN-γ stimulates the expression of VCAM-1 on endothelial cells, MHC-II on macrophages and SMCs, and class A scavenger receptors (SR-A) on SMCs (8, 11, 12), which are all potentially pro-atherogenic effects. Gupta et al. (13) demonstrated that atherosclerosis was significantly attenuated in double knockout mice, created by crossing IFN-γ receptor knockout mice with EKO mice. This also suggests a pro- atherogenic role for IFN-γ. In contrast, in vitro studies have demonstrated that IFN-γ decreases SR-A, the LDL receptor-related protein (LRP), and lipoprotein lipase (LPL) expression in macrophages and inhibits SMC col-

Abbreviations: LDL, low density lipoproteins; VLDL, very low density lipoproteins; HTG, hypertriglyceridemic; HLP, hyperlipoproteinemia; LPL, lipoprotein lipase; Ox, oxidation; apo, apolipoprotein; THL, tetrahydrolipstatin; CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride; LPDS, lipoprotein-deficient serum; DMEM, Dulbecco's modified Eagle's medium; ACAT, acyl-CoA:cholesterol acyltransferase; IFN-γ, interferon-γ; EKO, apoE-knockout; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; SMC, smooth muscle cells.

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lagen synthesis and proliferation (14–18), which are all potentially pro-atherogenic. In vitro studies have focused on the effects of IFN-γ on macrophage foam cell formation induced by LDL modified by acetylation or oxidation (15, 19). Despite a major role in regulating macrophage function, little is known about the impact of IFN-γ on foam cell formation induced by native lipoproteins.

Subjects with type IV HLP have elevated plasma levels of HTG-VLDL due to an overproduction of this lipoprotein by the liver (20–23). In some instances this elevation is further enhanced by a decrease in HTG-VLDL catabolism (21, 23). Although subjects with type IV HLP have elevated levels of VLDL and remnant particles, they often have normal levels of LDL (21, 23), a class of lipoprotein whose elevation is typically associated with atherogenesis (24).

Unlike LDL, hypertriglyceridemic very low density lipoproteins (HTG-VLDL), isolated from subjects with type IV hyperlipoproteinemia (HLP) can induce macrophage foam cell formation, without the requirement for oxidation (25–29). ApoE modulates the uptake of HTG-VLDL by macrophages (25, 26, 30–32), and previous studies from our laboratory have shown that uptake of HTG-VLDL by cultured macrophages requires receptor-binding competent apoE (25, 26).

While individuals with type IV HLP are at an increased risk of developing atherosclerosis (33–35), the basis for this increased risk is not fully understood. It is known that a proportion of S_f 60–400 VLDL in subjects with type IV HLP is directly removed from the circulation. In addition, after remnant formation via endothelial cell-bound LPL, the majority of remnants (S_f 12–60) are cleared, and not converted into LDL (36–39). Although the liver is the primary tissue-specific site responsible for the removal of HTG-VLDL and their remnants from the circulation (40), macrophages, residing within the vascular intima may also facilitate removal of HTG-VLDL and their remnants once these lipoproteins penetrate the arterial wall (41).

In the present study we examined the ability of IFN-γ to modulate uptake of type IV HTG-VLDL by cultured macrophages. The results demonstrated that: *1*) IFN-γ treatment of macrophages resulted in a marked enhancement in cellular CE, free cholesterol (FC), and triglyceride (TG) content upon incubation with native type IV HTG-VLDL; *2*) cell-secreted LPL was required for this uptake process despite the fact that IFN-γ treatment reduced cellular LPL activity; *3*) IFN-γ-induced CE accumulation required cellular ACAT activity; and *4*) enhanced uptake of HTG-VLDL and LDL mediated by IFN-γ was due to increased uptake via the LDL-receptor and did not involve the LDL receptor-related protein (LRP) or scavenger receptors.

EXPERIMENTAL PROCEDURES

Subjects

Study subjects were recruited from the Outpatient Lipid and Vascular Risk Protection Clinic at the London Health Sciences Center, University Campus, London, Ontario, and the Lipid Clinic at St. Michael's Hospital, Toronto, Ontario. Lipoprotein phenotypes of the type IIa, III, and IV HLP subjects were classified according to the criteria of Schaefer and Levy (42). All type III HLP subjects were homozygous for the apoE2 isoform as determined by isoelectric focusing gel electrophoresis and/or DNA restriction isotyping (43–45). All type III and IV HLP subjects had elevated plasma cholesterol and TG levels due to elevated VLDL cholesterol and TG concentrations (see Table 1), and none of these subjects displayed fasting chylomicronemia. None of the subjects used in these studies had evidence of hepatic, renal, or other detectable metabolic disorders and none of the patients were being treated with drugs known to exacerbate hypertriglyceridemia. These studies were approved by the University of Western Ontario Health Sciences Standing Committee on Human Research and all subjects gave informed consent prior to blood sampling.

Mice

EKO mice (46) bred into a C57BL/6J background (strain C57BL/6J-*Apo* e^{tmlUnc}, Jackson Laboratory, Bar Harbor, ME) were a generous gift from Dr. B. Singh, the University of Western Ontario, London, Ontario. Four- to six-month-old EKO mice were placed on a Western-type diet (21% (w/w) fat, 0.15% (w/w) cholesterol; Harlan/Teklad, Madison, WI) for 1 week prior to blood collection. The mouse experimental protocols were approved by the Animal Care Committee of the University of Western Ontario.

Lipoprotein isolation

After a 12–14-hour period of fasting, 60–180 ml of blood was collected from each subject. After a 6-h period of fasting, 1 ml of blood was collected from each EKO mouse. The blood was immediately placed in tubes containing ethylenediaminetetraacetic acid (EDTA) at a final concentration of 0.15% (w/v). Plasma was isolated by centrifugation (Sorvall IEC Centra-8R centrifuge) at 2500 rpm (1000 *g*) for 25 min at 4°C. Lipoproteins were collected and washed, as described previously (47), by ultracentrifugation in a Beckman L8 ultracentrifuge. From human plasma, the large HTG-VLDL (S_f 60–400) subclass was isolated using a Beckman 55.2 Ti rotor (1.75 h, 40,000 rpm, 12°C), and subsequently washed using a Beckman 70.1 Ti rotor (16 h, 40,000 rpm, 12°C). After isolation of intermediate density lipoproteins (d 1.006–1.019 g/ml), the LDL fraction (d 1.019–1.063 g/ml) was isolated from the infranatant using either a Beckman 50.4 Ti or 55.2 Ti rotor (16 h, 50,000 rpm, 12°C). The LDL was washed using a Beckman 70.1 Ti rotor (16 h, 50,000 rpm, 12°C). Due to the small volumes of plasma collected from each mouse, the entire VLDL subclass (S_f 20–400) was isolated using a Beckman 50.4 Ti rotor (16 h, 36,000 rpm, 12°C), and subsequently washed using the same rotor and running conditions. HTG-VLDL (S_f 60–400), LDL and EKO-VLDL (S_f 20–400) preparations were extensively dialyzed, in the dark and at 4°C, against a 200-fold excess volume of phosphate-buffered saline (PBS; 154 mm NaCl, 8 mm $Na_2HPO_4 \cdot 7H_2O$, 1.5 mm KH_2PO_4, 2.7 mm KCl, pH 7.4) containing 10 μm EDTA. After dialysis, the lipoprotein samples were sterilized by passage through 0.45-μm filters and stored at 4°C. VLDL and LDL samples were used in cell experiments within 2 and 4 weeks, respectively.

