Interleukin-1β and interleukin-6 increase levels of apolipoprotein B mRNA and decrease accumulation of its protein in culture medium of HepG2 cells

Keiko Yokoyama,* Toshiyuki Ishibashi,* Liang Yi-qiang,† Akira Nagayoshi,† Tamio Teramoto,† and Yukio Maruyama[1,*]

First Department of Internal Medicine,* Fukushima Medical College, 1 Hikarigaoka, Fukushima 960-12, Japan, and First Department of Internal Medicine,† Teikyo University School of Medicine, Tokyo, Japan

Abstract The purpose of the present study was to examine the regulation of levels of apolipoprotein B (apoB) mRNA and its protein by cytokines in HepG2 cells. A dose-dependent increase in apoB mRNA levels was observed in the presence of either interleukin-1β (IL-1β) or IL-6 alone. This increase occurred as early as 1 h after IL-1β or IL-6 stimulation. Exogenous addition of IL-1β (5 ng/ml) and IL-6 (50 ng/ml) induced 2.8- and 2.1-fold increases as a result of 18 h of culture, respectively. Co-stimulation with IL-1β and IL-6 significantly enhanced the increase in apoB mRNA levels stimulated with either cytokine alone. Treatment with cycloheximide prevented the induction of apoB mRNA by IL-1β, but not by IL-6. These findings suggest that enhancement of apoB mRNA levels by these cytokines is mediated through different pathways. Conversely, IL-1β and IL-6 lowered the accumulation of apoB protein levels in the culture medium. The pulse-chase study showed that addition of N-acetyl leucyl leucyl norleucinal to the medium induced a decrease in newly synthesized apoB in the cell lysate in response to IL-1β ($P < 0.05$) or IL-6 (not to a significant extent) compared with control. These findings demonstrated that the lower level of apoB in the medium was caused by the enhanced intracellular degradation. In addition, IL-1β increased LDL receptor mRNA levels as well as protein activity, although IL-6 did not, suggesting that the more marked decrease in apoB accumulation in the medium induced by IL-1β compared with that induced by IL-6 may reflect an increased uptake of apoB from the medium by IL-1β. The present study demonstrates that a cytokine network may be involved in the metabolism of apoB under certain conditions such as inflammation.—**Yokoyama, K., T. Ishibashi, L. Yi-qiang, A. Nagayoshi, T. Teramoto, and Y. Maruyama.** Interleukin-1β and interleukin-6 increase levels of apolipoprotein B mRNA and decrease accumulation of its protein in culture medium of HepG2 cells. *J. Lipid Res.* 1988. **39:** 103–113.

Supplementary key words cytokine • Northern blot analysis • intracellular degradation • LDL receptor

The liver secretes very low density lipoprotein (VLDL) which is enriched with triglycerides. Apolipoprotein B-100 (apoB), a major protein component of VLDL, is required for VLDL assembly and secretion (1–3). Measuring the rate of VLDL secretion is one of the most useful means of determining the concentration of low density lipoprotein (LDL) in plasma which closely correlates with cholesterol levels. ApoB is a ligand on LDL recognized by the LDL receptor which plays a pivotal role in cholesterol homeostasis (4). Increased levels of plasma apoB and LDL cholesterol are risk factors for coronary heart disease and are associated with atherosclerotic disease (5, 6).

It is known that various growth factors, cytokines, and adhesion molecules are involved in atherogenesis (7, 8). Some of these play a role in the initiation and stimulation of cell proliferation in the arterial wall, whereas others inhibit proliferation. Recently, it has been shown that macrophage colony-stimulating factor (M-CSF) lowers plasma cholesterol levels by increasing macrophage function, including the uptake of lipoproteins and cholesterol efflux from various tissues, for ultimate delivery to the circulation (9, 10). We also showed that granulocyte-macrophage colony-stimulating factor (GM-CSF) lowers plasma cholesterol and triglyceride levels and that this effect is partially mediated through the enhancement of macrophage function and the up-regulation of VLDL receptor mRNA (11).

Hepatic production of apoB-containing lipoproteins

Abbreviations: apoB, apolipoprotein B; IL-1β, interleukin-1β; IL-6, interleukin-6; VLDL, very low density lipoprotein; LDL, low density lipoprotein; TNF, tumor necrosis factor; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-3, interleukin 3; SCF, stem cell factor; CHX, cycloheximide; LPDS, lipoprotein-deficient serum; ALLN, N-acetyl leucyl leucyl norleucinal; TCA, trichloroacetic acid; HDL, high density lipoprotein.

[1]To whom correspondence should be addressed.

in humans is regulated by diet and other factors (12–14). HepG2 cells of a human hepatoma cell line mimic the in vivo synthesis of apolipoproteins including apoB (15). Thus, this cell line is useful as an in vitro model for apoB synthesis and secretion. Many investigators have used this cell line as an in vitro model for studying apoB and have shown that compounds such as oleate, albumin, and insulin modulate apoB secretion, mainly at the posttranslational level (16–19). However, there have been very few reports describing cytokine regulation of apoB synthesis (20, 21).

To extend our knowledge in this area, we evaluated the effects of cytokines on apoB mRNA and apoB protein levels in HepG2 cells. The present study suggests that IL-1β and IL-6 affect transcription and intracellular degradation of apoB and that these molecules may play a key role in abnormalities of lipid metabolism seen during inflammation.

MATERIALS AND METHODS

Cytokines

Recombinant human interleukin 1β (IL-1β, specific activity; 2×10^7 units/mg) and IL-6 (specific activity; 2×10^6 units/mg) were obtained from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan) and Ajinomoto Co. (Yokohama, Japan), respectively. Human recombinant tumor necrosis factor (TNF, specific activity; 2.55×10^6 units/mg) was a gift from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Human purified urinary macrophage colony-stimulating factor (M-CSF, specific activity; 4×10^8 units/mg) and *E. coli*-derived human granulocyte-macrophage colony-stimulating factor (GM-CSF, specific activity; 2×10^6 units/mg) were generously supplied by the Morinaga Milk Industry (Tokyo, Japan) and Hoechst Japan Co. (Tokyo, Japan), respectively. Human recombinant IL-3 (specific activity; 1×10^8 units/mg) and stem cell factor (SCF, specific activity; 2×10^6 units/mg) were provided by Kirin Brewery Co., Ltd. (Tokyo, Japan).

