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Immune complexes and IFN-γ decrease cholesterol 27-hydroxylase in human arterial endothelium and macrophages

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Abstract The enzyme cholesterol 27-hydroxylase, expressed by arterial endothelium and monocytes/macrophages, is one of the first lines of defense against the development of atherosclerosis. By catalyzing the hydroxylation of cholesterol to 27-hydroxycholesterol, which is more soluble in aqueous medium, the enzyme promotes the removal of cholesterol from the arterial wall. Prior studies have suggested that immune reactants play a role in the pathogenesis of atherosclerosis; we report here that immune reactants, IFN-γ and immune complexes bound to C1q, but not interleukin-1 and tumor necrosis factor, diminish the expression of cholesterol 27-hydroxylase in human aortic endothelial cells, peripheral blood mononuclear cells, monocyte-derived macrophages, and the human monocytoid cell line THP-1. In addition, our studies demonstrate that immune complexes down-regulate cholesterol 27-hydroxylase only after complement fixation via interaction with the 126-kD C1qRp protein on endothelial cells and THP-1 cells. These results are consistent with the prior demonstration that IFN-γ contributes to the pathogenesis of atherosclerosis and suggest a role for C1q receptors in the atherogenic process. Moreover, these observations suggest that one mechanism by which immune reactants contribute to the development of atherosclerosis is by down-regulating the expression of the enzymes required to maintain cholesterol homeostasis in the arterial wall.—Reiss, A. B., N. W. Awadallah, S. Malhotra, M. C. Montesinos, E. S. L. Chan, N. B. Javitt, and B. N. Cronstein. **Immune complexes and IFN-γ decrease cholesterol 27-hydroxylase in human arterial endothelium and macrophages.** ***J. Lipid Res.*** **2001.** 42: **1913–1922.**

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Immune and inflammatory cells and mediators contribute to the development of atherosclerosis (1, 2). Monocytes, recruited to the vessel wall by injured endothelium, accumulate lipoprotein cholesterol and transform into foam cells in the vessel wall. IFN-γ, a cytokine produced by macrophages and certain subsets of T cells, and T cell-endothelial interactions (mediated by CD40 ligand-CD40 interactions) are critical to the development of atherosclerosis in murine models (3). Additionally, immune complexes (IC) consisting of oxidized LDL and antibodies to oxidized LDL, stimulate macrophages to form foam cells within the arterial wall. We and subsequently others have demonstrated that endothelial cells express receptors for the complement component C1q (4–7) that, when occupied by IC that have fixed C1q (IC-C1q), stimulate endothelial expression of vascular cell adhesion molecule-1 (VCAM-1), an adhesion molecule involved in the recruitment of monocyte/macrophages and lymphocytes during the development of atherosclerosis (4). Immune reactants may also influence critical metabolic events in the arterial wall, although there is little evidence of this to date.

It has recently been appreciated that the metabolism of cholesterol to 27-hydroxycholesterol by endothelial cells and macrophages in the arterial wall by the action of the mitochondrial P450 enzyme cholesterol 27-hydroxylase may constitute one of the first lines of defense in the prevention of atherosclerosis (8–12). Cholesterol 27-hydroxylase catalyzes the first step in the extrahepatic metabolism of cholesterol. The increased solubility in aqueous solution of 27-hydroxycholesterol facilitates transport to the liver for excretion. Moreover, the oxygenated sterol 27-hydroxycholesterol has a number of potentially antiatherogenic effects, including potent inhibition of HMG-CoA reductase (13–15), down-regulation of cell surface LDL receptors with consequent suppression of receptor-mediated LDL

Abbreviations: EGM, endothelial growth medium; HAEC, human aortic endothelial cells; HIgG, heat aggregated human IgG; HIS, heat-inactivated human serum; IC, immune complexes; ICAM-1, intracellular adhesion molecule-1; IC-C1q, immune complexes that have fixed C1q; IL-1, interleukin-1; NHS, normal human serum; PBM, peripheral blood mononuclear cells; TNF, tumor necrosis factor; TTBS, Tween 20 TBS; VCAM-1, vascular cell adhesion molecule-1.