All lipoprotein samples were analyzed for protein content by a modification of the Lowry method (48), for free fatty acids (FFA) using enzymatic reagents (#990-75401) from Wako (Neuss, Germany; distributed by Immunocorp, Montreal, Quebec) and for TG, FC, and total cholesterol (TC) using enzymatic reagents from Boehringer Mannheim GmbH Diagnostica (Montreal, Quebec; TG: #450032 without free glycerol, FC: #310328 and TC: #1442350).

Oxidation of lipoproteins

Dialyzed and sterile HTG-VLDL and LDL preparations were oxidized in vitro for 48 h using $CuSO_4$ (5.0 μm) following a mod-

ification (49, 50) of the protocol described by Steinbrecher et al. (51). The oxidized VLDL (CuOx-VLDL) and LDL (CuOx-LDL) preparations were dialyzed and sterilized by filtration as stated above. Lipoprotein oxidation reactions were monitored at the early stage of modification by assaying for conjugated diene formation. The conjugated diene assays were conducted in parallel with the oxidation reactions by following the protocol of Kleinveld et al. (52), as described previously (49, 50). End-stage modification of the lipoproteins was monitored by assaying for changes in the relative electrophoretic mobility of the modified lipoproteins as described previously (49, 50).

Cell culture

J774A.1 cells, a murine macrophage-like cell line that secretes LPL but not apoE (53, 54), were used in this study. J774A.1 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in culture and set up for experiments as outlined previously (26, 49). Lipoprotein-deficient serum (LPDS) was prepared as outlined previously (26) and was confirmed to be deficient in apoC-II and apoE (36). The final albumin concentration in the medium was 0.13%. For each lipoprotein preparation, 50 μg of total lipoprotein cholesterol per ml medium was added to duplicate wells of cells and incubated for 16 h at 37°C. The LPL inhibitor tetrahydrolipstatin (THL) (Orlistat™; provided by Hoffmann-LaRoche Pharmaceuticals Ltd, Montreal) was used at a concentration of 1.0 μm. At this concentration, THL inhibited the activity of 0.25 units of bovine milk LPL by greater than 95% (data not shown). Previous studies demonstrated that J774A.1 macrophages secrete 0.25 units of LPL activity/24 h (26). The THL stock solution was made up in dimethyl sulfoxide (DMSO) and then diluted with low glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Burlington, Ontario) plus LPDS prior to being added to the cells. Control dishes received an equal volume (not exceeding 10 μl/well) of DMSO alone. In experiments in which the ACAT inhibitor DuP 128 (provided by DuPont Merck Pharmaceutical Co., Wilmington, Delaware) was used, 10 μm (final concentration) was added in a volume of 5 μl DMSO/ml medium. Cells receiving IFN-γ (murine, Life Technologies) were exposed to the cytokine for a total of 40 h. For the first 24 h cells were pretreated with the cytokine alone. For the remaining 16 h, fresh DMEM plus LPDS and fresh IFN-γ (± lipoproteins) were added. Stock solutions of IFN-γ were prepared by dissolving the lyophilized INF-γ in sterile water (5,000 U/50 μl) which were stored at −80°C. For experiments, stock solutions of IFN-γ were diluted in DMEM plus LPDS and added to cells at a concentration of 50 U/ml medium. The IFN-γ working solution, stored at 4°C, was not used if it was >48 h old. Several lots of IFN-γ were used over the course of these studies and no differences in response were observed. Murine IFN-γ was also obtained from R&D technologies (Minneapolis, MN). No differences were observed in response for IFN-γ obtained from the two sources.

Quantitative analysis of cellular lipids and cholesterol esterification

The cell/lipoprotein incubations were terminated by two washes of buffer A (0.15 m NaCl, 50 mm Tris, 0.2% (w/v) fatty acid-free bovine serum albumin (BSA fraction V, Sigma, St. Louis, MO), pH 7.4) and two additional washes with buffer A without fatty acid-free BSA. Cell lipids were extracted in situ with two 30-min incubations using 1.0 ml of hexane–isopropanol 3:2 (v/v). The solvents from each extraction were pooled for analysis. To each dish, 1.0 ml of 0.1 N NaOH was added and incubated overnight at room temperature to digest the cells. Cell protein was determined by a modification of the Lowry method (48). Cellular TC, FC, and TG mass were determined spectrophotometrically by a modification (50) of the protocol described by Carr, Andresen, and Rudel (55), using enzymatic reagents from Boehringer Mannheim (see above) and a V_{max} Kinetic 96 multi-well microplate reader (Molecular Devices, Mississauga, Ontario). Cellular lipid results are reported as μg of cellular lipid (FC, CE, or TG) per mg of cell protein. The incorporation of [^{14}C]oleic acid into CE was determined as described previously (25). Values were calculated as nmol of [^{14}C]cholesteryl oleate per mg cell protein/5 h and reported as percent of control cell values.

Cellular LPL activity

J774A.1 cells, approximately 80% confluent, were first grown for 24 h in the absence or presence of 50 U/ml of IFN-γ, and subsequently incubated for an additional 16 h with DMEM containing 5% LPDS in the presence or absence of 50 U/ml IFN-γ and/or 10 U/ml of heparin (Hepalean, Organon Canada Ltd., Toronto, Ontario). The effect of IFN-γ on LPL activity secreted by J774A.1 cells was assessed as described below. The conditioned medium was collected in sterile tubes and stored at −80°C until used in the assay. Triplicate 200-μl aliquots of conditioned media were used in the assay, with 200-μl aliquots of unconditioned medium serving as control. As a negative control, the conditioned medium was also assayed in the presence of 1 m NaCl, conditions known to inhibit LPL (56). The cells were washed 3 times with buffer B (0.15 m NaCl, 50 mm Tris, pH 7.4) and the cell protein was measured as described above.

J774A.1 secreted LPL activity was determined by measuring the amount of FFA released from a predetermined amount of a commercially obtained TG emulsion (Intralipid, Pharmacia Inc., Mississauga, Ontario). The LPL activity assay was conducted in 16 × 100 mm borosilicate glass tubes (Fisher Scientific, Pittsburgh, PA) by adding, in order, the following: 200 μl LPL buffer C (0.225 m NaCl, 0.3 m Tris, pH 8.2), 7.5% (w/v) fatty acid-free BSA, 18% (v/v) normolipidemic human plasma as a source of apoC-II, 200 μl of media, and 20 μl Intralipid containing 1.82 μmol of TG. After incubation for 1 h at 37°C in a shaking water bath, the reaction was stopped by adding 2 ml of isopropyl alcohol–3 N H_2SO_4, 40:1 (v/v), 1 ml deionized H_2O, and 2.5 ml hexane, with vigorous vortexing for 1 min. The phases were separated by standing for 20 min at room temperature. The hexane layer was removed into new 16 × 100 mm glass test tubes, and the hexane extraction step was then repeated. The pooled hexane fractions were evaporated under N_2. Chloroform (1 ml) was added to each tube, and a 200-μl aliquot taken for subsequent fatty acid determination. To this aliquot, 750 μl of a 1% (v/v) Triton X-100 in chloroform solution was added; the solution was mixed and dried under N_2. Fifty μl of deionized H_2O was then added and the samples were mixed by shaking for 30 min in a 37°C water bath. The amount of FFA released was determined spectrophotometrically using FFA enzymatic reagents. LPL activity was expressed in units/mg of cell protein, where one unit of activity was defined as 1 μmol of FFA released per ml of medium per h.