Cell culture

HepG2 cells of a hepatoblastoma cell line that mimic normal hepatocytes were grown in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 100 units/ml penicillin–streptomycin (GIBCO) in 75 cm^2 cell culture flasks (Costar, Cambridge, MA) (15). These cells were used for Northern blot analysis, the apoB protein assay, and immunoprecipitation, as described below. Cell viability was determined by trypan blue exclusion method.

Northern blot analysis

Nearly confluent cells were cultured in the presence of 5 ng/ml IL-1β or 50 ng/ml IL-6 and harvested at periodic time intervals. The cells were also stimulated for 18 h with 0.1, 1, and 5 ng/ml IL-1β, 2, 10, and 50 ng/ml IL-6, 100 ng/ml TNF, 1000 units/ml M-CSF, 200 units/ml GM-CSF, 100 units/ml IL-3, or 100 ng/ml SCF. The cells were then washed three times with phosphate-buffered saline (PBS, pH 7.4), and RNA was then prepared. In addition, cells were pretreated with 5 μg/ml of cycloheximide (CHX, Sigma Chemical Co., St. Louis, MO) for 30 min to block protein synthesis, and 5 ng/ml IL-1β or 50 ng/ml IL-6 was subsequently added to the medium in the presence of CHX and incubated for 3 h, followed by RNA preparation.

Total RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method, followed by selection of poly(A)$^+$ RNA using an oligo(dT) cellulose column (22). Poly(A)$^+$ RNA samples (2 μg each lane) were electrophoresed on an agarose gel (1%, FMC Bio Products, Rockland, ME) and transferred to a nylon membrane (Hybond N+, Amersham, UK) in 20 × SSPE. Northern hybridization was performed as described previously (23). Briefly, the membrane was prehybridized at 42°C for 3 h in a buffer containing 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 1% SDS, and 200 μg/ml denatured salmon sperm DNA (Sigma), followed by hybridization at 42°C for 16 h in fresh buffer plus a ^{32}P-labeled probe. After sequential washings (0.1 × SSPE, 0.1% SDS at 65°C for 15 min), the membrane was air-dried and exposed to an X-ray film and an intensifying screen at −70°C. After autoradiography, the nylon membrane was treated with boiling water containing 0.5% SDS and used for rehybridization. The probes used in this study were a 1.2 Kb EcoRI fragment of rat apoB cDNA (24), a 0.75 Kb XbaI-EcoRI fragment of human LDL receptor cDNA (25), and a 1.9 Kb BamHI fragment of β-actin cDNA (26). Each probe was labeled with [^{32}P]dCTP by random hexanucleotide priming (27).

Quantitative measurement of transcripts

Signals of Northern blots were quantified by densitometric scanning after autoradiography. The levels of apoB and LDL receptor mRNA in each experiment were normalized to β-actin mRNA levels. The ratio of tested sample to the control was determined.

Protein measurement of apoB in cell suspensions

Various concentrations of IL-1β or IL-6 were added to nearly confluent HepG2 cells in a 24-well culture plate filled with fresh medium supplemented with 2 mg protein/ml of human lipoprotein-deficient serum (LPDS) instead of with FBS. LPDS was obtained by ultracentrifu-

gation as the density ≥1.21 g/ml from normal volunteers. After 18 h of culture, the supernatant was used for measurement of apoB and the cells were solubilized in 0.1 N NaOH to determine the protein concentration. Concentrations of apoB in the cell suspensions were measured by a one-step sandwich ELISA method as previously described (28). Briefly, 300 μl of first antibody against human apoB (Chemicon International Inc., Temecula, CA) was dispensed into a flat-bottomed micro ELISA plate (Sumitomo Bakelite, Tokyo, Japan) and incubated at room temperature for 1 h. The plate was then washed three times with PBS, 100 μl of standard or sample solution, and 100 μl of 2nd peroxidase coupled antibody (The Binding Site Ltd., Birmingham, England) was added and allowed to react for 1 h at room temperature. After washing, the substrate (2.5 mg/ml ortho-phenylene diamine 0.018% H_2O_2 in 0.01 M Na_2HPO_4–0.044 M sodium citrate buffer, pH 5.4) was added to each well. The reaction was terminated by the addition of 100 μl of 1 M H_2SO_4. Absorbance was determined at 490 nm using a microplate reader (Model 3550, Bio-Rad, Richmond, CA). The apoB concentration in standard samples was linear between 0 and 4 μg/ml (data not shown). Cellular protein concentrations were measured by the method of Lowry et al. (29).

Pulse-chase study

The cells (2.5×10^4 cells) were seeded in 0.5 ml of medium in a 24-well tissue culture plate for 4 days. After washing twice with 0.5 ml of PBS, 0.5 ml of RPMI 1640 containing 10% LPDS and 5 ng/ml IL-1β or 50 ng/ml IL-6 was added to the cells. After 18 h of culture, the cells were washed with PBS twice and the medium was changed to methionine-free Minimum Essential Medium (GIBCO) containing 1.5% bovine serum albumin (BSA, Sigma). L-[^{35}S]methionine (Amersham, Arlington Heights, IL) was added to the cells at the concentration of 100 μCi, incubated for 10 min at 37°C, and removed. After washing, the cells were then treated with or without 40 μg/ml N-acetyl leucyl leucyl norleucinal, a cysteine proteinase inhibitor (ALLN, Sigma) (30) for 60 min in the same medium containing 1.5% BSA. After chasing, the medium was removed and the cells were extracted by addition of 0.5 ml of the lysis buffer containing 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100 (Sigma), and 0.05 M Tris-HCl (pH 7.4). Newly synthesized apoB in the medium and cell lysate was immunoprecipitated using a specific antiserum, as previously described (31). This anti-human apoB antiserum was raised in a rabbit in the First Department of Internal Medicine, Teikyo University School of Medicine, and was determined to be monospecific to human apoB by Western blotting analysis (data not shown). After addition of 10 μl nonimmune rabbit serum to 100 μl of the medium or the cell extract, 10 μl Protein A Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) dissolved in PBS (pH 7.4) was added to the medium to remove the nonimmune immunoglobulins. Twenty μl of rabbit anti-apoB serum was added to the medium or to the cell extracts and the mixture was incubated overnight at 4°C. Subsequently, 20 μl of Protein A Sepharose was added and the samples were incubated for another 2 h at 4°C. Precipitates were then pelleted and washed twice with 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, and 0.05 M Tris-HCl (pH 8.0) buffer, then once with distilled water, and solubilized in 50 μl of 2.3% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.625 M Tris-HCl (pH 8.2) buffer. The immunoprecipitated samples were boiled for 5 min and applied to the polyacrylamide gel electrophoresis containing SDS (SDS-PAGE). The resolving gel contained a 5–22.5% gradient of acrylamide. The gels were fixed, stained with Coomassie Blue (Bio-Rad Laboratories), destained, dried, subjected to autoradiography, and analyzed with a Fujix BAS1000 Bioimage analyzer system (Fuji Photo Film Co. Ltd., Tokyo). The ratio of synthesized apoB to trichloroacetic acid (TCA)-precipitable protein was determined.

Lipoproteins and ^{125}I-labeled LDL

Human very low density lipoprotein (VLDL, d < 1.006 g/ml), LDL (d 1.019–1.063 g/ml), high density lipoprotein (HDL, d 1.063–1.215 g/ml), and LPDS were isolated from serum of fasting normolipidemic volunteers by sequential ultracentrifugation (32). Isolated fractions were extensively dialyzed against a buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4, at 4°C. LPDS was defibrinated and sterilized by filtration (33). After sterilization, LDL was trace-labeled with ^{125}I by the method of McFarlane (34) as modified by Shimada et al. (35). The specific activity of ^{125}I-labeled LDL was 167 counts/min per ng protein, and more than 98% of the radioactivity was precipitable with 5% hot trichloroacetic acid.

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

HepG2 cells were plated at a density of 2.5×10^4 cells/well in 24-well plates. After 3 days of culture, cells received fresh medium containing 2 mg protein/ml of LPDS instead of FBS and were further incubated for 18 h at 37°C in the presence of 5 ng/ml IL-1β or 50 ng/ml IL-6. The medium was removed and replaced with 0.25 ml of fresh LPDS-supplemented medium containing varying amounts of ^{125}I-labeled LDL. After 2 h of incubation at 37°C, the medium was collected for determination of proteolytic degradation of LDL as previously described (35). The cells were washed five times with 50 mM Tris buffer containing 0.15 M NaCl and 2 mg/ml of bovine serum albumin (BSA, Sigma), pH 7.4, and twice

with the Tris buffer without BSA. The cells were incubated at 4°C with shaking in 0.25 ml of heparin (10 mg/ml) containing medium and the amounts of LDL bound were determined from the heparin-releasable radioactivity. The remaining cells were dissolved in 0.1 N NaOH for measurement of internalized LDL. Cellular protein was measured by the method of Lowry et al. (29).

Statistical analysis

Statistical analysis was performed using the paired *t* test or the Newman-Keuls multiple range test as appropriate. Data are expressed as means ± SD unless otherwise indicated. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Effect of cytokines on levels of apoB mRNA

IL-1β and IL-6 induced an increase in apoB mRNA in HepG2 cells. **Figure 1 A** shows the periodic profile of lev-

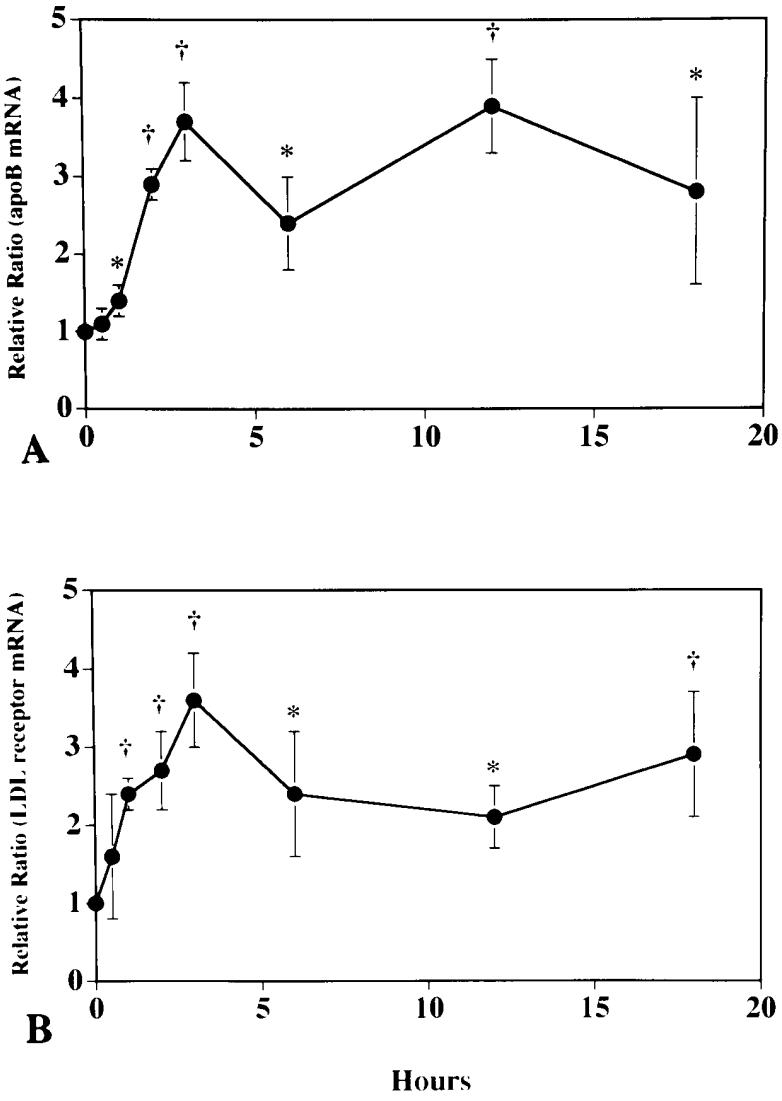

Fig. 1. The time-course of apoB and LDL receptor mRNA levels after IL-1β or IL-6 stimulation. IL-1β (5 ng/ml) was added to nearly confluent HepG2 cells that were harvested at periodic intervals, followed by isolation of poly(A)$^+$ RNA. The levels of mRNA for apoB (A) and LDL receptor (B) were determined as described in the Methods. Data are expressed as means ± SD of three experiments. *$P < 0.05$; †$P < 0.01$ vs. control.