Cellular binding of α2-Macroglobulin

Purified α2-macroglobulin (a generous gift from Dr. J. LaMarre, University of Guelph, Ontario) was activated with methylamine (57) and radioiodinated with Iodobeads, as described by the manufacturer (Pierce Chemical, Rockford IL). To determine specific activity, samples were counted and protein was determined (48). J774A.1 cells were plated in 12-well culture dishes in DMEM plus 10% FBS (1 ml/well). When cells were 90% confluent, the medium was changed to DMEM–HEPES with 5% LPDS (0.5 ml/well). All binding experiments were performed for 3 h at 4°C, with each condition performed in triplicate. Nonspecific binding was determined by the addition of a 50-fold excess of un-

labeled competitor. At the completion of each experiment, all wells were washed twice with 1 ml/well of PBS containing 0.2% fatty acid-free BSA, followed by two washes of PBS alone. One ml of 0.1 N NaOH was then added to all wells to digest cells and cell protein was determined (48).

Cellular binding uptake and degradation of ^{125}I-labeled LDL

^{125}I-labeled LDL binding, uptake, and degradation studies were performed essentially as described by Goldstein, Basu, and Brown (58). Briefly, J774A.1 cells were grown as above, in 6-well culture plates, and pre-incubated for 24 h in DMEM containing 10% FBS with or without IFN-γ (50 U/mL media). For binding studies, cells were then incubated at 4°C for 30 min. The media was changed to ice-cold DMEM containing 5% lipoprotein-deficient serum (LPDS). IFN-γ was re-added along with ^{125}I-labeled LDL (10 μg/mL of media) in the absence and presence of unlabeled LDL (500 μg/mL of media). The cells were incubated for 5 h at 4°C. The media were collected and cells were extensively washed with Tris/BSA followed by a Tris only solution. Cells were incubated for 1 h at 4°C with heparin (0.5 mg/mL media). The media were then collected and the content of ^{125}I radioactivity was determined as a measure of ^{125}I-labeled LDL bound. The cells were subsequently lysed with 0.1 N NaOH and an aliquot was counted for ^{125}I radioactivity to determine ^{125}I-labeled LDL uptake. For uptake and degradation experiments, the procedure was the same except that cells were incubated for 5 h at 37°C. After the heparin release step, 0.25 mL of 50% trichloroacetic acid (TCA) was added to 1 mL of media and tubes were incubated at 4°C for 30 min. After centrifugation, 5 μL of 4% potassium iodide and 20 μL of 30% hydrogen peroxide were added to 0.5 mL of the supernatant, and incubated for 10 min at room temperature. To extract [^{125}I]iodides, 1 mL of chloroform was added and tubes were vortexed and incubated for 15 min. An aliquot of the aqueous phase was counted for ^{125}I radioactivity as a measure of ^{125}I-labeled LDL degradation. All conditions were corrected for a 'no cell' blank containing only ^{125}I-labeled LDL and media and all conditions were standardized to the amount of cell protein (48).

Statistical analysis

In each experiment, unless stated otherwise, duplicate cell culture wells were used for each specific lipoprotein preparation, with the resulting values combined to give a mean value. Mean values from separate experiments were then used to calculate a group mean ± SEM for each condition. The "n" referred to for each experiment indicates the number of different patients' samples or EKO mouse samples that were used to determine each experimental parameter. Statistical significance between control and experimental group mean values was assessed using a Student's *t*-test. A two-tailed $P \leq 0.05$ was considered statistically significant.

RESULTS

Efect of IFN-γ on HTG-VLDL-induced macrophage lipid accumulation

The baseline data for the subjects from whom lipoproteins were obtained are listed in **Table 1**. Incubation of J774A.1 cells with native type IV HTG-VLDL induced a 17.4-fold and a 4.3-fold increase in cellular CE and TG content, respectively, compared to cells incubated in the absence of lipoproteins (control cells) (both $P = 0.0002$; **Fig. 1**, **A** and **C**). No change in cellular FC content was observed (Fig. 1B). In contrast, pretreatment of cells with IFN-γ, followed by incubation with type IV HTG-VLDL, caused a marked enhancement in cellular CE and TG (27- and 6-fold over controls, respectively, $P = 0.0001$) and a 1.5-fold increase in FC ($P = 0.0001$) (Fig. 1, A, B, and C). This enhancement in cellular CE, FC, and TG content was statistically significant ($P \leq 0.01$) when compared to cells incubated with type IV HTG-VLDL in the absence of IFN-γ (Fig. 1, A, B, and C). In marked contrast, pretreatment of cells with IFN-γ, followed by incubation with type III HTG-VLDL from apoE2/E2 subjects or EKO-VLDL had no significant effect on cellular CE, FC, and TG content (Fig. 1, A, B, and C).

A concentration–response curve showed that IFN-γ, at concentrations ⩾12.5 U/ml medium, significantly enhanced cellular TG and FC content ($P \leq 0.04$) upon coincubation with type IV HTG-VLDL, and that ⩾25 U/ml medium was needed in order to achieve a significant increase in cellular CE content ($P \leq 0.029$; **Fig. 2**, **A**, **B**, and **C**). Furthermore, an IFN-γ concentration of 50 U/ml was found to be the lowest concentration that would achieve optimal enhancement in cellular CE, FC, and TG content, upon incubation of cells with native type IV HTG-VLDL (Fig. 2, A, B, C).

IFN-γ enhanced macrophage uptake of LDL

Incubation of J774A.1 cells with native LDL induced an 8.7-fold increase in cellular CE content, compared to control cells ($P = 0.0004$; **Fig. 3A**). No change in cellular FC or TG content was observed (Fig. 3, B and C). Pretreatment of cells with IFN-γ (50 U/ml), followed by incubation with LDL, induced an even greater increase in cellular CE content (15.6-fold over control cells) compared to incubation with LDL alone ($P = 0.001$; Fig. 3A). Treatment of cells with IFN-γ, followed by incuba-

TABLE 1. Plasma and lipoprotein lipid concentrations of lipoprotein donors

Lipoprotein Phenotype	Plasma		VLDL			
	TC	TG	TC	TG	LDL-TC	HDL-TC
	mM		mM		mM	mM
Type IV (n = 14)	7.36 ± 0.56	6.86 ± 1.05	4.26 ± 0.54	6.26 ± 1.03	2.41 ± 0.30	0.68 ± 0.02
Type III[a] (n = 3)	9.94 ± 1.10	7.23 ± 0.92	5.57 ± 1.31	6.89 ± 0.88	1.61 ± 0.72	2.47 ± 0.28
Type IIA (n = 4)	10.06 ± 0.01	1.96 ± 0.47	0.81 ± 0.11	1.21 ± 0.33	8.27 ± 0.06	0.98 ± 0.04
Normal (n = 1)	4.15	0.66	0.01	0.38	3.02	1.12

Values are expressed as means ± SEM; TC, total cholesterol (free + esterified); TG, triglyceride; VLDL, very low density lipoprotein, S_f 60–400; LDL, low density lipoprotein; HDL, high density lipoprotein.

[a] The type III HLP subjects used in these studies were all homozygous for apoE2.