els in the cells stimulated with 5 ng/ml IL-1β. A significant increase in apoB mRNA was observed as early as 1 h after IL-1β stimulation with further increments at longer time intervals. Maximum levels were reached 3 h after stimulation and persisted for up to 18 h (Fig. 1A). A similar time course of apoB mRNA levels was observed after stimulation with 50 ng/ml of IL-6 (data not shown). HepG2 cells were then cultured for 3 or 18 h in the presence of various cytokines. **Figure 2A** shows the effect of each cytokine on apoB mRNA after 18 h of culture. Among the cytokines tested, IL-1β and IL-6 independently brought about an increase in the levels of apoB mRNA. TNF, M-CSF, GM-CSF, IL-3, or SCF treatment of HepG2 cells all failed to alter levels of apoB mRNA after 3 or 18 h of culture. Five nanograms per milliliter IL-1β and 50 ng/ml IL-6 increased apoB mRNA levels 2.8 ± 1.2 ($P < 0.01$) and 2.1 ± 0.1 ($P < 0.01$)-fold after 18 h of culture, respectively (Fig. 2A). Both of these cytokines raised levels of apoB transcripts in a dose-dependent fashion (**Fig. 3**). Significant increases in apoB mRNA were observed at concentrations of 0.1 ng/ml IL-1β ($P < 0.01$) and 10 ng/ml IL-6 ($P < 0.05$) after 18 h of stimulation. In addition, co-stimulation with IL-1β (5 ng/ml) and IL-6 (50 ng/ml) significantly enhanced the increase induced by either cytokine alone ($P < 0.05$, **Fig. 4**).

Effect of cycloheximide treatment on apoB mRNA

To determine whether protein synthesis contributes to induction of apoB mRNA by IL-1β or IL-6 alone, we measured the mRNA levels after initial cycloheximide pretreatment of 30 min and subsequent 3-h incubation in the presence of 5 ng/ml IL-1β or 50 ng/ml IL-6. There was no significant difference in cell viability between the cycloheximide-treated cells (94 ± 5%) and control cells (97 ± 3%). This treatment did not significantly change the levels of apoB mRNA in cytokine-untreated cells (data not shown). ApoB mRNA levels of cells stimulated with 5 ng/ml IL-1β were reduced by cycloheximide treatment (23 ± 11% of CHX-untreated cells, n = 3, $P < 0.01$), whereas this treatment did not significantly alter the levels of apoB mRNA induced by IL-6 (91 ± 32% of CHX-untreated cells, n = 3). **Figure 5** shows a representative autoradiogram of the results. These data suggest that IL-1β requires additional protein synthesis for the increase in apoB mRNA levels, although IL-6 does not.

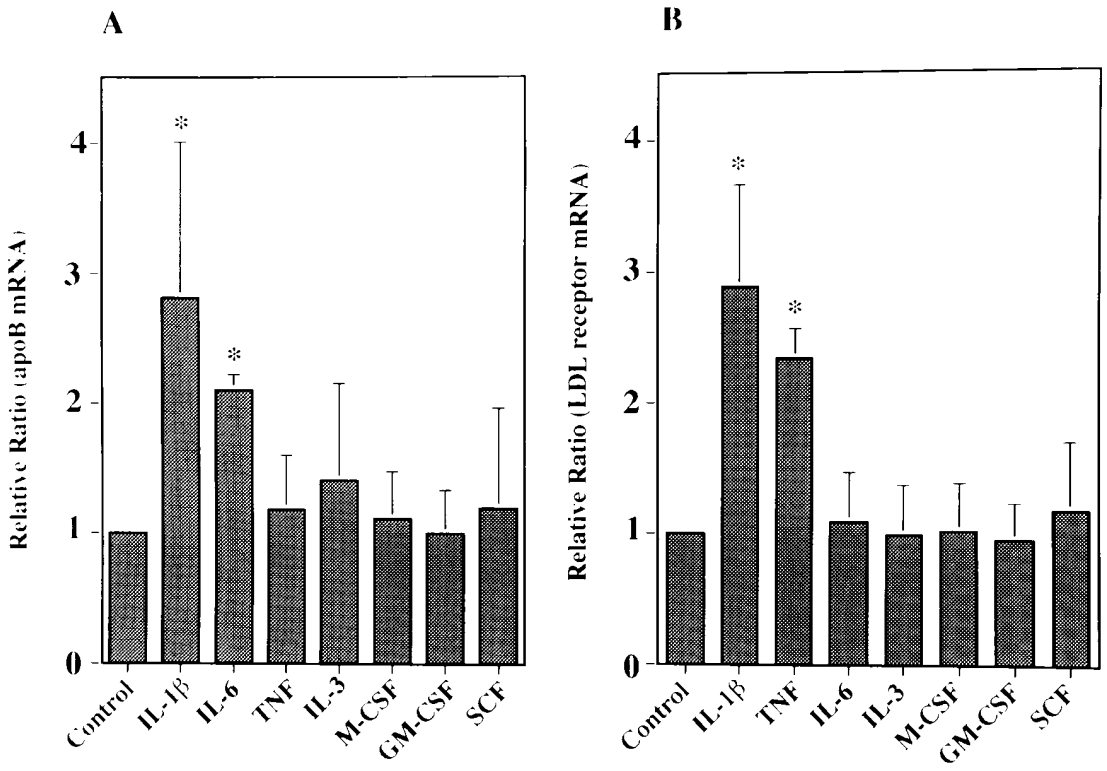

Fig. 2. Effect of various cytokines on apoB and LDL receptor mRNA levels. HepG2 cells were grown in flasks to near confluency in media containing 10% FBS and then treated with various cytokines (5 ng/ml IL-1β, 50 ng/ml IL-6, 100 ng/ml TNF, 1000 U/ml M-CSF, 200 U/ml GM-CSF, 100 U/ml IL-3, or 100 ng/ml SCF) for 18 h. After harvesting, total RNA was extracted from the cells and poly(A)$^+$RNA was then isolated. The levels of apoB (A) and LDL receptor (B) mRNA were determined as described in the Methods. Data are expressed as means ± SD of four experiments. *$P < 0.01$ vs. control.

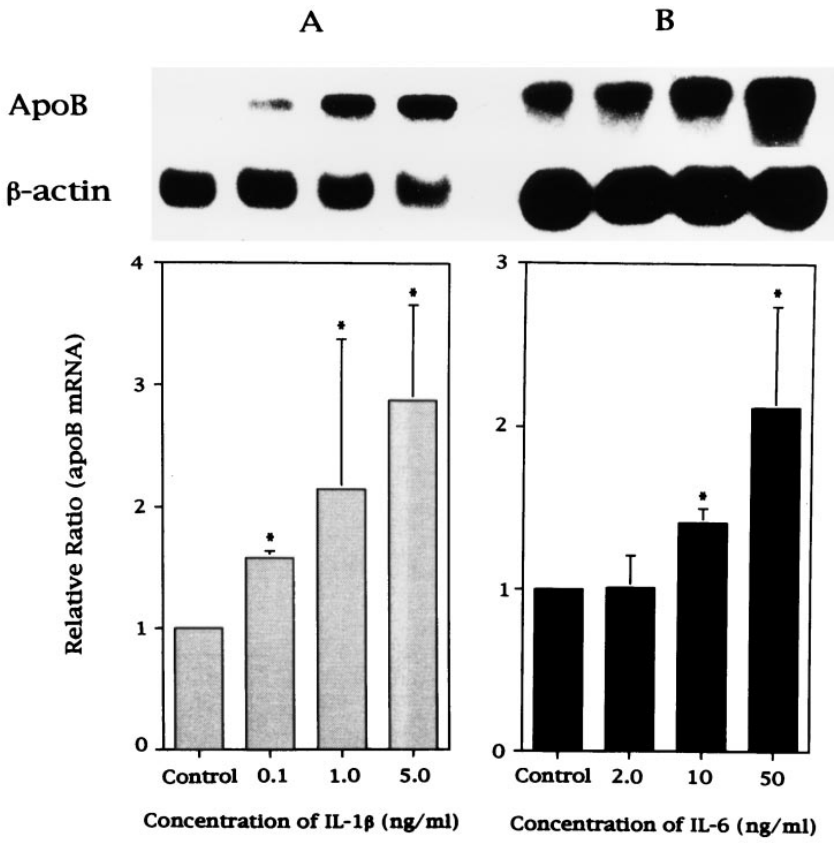

Fig. 3. Effects of various concentrations of IL-1β (A) or IL-6 (B) on apoB mRNA levels. After HepG2 cells were grown to near confluency, 0.1, 1, and 5 ng/ml rhIL-1β or 2, 10, and 50 ng/ml rhIL-6 were added and cells were cultured for 18 h at 37°C. Poly(A)$^+$RNA was isolated from the treated and control cells for Northern blot analysis. The apoB mRNA was quantified as described in the Methods. Representative autoradiograms are presented at the top and each histogram shows the mean ± SD of four experiments for each group. *$P < 0.05$ vs. control.

ApoB protein accumulation in the medium

As shown in **Fig. 6,** there was a dose-dependent decrease in the concentration of apoB after stimulation with IL-1β at concentrations of 0.1 to 5 ng/ml (control; 12.1 ± 6.7 μg/mg, 0.1 ng/ml; 63.1 ± 14.6%, $P < 0.05$ vs. control, 1 ng/ml; 43.8 ± 12.6%, $P < 0.01$ vs. control, 5 ng/ml; 42.1 ± 18.3%, $P < 0.01$ vs. control), whereas IL-6 caused less of a decrease in apoB protein accumulation when used at concentrations of 2 to 50 ng/ml, but this decrease was not dose-dependent (control; 10.1 ± 1.6 μg/mg, 2 ng/ml; 71.0 ± 12.7%, $P < 0.05$ vs. control, 10 ng/ml; 71.4 ± 20.6%, 50 ng/ml; 66.9 ± 0.8%, $P < 0.05$ vs. control).

Effect of cytokines on newly synthesized apoB in medium and cells

To determine the effect of IL-1β and IL-6 on intracellular degradation of newly synthesized apoB, pulse-chase studies using ALLN were performed. **Figure 7** shows the result of pulse-chase experiment of 60-min exposure to 40 μg/ml ALLN after 10 min of labeling. A significant increase in newly synthesized apoB in the cell lysate was observed in the presence of ALLN in comparison to the absence of ALLN in control, IL-1β- and IL-6-stimulated cells (control; $P < 0.05$, IL-1β; $P < 0.01$, IL-6; $P < 0.01$). The ratio of newly synthesized apoB in the cell lysate of non-ALLN-treated cells to ALLN-treated cells was decreased by 5 ng/ml IL-1β ($P < 0.05$) or 50 ng/ml IL-6 (not significantly), whereas IL-1β or IL-6 treatment tended to reduce the ratio of the medium, but not significantly (Fig. 7). The data indicated that these cytokines enhance intracellular degradation of apoB.