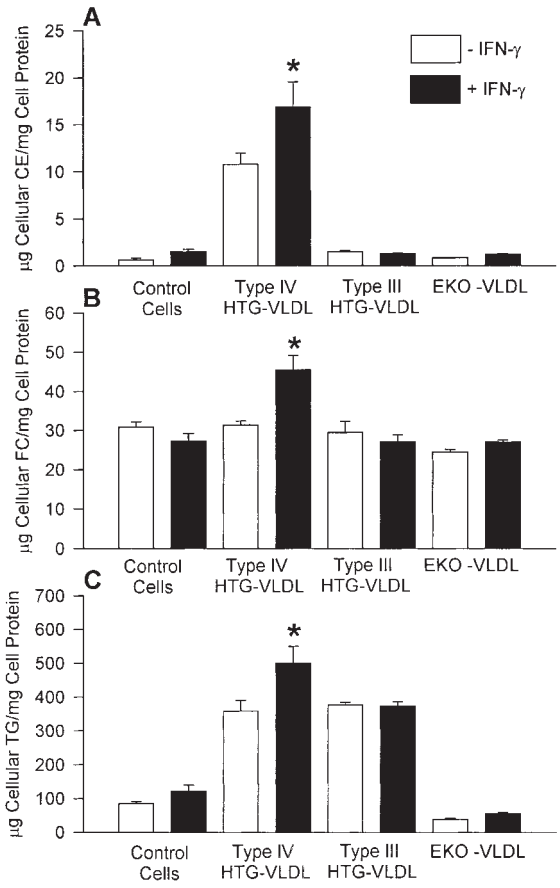

Fig. 1. Cholesteryl ester, free cholesterol, and triglyceride content of J774A.1 cells incubated with type IV HTG-VLDL, type III HTG-VLDL, and EKO-VLDL in the absence or presence of interferon-γ. Cholesteryl ester (A), free cholesterol (B), and triglyceride (C) content of J774A.1 macrophages incubated for 16 h with either type IV HTG-VLDL (n = 11), type III HTG-VLDL (n = 3), or EKO-VLDL (n = 3) in the absence (□) or presence (■) of interferon-γ (IFN-γ; 50 U/ml of medium). Incubation conditions were conducted at 50 μg lipoprotein cholesterol/ml medium. Control cells indicates incubations in the absence of lipoproteins. Values are expressed as mean ± SEM. (A) *$P = 0.01$ relative to incubation in the absence of IFN-γ. (B) *$P = 0.004$ relative to incubation in the absence of IFN-γ. (C) *$P = 0.0007$ relative to incubation in the absence of IFN-γ. Measurements of cellular lipid content were performed as described in the Experimental Procedures.

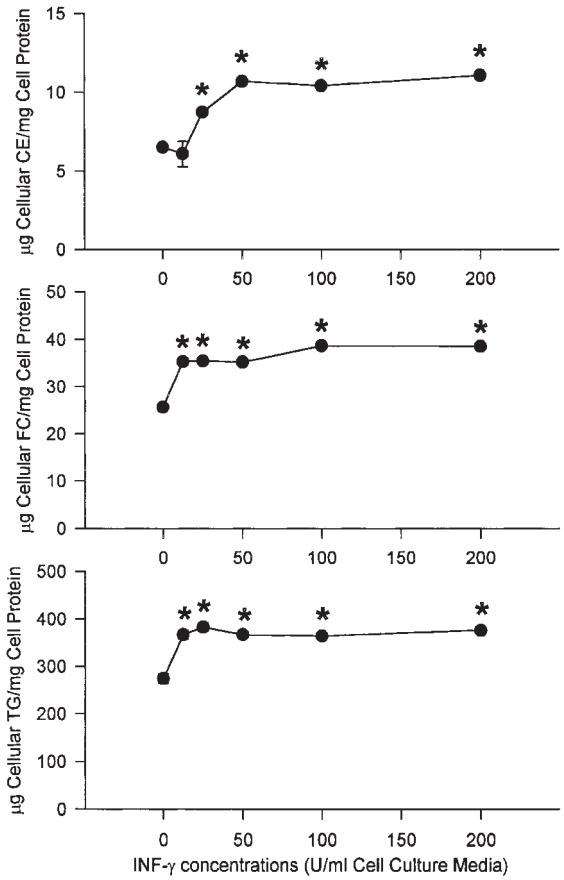

Fig. 2. Cholesteryl ester, free cholesterol, and triglyceride content of J774A.1 cells incubated with type IV HTG-VLDL in the presence of increasing concentrations of interferon-γ. Cholesteryl ester (A), free cholesterol (B), and triglyceride (C) content of J774A.1 macrophages incubated for 16 h with type IV HTG-VLDL (50 μg lipoprotein cholesterol/ml medium; n = 2 for each) in the absence (○) or presence (●) of interferon-γ (IFN-γ; 12.5–200 U/ml of medium). Values are expressed as mean ± SEM. (A) *$P \leq 0.029$, relative to incubation of type IV HTG-VLDL in the absence of IFN-γ. (B) *$P \leq 0.01$, relative to incubation of type IV HTG-VLDL in the absence of IFN-γ. (C) *$P \leq 0.04$, relative to incubation of type IV HTG-VLDL in the absence of IFN-γ. Measurements of cellular lipid content were performed as described in the Experimental Procedures.

tion with LDL, also induced a small but significant increase in cellular FC content compared to control cells ($P = 0.04$), and when compared to cells incubated with LDL in the absence of IFN-γ ($P = 0.014$; Fig. 3B). Treatment of cells with IFN-γ, followed by incubation with LDL, had no effect on cellular TG content (Fig. 3C).

Effect of IFN-γ on macrophage LPL activity

The role of macrophage-secreted LPL in IFN-γ-mediated enhanced uptake of type IV HTG-VLDL was next examined. Co-incubation of type IV HTG-VLDL with the lipid active-site inhibitor, THL, reduced cellular CE and TG content by 68% and 85%, respectively, ($P \leq 0.01$; **Fig. 4**, **A** and **C**). Similarly, co-incubation of type IV HTG-VLDL plus THL with IFN-γ-treated cells significantly decreased cellular CE and TG content by 62% and 81%, respectively ($P \leq 0.01$; Fig. 4, A and C). In the absence of THL, cellular CE and TG content was significantly elevated in the IFN-γ-treated cells incubated with type IV HTG-VLDL ($P \leq 0.02$; Fig. 4, A and C). THL had no effect on the FC content of cells incubated with HTG-VLDL in the absence or presence of IFN-γ. The FC content remained significantly elevated in IFN-γ-treated cells ($P = 0.01$; Fig. 4B). Treatment of J774A.1 cells with IFN-γ, in the absence of lipoproteins, caused a 53% reduction ($P = 0.001$) in total LPL activity, which was due to a 36% reduction ($P = 0.01$) in LPL activity found in the medium, and a 53% reduction ($P = 0.001$) in LPL activity bound to the cell surface (heparin-released LPL activity) (**Fig. 5**).