Effect of cytokines on LDL receptor mRNA

The time-course experiment demonstrated a significant increase in LDL receptor mRNA as early as 1 h after IL-1β stimulation with further increases at longer intervals (Fig. 1B). Levels reached a maximum 3 h after stimula-

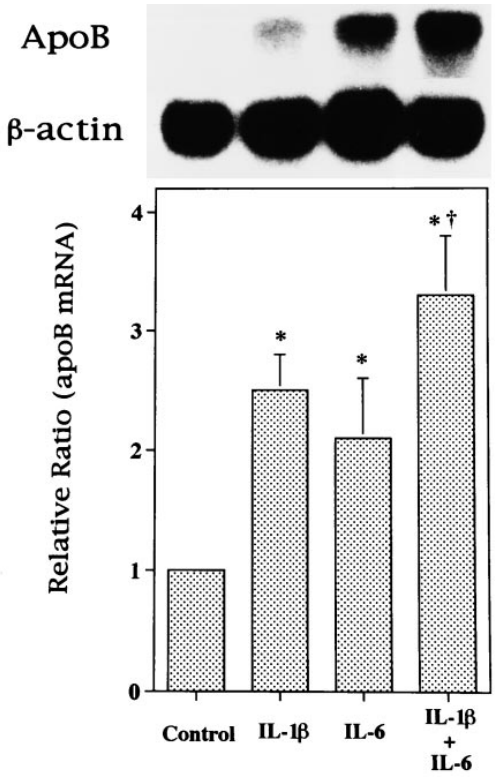

Fig. 4. Effect of co-stimulation with IL-1β and IL-6 in increasing apoB mRNA levels. HepG2 cells at near confluency were treated with 5 ng/ml IL-1β or 50 ng/ml IL-6 alone, or with a combination of both, for 18 h. Poly(A)$^+$RNA was isolated from the cells and apoB mRNA was quantified as described in the Methods. A representative autoradiogram is presented at the top and the histogram shows mean ± SD of six experiments. *$P < 0.05$ vs. control; †$P < 0.05$ vs. IL-1β or IL-6 alone.

tion and persisted for up to 18 h, as shown in Fig. 1B. Figure 2B shows the effects of various cytokines on the levels of LDL receptor mRNA after 18 h of culture. Among the cytokines tested, exogenous addition of IL-1β or TNF induced a significant increase in LDL receptor mRNA levels. IL-6 did not significantly alter mRNA levels of this receptor. IL-3, M-CSF, GM-CSF, and SCF treatment had no effect on LDL receptor transcripts after 3 or 18 h of culture (Fig. 2B). IL-1β (5 ng/ml) and TNF (100 ng/ml) increased the levels of LDL receptor mRNA by 2.9 ± 0.8 and 2.3 ± 0.2-fold as observed at 18 h of stimulation, respectively ($P < 0.01$), and with IL-1β at concentrations ranging from 0.1 to 5 ng/ml, the induced increase was dose-dependent (data not shown). Cycloheximide treatment did not prevent induction of LDL receptor mRNA by IL-1β (data not shown).

^{125}I-labeled LDL assay

The effect of cytokines on LDL receptor activity in HepG2 cells is shown in **Fig. 8.** IL-1β (5 ng/ml) increased LDL binding, internalization and degradation by 2.7-, 1.7-, and 1.8-fold, respectively, at an added concentration of 10 mg/ml ^{125}I-labeled LDL, compared with the control, whereas IL-6 (50 ng/ml) had no effect (Fig. 8). The data were consistent with LDL receptor mRNA levels after IL-1β or IL-6 stimulation. These findings suggest the enhanced reuptake of apoB-containing lipoproteins from the medium in cells treated with IL-1β.

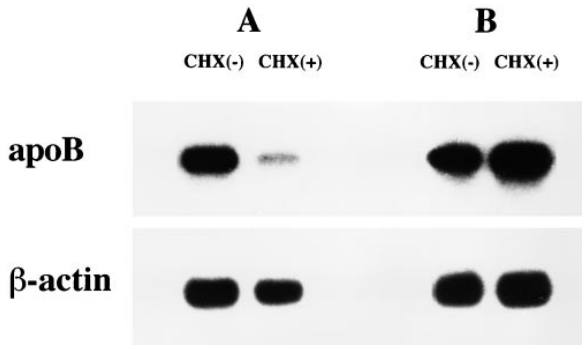

Fig. 5. Effect of cycloheximide treatment on IL-1β and IL-6 induction of apoB mRNA. After preincubation with 5 mg/ml cycloheximide for 30 min, 5 ng/ml IL-1β or 50 ng/ml IL-6 was subsequently added to the medium in the presence of cycloheximide and incubated for 3 h. Poly(A)$^+$RNA was isolated for Northern blot analysis. The levels of apoB mRNA were determined by comparing the levels of control cells treated with cycloheximide. A representative autoradiogram of IL-1β (A) and IL-6 (B) is shown. CHX indicates cycloheximide.

DISCUSSION

The production of apoB is affected by transcription, mRNA stability, translation, posttranslational processing, secretion, and reuptake. Little is known about the association between regulation of apoB production and enhanced cytokine levels in vivo or how production is affected by the exogenous addition of cytokines in vitro. The present study was undertaken to assess the effects of cytokines on apoB mRNA and protein levels in HepG2 cells. We demonstrated that IL-1β and IL-6 induced a marked increase in apoB mRNA levels and that this increase was mediated via different signal transduction pathways. We also found that apoB protein levels in culture medium and newly synthesized apoB in the cell decreased after IL-1β or IL-6 stimulation. These findings suggest that the increase in apoB mRNA levels induced by the cytokines may be caused by enhanced intracellular degradation.