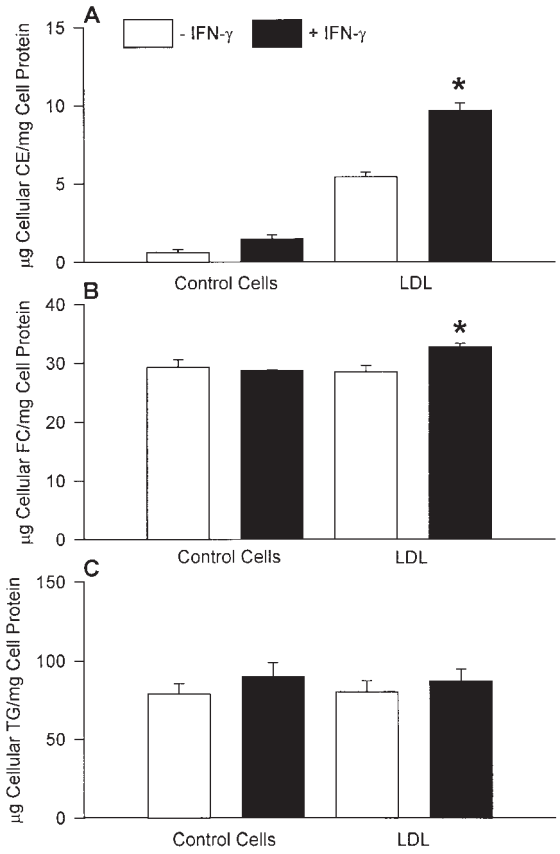

Fig. 3. Cholesteryl ester, free cholesterol, and triglyceride content of J774A.1 cells incubated with LDL in the absence or presence of interferon-γ. Cholesteryl ester (A), free cholesterol (B), and triglyceride (C) content of J774A.1 macrophages incubated for 16 h with LDL (150 μg lipoprotein cholesterol/ml medium; n = 6 for each) in the absence (□) or presence (■) of interferon-γ (IFN-γ; 50 U/ml of medium). Control cells indicates incubations in the absence of lipoproteins. Values are expressed as mean ± SEM. (A) $*P = 0.001$ relative to incubation in the absence of IFN-γ. (B) $*P = 0.014$ relative to incubation in the absence of IFN-γ. Measurements of cellular lipid content were performed as described in the Experimental Procedures.

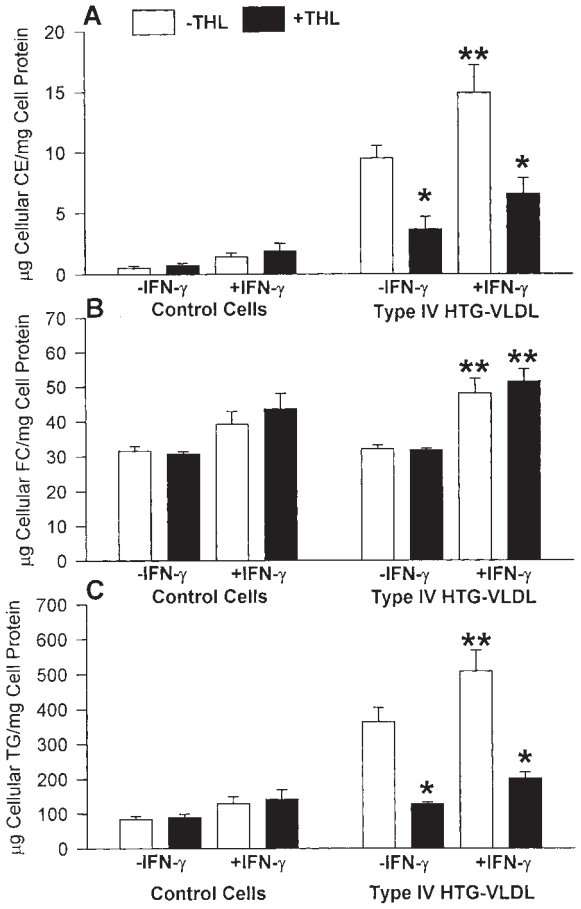

Fig. 4. Cholesteryl ester, free cholesterol, and triglyceride content of J774A.1 cells incubated with type IV HTG-VLDL in the absence or presence of interferon-γ and tetrahydrolipstatin. Cholesteryl ester (A), free cholesterol (B), and triglyceride (C) content of J774A.1 macrophages, untreated or treated with interferon-γ (IFN-γ; 50 U/ml of medium), after incubation for 16 h with type IV HTG-VLDL (50 μg lipoprotein cholesterol/ml medium; n = 7 for each) in the absence (□) or presence (■) of tetrahydrolipstatin (THL; 1 μm), a competitive inhibitor of LPL. Control cells indicates incubations in the absence of lipoproteins. Values are expressed as mean ± SEM. (A) $*P \leq 0.01$, relative to incubation in the absence of THL. $**P \leq 0.02$ relative to incubation in the absence of IFN-γ. (B) $**P \leq 0.01$ relative to incubation in the absence of IFN-γ. (C) $*P \leq 0.003$, relative to incubation in the absence of THL. $**P \leq 0.016$ relative to incubation in the absence of IFN-γ. Measurements of cellular lipid content were performed as described in the Experimental Procedures.

Role of cholesterol esterification in INF-γ-enhanced macrophage CE accumulation

Experiments using an inhibitor of ACAT, DuP 128, were carried out in J774A.1 cells exposed to type IV HTG-VLDL after pretreatment with or without IFN-γ for 24 h, prior to the 5 h cholesterol esterification assay. Incubation of cells with type IV HTG-VLDL in the absence or presence of IFN-γ significantly increased the incorporation of [^{14}C]oleate into cellular CE by 56% and 91%, respectively ($P \leq 0.003$; **Fig. 6**). The difference in cholesterol esterification between cells incubated in the absence versus presence of IFN-γ was significant ($P = 0.039$; Fig. 6). A time course for the effect of IFN-γ on cholesterol esterification after incubation of cells with Type IV HTG-VLDL was also determined. Cells were pre-incubated with IFN-γ for 5, 24, and 40 h prior to the cholesteryl esterification assay. The increases in cholesteryl esterification were 1.2-, 2.0-, and 2.3-fold over control, respectively (data not shown). This demonstrates that the effect of IFN-γ was not transient.

Addition of DuP 128 plus type IV HTG-VLDL, in the absence or presence of IFN-γ, completely inhibited (both 100%, $P \leq 0.002$) [^{14}C]oleate incorporation into cellular CE (Fig. 6). DuP 128 did not affect the incorporation of [^{14}C]oleate into cellular TG (data not shown). Co-incubation of cells with DuP 128 plus type IV HTG-VLDL, in the absence or presence of IFN-γ, resulted in a 95% and a 93% reduction, respectively, in cellular CE content ($P \leq 0.01$; **Fig. 7A**), which was similar in magnitude to the reduction in cholesterol esterification. DuP 128, in the absence and presence of IFN-γ, caused a significant increase of 1.1-fold

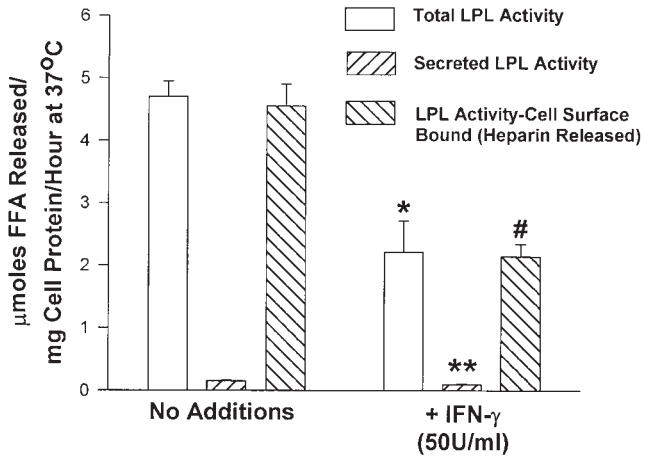

Fig. 5. Measurement of lipoprotein lipase activity secreted into the medium and bound to the cell surface (heparin-released) by J774A.1 macrophages incubated in the absence or presence of interferon-γ. J774A.1 conditioned medium, incubated in the absence or presence of heparin (10 U/ml medium) and IFN-γ (50 U/ml medium), was assayed for LPL activity as described in the Experimental Procedures (n = 1, run in quadruplicate). Values are expressed as mean ± SEM. *P = 0.001, **P = 0.01, #P = 0.001, relative to incubation in the absence of IFN-γ.