With the onset of infection, a complex series of reactions take place ending in tissue damage which is initi-

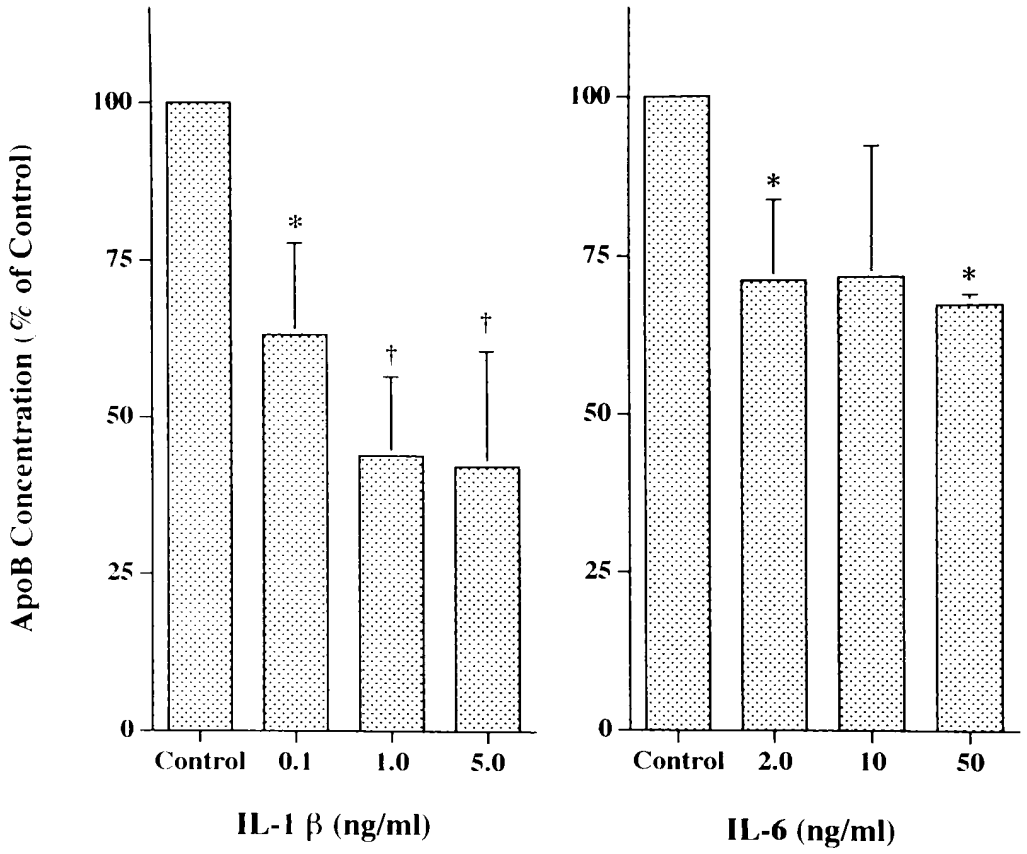

Fig. 6. Effect of IL-1β and IL-6 on accumulation of apoB in medium from HepG2 cells. Various concentrations of IL-1β or IL-6 were added to nearly confluent cells in medium containing LPDS. After 18 h of culture, concentrations of apoB in medium were measured by a one-step sandwich ELISA method. Data represent amounts of apoB per mg of cellular protein. Each data bar is the mean ± SD of five culture dishes. $^{*}P < 0.05$; $^{\dagger}P < 0.01$ vs. control.

ated by both local and systemic changes. A profound disturbance in lipid metabolism is one of the systemic manifestations of infection (36). The homeostatic immune response, hematopoiesis and the nervous system act to prevent further damage after infection, and an acute phase reaction is immediately evoked, resulting in an increase in plasma levels of acute phase protein (37). During this process, various cytokines, such as TNF, IL-1β, IL-6, interferons, GM-CSF, and M-CSF, are released into local tissue as well as into the circulation. Thus, the turbulent changes in lipid metabolism observed with infection may be related to an increase in cytokine levels caused by a systemic reaction. In addition, Feingold and his colleagues (38–40) have shown that administration of inflammatory cytokines such as TNF, IL-1β, and IL-6 to animal models induces a change in lipid metabolism.

In the present study, we found that a significant increase in apoB mRNA levels appeared as early as 1 h after IL-1β or IL-6 stimulation (Fig. 1A). Pullinger et al. (16) reported that the half-life of apoB mRNA was 16 h, as determined by the kinetics of the decay of [^{3}H] uridine-labeled apoB mRNA. Thus, it is conceivable that IL-1β or IL-6 may enhance transcription of the constitutively expressed apoB gene in HepG2 cells.

Our cycloheximide experiment also demonstrated that additional protein synthesis is necessary for IL-1β induction of apoB mRNA, but not for induction by IL-6 (Fig. 5). We postulate that the mechanism responsible for the enhancement of apoB mRNA levels induced by IL-1β or IL-6 may mediate distinct signal transduction pathways. This may partially explain why co-stimulation with IL-1β and IL-6 induced an additive increase in apoB mRNA levels compared with results obtained using IL-1β or IL-6 alone (Fig. 4).

We simultaneously measured protein concentrations of apoB in culture media from HepG2 cells (Fig. 6). IL-1β and IL-6 lowered levels of apoB accumulation in the medium despite a marked increase in the mRNA levels. Our finding is consistent with a previous report by Et-

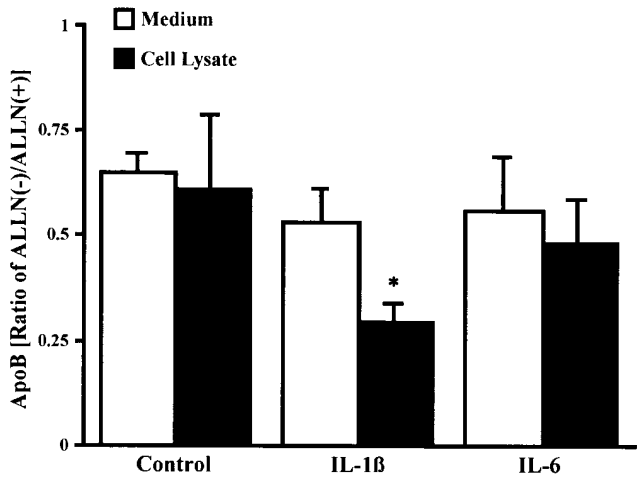

Fig. 7. Result of pulse-chase study of 60-min exposure to 40 μg/ml ALLN after 10 min of labeling. The cells were cultured in the presence of 5 ng/ml IL-1β or 50 ng/ml IL-6 for 18 h and labeled with 100 μCi L-[^{35}S]methionine for 10 min in methionine-free medium. After labeling, the cells were treated with or without 40 μg/ml ALLN for 60 min and newly synthesized apoB was measured as described in the Methods. Data are expressed as means ± SD of four experiments. *$P < 0.05$ vs. control; □ medium; ■ cell lysate.