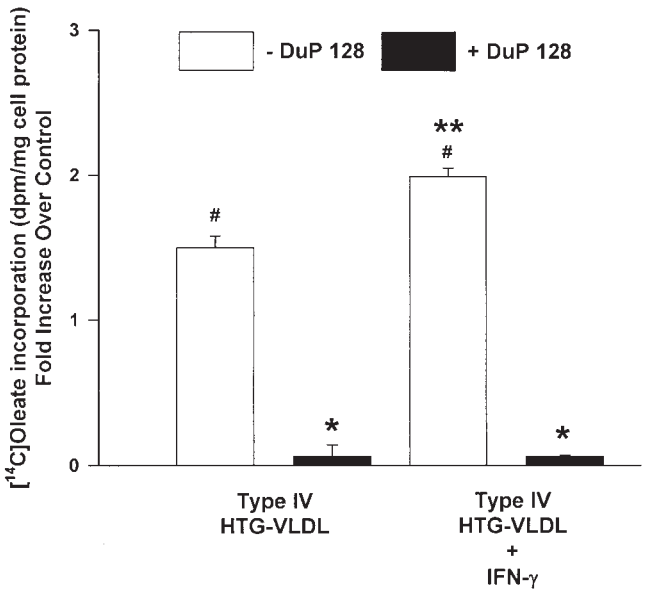

Fig. 6. [^{14}C]oleate esterification to cholesterol in J774A.1 cells incubated with native type IV HTG-VLDL in the absence or presence of interferon-γ and the ACAT inhibitor DuP 128. Cholesterol esterification in J774A.1 macrophages, untreated or treated with interferon-γ (IFN-γ; 50 U/ml of medium), after incubation with type IV HTG-VLDL (50 μg lipoprotein cholesterol/ml medium; n = 2 for each) in the absence (□) or presence (■) of DuP 128 (10 μm), a competitive inhibitor of ACAT. Lipoproteins were added to the cells along with a [^{14}C]oleic acid–albumin complex and incubated for 5 h. Cholesterol esterification is expressed as fold increase in [^{14}C]oleate incorporation over control. Values are expressed as mean ± SEM. *$P \leq 0.002$ relative to incubation in the absence of DuP 128. **P = 0.039 relative to incubation in the absence of IFN-γ. #$P \leq 0.003$, relative to control cells. Incorporation of [^{14}C]oleate into cellular cholesteryl esters was determined as described in the Experimental Procedures.

and 1.2-fold, respectively, in cellular FC content ($P \leq 0.047$; Fig. 7B). DuP 128 did not affect the ability of type IV HTG-VLDL, incubated in the absence or presence of IFN-γ, to induce cellular TG accumulation (Fig. 7C).

α2-Macroglobulin binding to IFN-γ-treated macrophages

α2-Macroglobulin cell-binding experiments were performed in order to examine whether IFN-γ-mediated enhanced uptake of type IV HTG-VLDL occurred via an increase in cell surface LRP expression. As shown in **Fig. 8**, pretreatment of cells with IFN-γ inhibited, by as much as 49% ($P \leq 0.027$), the specific binding of activated ^{125}I-labeled α2-macroglobulin, which like apoE3-containing VLDL, is a ligand for the LRP (59, 60). A decrease, rather than an increase, was observed in LRP expression in the presence of IFN-γ.

Uptake of oxidized HTG-VLDL and oxidized LDL by IFN-γ-treated macrophages

Lipoproteins can be oxidized by incubation with cells, including macrophages, which in turn increases their uptake by macrophages by a process involving scavenger receptors (61). To assess whether the IFN-γ-enhanced uptake of HTG-VLDL was due to an increase in scavenger receptor expression, we determined the uptake of HTG-VLDL and LDL after copper oxidation in vitro. Copper-mediated modification of the lipoproteins was confirmed by observing both an increase in the formation of conjugated dienes (data not shown) and an increase in the relative mobility of CuOx–type IV HTG-VLDL and CuOx–LDL (a 1.8-fold and 2.1-fold increase, respectively) when compared to their native counterparts ($P \leq 0.001$). Pretreatment of macrophages with IFN-γ completely inhibited cellular CE accumulation induced by CuOx–type IV HTG- VLDL and CuOx–LDL ($P \leq 0.002$). In contrast, IFN-γ pretreatment significantly increased cellular CE accumulation induced by native type IV HTG-VLDL and LDL ($P \leq 0.05$; **Fig. 9**).

Binding, uptake, and degradation of ^{125}I-labeled LDL in macrophages incubated with IFN-γ

The interaction of LDL cell-binding, cell-association, and degradation experiments were performed in order to examine whether IFN-γ-mediated enhanced uptake of type IV HTG-VLDL and LDL occurred via an increase in cell surface LDL receptor expression. As shown in **Fig. 10**, pretreatment of cells with IFN-γ increased by 2-fold the specific binding of ^{125}I-labeled LDL at 4°C ($P \leq 0.05$). IFN-γ did not significantly affect cell-associated ^{125}I-labeled LDL; however, IFN-γ increased the degradation of ^{125}I-labeled LDL by 30% ($P < 0.005$). This observation suggests that the IFN-γ-induced increase in lipoprotein uptake was mediated by an increased expression of the LDL receptor.

DISCUSSION

The present study demonstrated that pretreatment of cultured macrophages with the cytokine, IFN-γ, resulted

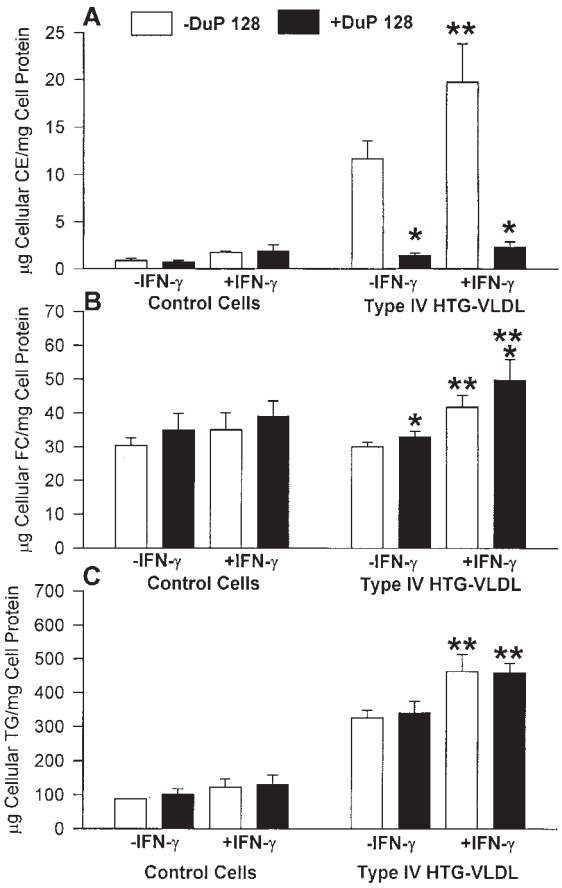

Fig. 7. Cholesteryl ester, free cholesterol, and triglyceride content of J774A.1 cells incubated with type IV HTG-VLDL in the absence or presence of interferon-γ and the ACAT inhibitor DuP 128. Cholesteryl ester (A), free cholesteryl (B), and triglyceride (C) content of J774A.1 macrophages untreated or treated with interferon-γ (IFN-γ; 50 U/ml of medium), after incubation for 16 h with type IV HTG-VLDL (50 μg lipoprotein cholesterol/ml medium; n = 5 for each) in the absence (□) or presence (■) of DuP 128 (10 μm), a noncompetitive inhibitor of ACAT. Control cells indicates incubations in the absence of lipoproteins. Values are expressed as mean ± SEM. (A) $*P \leq 0.01$, relative to incubation in the absence of DuP 128. $**P = 0.02$ relative to incubation in the absence of IFN-γ. (B) $*P \leq 0.047$, relative to incubation in the absence of DuP 128. $**P \leq 0.04$ relative to incubation in the absence of IFN-γ. (C) $**P \leq 0.02$ relative to incubation in the absence of IFN-γ. Measurements of cellular lipid content were performed as described in the Experimental Procedures.

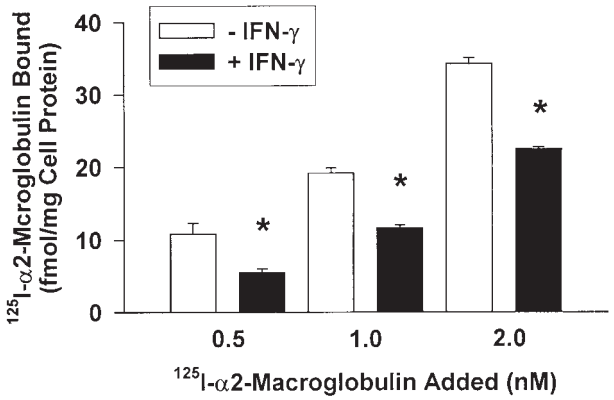

Fig. 8. Binding of ^{125}I-labeled α2-macroglobulin to J774A.1 cells in the absence and presence of interferon-γ. Binding of ^{125}I-labeled α2-macroglobulin to J774A.1 cells, that had been pre-incubated in the absence (□) or presence (■) of interferon-γ (IFN-γ; 50 U/ml of medium), was carried out for 3 h at 4°C (n = 1 performed in triplicate). $*P \leq 0.027$ relative to incubation in the absence of IFN-γ. Binding experiments were performed as described in the Experimental Procedures.

in an enhancement of cellular CE, FC, and TG content upon exposure of these cells to native type IV HTG-VLDL. In addition, this study revealed four new findings. First, cell surface and secreted LPL activity was required for the IFN-γ-enhanced uptake process despite the fact that IFN-γ treatment reduced cellular LPL activity. This implies that LPL activity was not rate-limiting with respect to the increased cellular lipid accumulation. Second, enhanced CE accumulation, induced by IFN-γ, involved cellular ACAT activity. This indicates that the increased cellular CE was not due to trapping of lipoproteins within the lysosome. Third, IFN-γ increased the binding and degradation of iodinated LDL in macrophages, suggesting that the enhanced lipoprotein uptake was mediated by the LDL-receptor. Fourth, IFN-γ-enhanced uptake was not mediated by an increased expression of the LRP or scavenger receptors.

T lymphocytes have been identified in human atherosclerotic plaques at all stages of lesion development (1–4). The presence of these T cells, many of them activated (1, 4), supports the idea that an immune response may

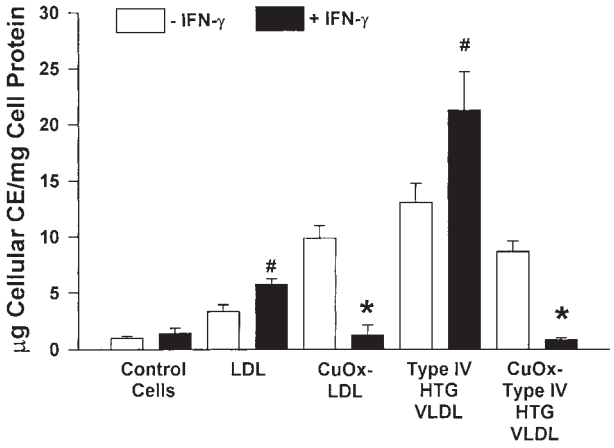

Fig. 9. Cholesteryl ester content of J774A.1 cells incubated with native or copper-oxidized LDL and type IV HTG-VLDL in the absence and presence of interferon-γ. Cholesteryl ester content of J774A.1 macrophages exposed for 16 h to native and copper-oxidized (CuOx-) LDL (n = 3) and type IV HTG-VLDL (n = 3). Incubation conditions were conducted at 50 μg lipoprotein cholesterol/ml medium in the absence (□) or presence (■) of interferon-γ (IFN-γ; 50 U/ml of medium). Control cells indicates incubations in the absence of lipoproteins. Values are expressed as mean ± SEM. $*P \leq 0.002$ and $^{\#}P \leq 0.05$, relative to incubation in the absence of IFN-γ. $CuSO_4$-mediated oxidation of the lipoproteins and measurement of cellular cholesteryl ester content were performed as described in the Experimental Procedures.

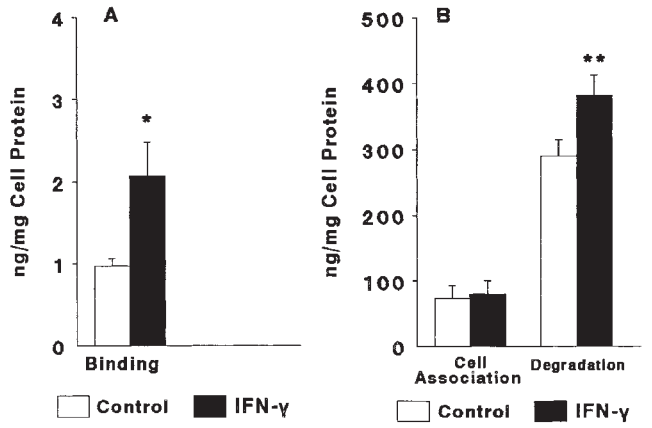

Fig. 10. Binding, cell association, and degradation of ^{125}I-labeled LDL to J774A.1 cells in the absence and presence of interferon-γ. (A) Binding of ^{125}I-labeled LDL (10 μg/ml of medium) to J774A.1 cells, that had been pre-incubated (24 h) in the absence (□) or presence (■) of interferon-γ (IFN-γ; 50 U/ml of medium), was carried out for 5 h at 4°C (n = 3 performed in triplicate). Non-specific binding was assessed by the addition of 50-fold excess of unlabeled LDL. (B) Cell association and degradation of ^{125}I-labeled LDL in J774A.1 cells, that had been pre-incubated (24 h) in the absence (□) or presence (■) of interferon-γ (IFN-γ; 50 U/ml of medium), were carried out for 5 h at 37°C (n = 4, performed in triplicate). Values are expressed as mean ± SEM. $*P \leq 0.05$; $**P < 0.005$ relative to incubation in the absence of IFN-γ. Experiments were performed as described in the Experimental Procedures.

play an active role in the pathogenesis of atherosclerosis. IFN-γ is a potent modulator of cellular activity involved in immunoregulation (62–66), and may modulate atherogenesis. A potential role for IFN-γ in the disease process is supported by studies that have detected this cytokine in T lymphocyte-rich regions of human plaques (4, 7). We, and others, have shown that IFN-γ can be considered to be anti-atherogenic as it can down-regulate macrophage SR-A expression and inhibit uptake of oxidized lipoproteins.