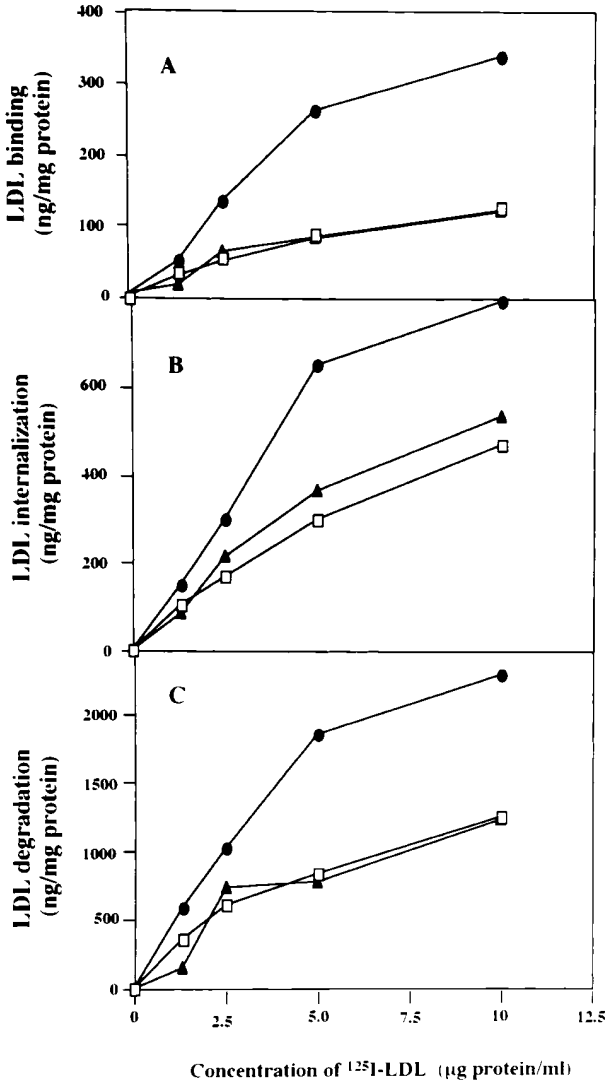

Fig. 8. Saturation kinetics of binding (A), internalization (B), and degradation (C) of ^{125}I-labeled LDL in HepG2 cells. Cells were stimulated in culture for 18 h with 5 ng/ml IL-1β or 50 ng/ml IL-6 and were then incubated with the indicated concentrations of ^{125}I-labeled LDL for 2 h at 37°C. Binding, internalization, and degradation of ^{125}I-labeled LDL were determined as described in the Methods. Data presented are from one of three similar experiments. Control, □; IL-1β, ●; IL-6, ▲.

tinger et al. (21) who found that protein levels of apoB in culture medium decreased in response to the addition of IL-1β and IL-6 to HepG2 cells. However, these investigators reported that neither cytokine had a significant effect on the apoB mRNA level. We performed our experiments using poly(A)$^+$RNA and various doses of cytokines to examine these levels, whereas they used a single cytokine dose. One explanation for the discrepancy in results between the two laboratories may have been that different doses of cytokines were used.

There are two reports that describe significant increases in apoB mRNA levels resulting from the use of two materials: in one case, a 55% increase was noted in response to the addition of 25-hydroxycholesterol (41); and in another case, a 20–30% increase was obtained with the addition of VLDL (42). Ours is the first study showing a marked increase (2- to 3-fold) in apoB mRNA levels induced by compounds such as IL-1β or IL-6 (Fig. 2A). Treatment with either 25-hydroxycholesterol or VLDL resulted in an increase in the net accumulation of apoB that was mediated through a dominant posttranslational mechanism (41, 42). However, in our study, IL-1β and IL-6 lowered the net accumulation in medium in spite of a marked increase in apoB mRNA levels. These findings suggest that IL-1β and IL-6 accelerate the intracellular degradation of apoB and subsequently increase levels of apoB mRNA, as intracellular degradation of apoB plays an important role in apoB synthesis (43, 44).

Therefore, we performed the pulse-chase experiments using ALLN to examine the effect of the two cytokines on newly synthesized apoB in the medium and cell lysate in the presence or absence of ALLN, which directly protects apoB from rapid intracellular degradation (30). A significant increase in newly synthesized apoB was observed in the presence of ALLN compared to the absence of ALLN in control, IL-1β- or IL-6-stimulated cells at 60 min after labeling (Fig. 7). The ratio of newly synthesized apoB in the absence of

ALLN to that in the presence of ALLN decreased in cells stimulated by IL-1β ($P < 0.05$) or IL-6 (not to a significant extent) when compared with control cells (Fig. 7). Alternatively, both cytokines tended to reduce the ratio of apoB in the medium of non-ALLN-treated cells to ALLN-treated cells. Net accumulation of apoB in medium reflects the balance of secretion and reuptake. We cultured the cells for 18 h, labeled the cells for 10 min, and chased them. In this condition, although a significant effect of the cytokines on newly synthesized apoB in medium was not observed, our findings suggest that IL-1β and IL-6 affect the secretion of apoB. The present study suggests that the decrease in apoB accumulation in the medium induced by the cytokines is caused by enhanced intracellular degradation, which may result in the increased apoB mRNA levels.

IL-1β induced more profound decrease in apoB accumulation in supernatants than did IL-6. This molecule also raised levels of LDL receptor mRNA and LDL receptor activity, whereas IL-6 did not (Fig. 8 and ref. 45). Therefore, the greater decrease in apoB accumulation in medium containing IL-1β-stimulated cells can be partly explained by the increased reuptake of apoB-containing lipoproteins.

In conclusion, apoB mRNA levels increased and accumulation of apoB protein decreased after IL-1β or IL-6 stimulation of HepG2 cells, suggesting that these cytokines affect transcription and intracellular degradation of apoB. The regulation of apoB and LDL receptor by various cytokines may be one of the mechanisms underlying the hypolipidemia that occurs with acute or chronic inflammation.

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