Despite the ability of IFN-γ to inhibit expression of the SR-A and the LRP, we now show that under certain circumstances, IFN-γ can be pro-atherogenic by further enhancing cellular uptake of not only native LDL but also native type IV HTG-VLDL, a lipoprotein capable of inducing foam cell formation without the requirement for modification (26, 50). The findings of Gupta et al. (13) support this pro-atherogenic role for IFN-γ by showing that IFN-γ potentiates atherosclerosis in EKO mice. In this study, EKO mice were crossed with mice deficient for the IFN-γ receptor, and the double knockout progeny were placed on a Western-type diet for 3 months. Compared to EKO mice, the compound knockout mice exhibited a substantial reduction in atherosclerotic lesion size, lesion lipid accumulation, and lesion cellularity. As the present study demonstrated that uptake of EKO-VLDL by J774A.1 cells was not enhanced by IFN-γ, apoE-deficient VLDL and VLDL remnants may not be directly involved in the IFN-γ effect on lesion enhancement as observed Gupta et al. (13). In addition to VLDL-remnants, LDL has also been shown to be significantly elevated in EKO mice fed a high fat, high cholesterol diet (67). Therefore, IFN-γ-mediated enhanced uptake of LDL rather than VLDL-remnants may explain the increase in lesion formation in EKO mice, as compared to IFN-γ receptor/apoE double knockout mice.

Four receptors are possible candidates to account for the IFN-γ-mediated enhanced uptake of both type IV HTG-VLDL and LDL: *1*) the LDL receptor; *2*) the VLDL/apoE receptor; *3*) the LRP and *4*) scavenger receptors. Of these, the LDL receptor appears to be the most plausible. Support for the LDL receptor is provided by binding and degradation studies, comparison of known ligands, and the extent of regulation exhibited by these receptors. Each of these receptors is known to bind apoE-containing lipoproteins with high affinity; however, only the LDL receptor will bind LDL as LDL contains only apoB-100. Because recent evidence suggests that the LRP and the VLDL receptor may be involved in macrophage foam cell formation (68) and that HTG-VLDL is a possible ligand for these receptors, it was important to exclude a potential role for the VLDL receptor and the LRP in mediating the enhanced CE accumulation induced by native HTG-VLDL in macrophages exposed to IFN-γ.

The VLDL/apoE receptor is an unlikely candidate because IFN-γ did not enhance the uptake of the apoE2-containing type III HTG-VLDL, even though apoE2 is a known ligand for this receptor (59, 60). Although it has not been demonstrated directly that type III (E2/E2) VLDL interacts with the VLDL receptor, Takahashi et al. (59, 60) reported that VLDL from normolipidemic subjects homozygous for either E3 or E2 bound similarly to the VLDL receptor. In addition, van Dijk et al. (69) found that overexpression of the VLDL receptor significantly reduced VLDL concentrations in apoE knockout mice overexpressing human apoE2. Therefore, if the increase in macrophage CE accumulation in the presence of IFN-γ was mediated by the VLDL receptor, we would have expected to observe an increased uptake of type III (E2/E2) HTG-VLDL similar to that observed for type IV HTG-VLDL. Also, the IFN-γ-induced increase in LDL uptake would be difficult to ascribe to the VLDL receptor as LDL is not a ligand for this receptor.

The involvement of the LRP is also unlikely, based on the finding that IFN-γ treatment of J774 macrophages inhibited, rather than inducing an increase in LRP expression, as demonstrated by a reduction of α2-macroglobulin binding. This result is consistent with the findings of La Marre et al. (14) who observed an IFN-γ-mediated decrease in LRP expression RAW macrophages. Although it has not been shown directly that HTG-VLDL binds to the LRP, Kuchenhoff et al. (70) demonstrated that a subfraction of rabbit β-VLDL bound to the LRP without additional apoE being added. Although rabbit β-VLDL is enriched in cholesteryl esters and apoE compared to whole, native type IV HTG-VLDL, these results suggest that subfractions of human HTG-VLDL may also interact with the LRP, in the absence of additional apoE. However, our observation that IFN-γ decreased LRP expression argues

against it as a possible receptor mediating the IFN-γ-enhanced lipoprotein uptake.

Lipoproteins can be modified during incubation with cultured macrophages which are then taken up by scavenger receptors, including the SR-A (61). Our results demonstrate that the IFN-γ-enhanced lipoprotein uptake was not due to an increase in scavenger receptor-mediated uptake of modified lipoproteins as IFN-γ almost completely inhibited oxidized LDL uptake. IFN-γ has been shown to decrease macrophage SR-A expression (15, 19). Recently, Lougheed et al. (71) estimated that in mouse peritoneal macrophages, approximately 30% of ox-LDL uptake was due to the SRA. Therefore, as we observed that the macrophage CE accumulation induced by oxLDL was completely inhibited in the presence of IFN-γ, our results suggest that only the SR-A is expressed by J774 cells, or more likely, that other scavenger receptors, such as CD36 (which are expressed by J774 cells (72)) and/or CD68, were also decreased by IFN-γ treatment. Nevertheless, we observed a decrease rather than an increase in oxLDL uptake indicating that scavenger receptors were not involved in the IFN-γ-enhanced uptake of native lipoproteins.

Treatment of cells with IFN-γ significantly increased the binding and degradation of radiolabeled LDL. These observations, together with the finding that IFN-γ had no effect on type III E2/E2 VLDL or on EKO VLDL, strongly suggest that the IFN-γ-induced increase in type IV HTG-VLDL and LDL uptake was mediated by an increased expression of the LDL receptor. The mechanism(s) of how IFN-γ modulate LDL receptor expression remain to be elucidated.

In conclusion, the results of the present study demonstrate another mechanism whereby the uptake of both type IV HTG-VLDL and LDL by macrophages can be further enhanced. A role for IFN-γ in lesion development has been proposed by others, and is based on the in vivo detection of this cytokine in T lymphocyte-rich regions of human plaques. In addition to an enhancement in cellular CE and TG levels, the finding that treatment of cultured macrophages with type IV HTG-VLDL and IFN-γ induced a significant increase in cellular FC content could have very significant ramifications in vivo. An IFN-γ-mediated enhancement of HTG-VLDL accumulation by intimal macrophages may represent a mechanism for FC-induced cytotoxicity, a potential contributing factor to the development of the necrotic core, a characteristic feature of more advanced lesions (73). The in vitro findings presented in this study further support the idea that IFN-γ can significantly influence lesion development.

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