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uptake in the periphery (16), and suppression of smooth muscle cell proliferation (17–19). Genetic deficiency of cholesterol 27-hydroxylase has been linked to the development of premature atherosclerosis (20, 21).

We report here evidence that immune reactants down-regulate the expression of cholesterol 27-hydroxylase in cells relevant to atherogenesis. Exposure of human aortic endothelial cells or the human monocytoid cell line THP-1 to either IC-C1q or IFN-γ, but not interleukin-1 (IL-1) or tumor necrosis factor (TNF)-α, significantly down-regulates the expression of cholesterol 27-hydroxylase, thereby diminishing the capacity of the vessel wall to metabolize cholesterol. Furthermore, IC-C1q decrease expression of the cholesterol 27-hydroxylase in human peripheral blood mononuclear cells (PBM) and monocyte-derived macrophages.

MATERIALS AND METHODS

Cell culture

THP-1 cells (kind gift of Dr. Frank Martiniuk) were grown at 37°C in a 5% CO_2 atmosphere in RPMI1640 (GIBCO BRL, Grand Island, NY) with 10% fetal calf serum (FCS) (GIBCO BRL) to a concentration of 10^6 cells/ml.

Human aortic endothelial cells (HAEC) were obtained from Clonetics Corp. (San Diego, CA) and were grown at 37°C in a 5% CO_2 atmosphere in endothelial growth medium (EGM; Clonetics Corp.) with 2% fetal calf serum (FCS). HAEC were seeded at 5,000 cells/cm^2 in 100-mm dishes and grown to 90% confluence. HAEC were between passages 3 and 6 when the experiments were performed.

Experimental conditions

WhenTHP-1 cells had reached 10^6 cells/ml and HAEC had attained 90% confluence, all cells were rinsed twice with PBS and then incubated for 3 h (37°C, 5% CO_2) in fresh medium under the following conditions (conditions for THP-1 and HAEC are identical except that medium for THP-1 is RPMI1640 and medium for HAEC is EGM): *a*) medium containing 10 U/ml IL-1β (R&D Systems, Minneapolis, MN); *b*) medium containing 100 U/ml TNF-α (R&D Systems); *c*) medium containing 500 U/ml of IFN-γ (kind gift of Dr. Jan Vilcek, Genentech, South San Francisco, CA); *d*) medium with 50% normal human serum (NHS) or 50% heat-inactivated human serum (HIS); *e*) medium with 50% NHS (or HIS) + IC [either BSA-rabbit anti-BSA, 0.48 mg/ml, or heat-aggregated human IgG (HIgG), 900 μg/ml]; *f*) preincubation for 1 h in medium with saturating solutions (10 μg/ml) of each of three antibodies directed at C1q binding proteins: R139, a126-kD C1qRp protein [kind gift of Dr. A. Tenner, (22)]; anti-gC1qR, a 33-kD binding protein (23); and anti-cC1qR, a 68-kD binding protein (24) (the latter two were kind gifts of Dr. B. Gebrehiwet) followed by a 3-h incubation in medium with 50% NHS + IC; *g*) preincubation for 1 h in medium with antibody against the cell surface molecule intracellular adhesion molecule-1 (ICAM-1, 28 μg/ml) (#YM1133F; Accurate Chemical & Scientific Corp., Westbury, NY; used as a negative control), followed by a 3-h incubation in medium with 50% NHS + IC.

Immediately after the incubation period, cell protein and RNA were collected directly from the culture dishes using the Trizol reagent (GIBCO BRL) according to manufacturer's directions. Concentrations of total RNA from each condition were measured by ultraviolet spectrophotometry. Protein concentrations were measured using the BCA protein assay reagent (Pierce, Rockford, IL).

C1q binding protein blocking antibodies

R139 (126-kD C1qRp binding protein). R139 is an IgG2b isotype monoclonal antibody against the 126-kD C1qRp binding protein. R139 was purified from mouse ascites using protein A/G-agarose (Schleicher & Schuell, Keene, NH) (22).

Anti-gC1qR (33-kD C1q binding protein). Rabbit polyclonal antibody 237 recognizes the native gC1q-R (23). The IgG fraction from the rabbit antiserum was prepared using the ImmunoPure A/G IgG purification kit (Pierce) according to the manufacturer's directions.

Anti-cC1qR (68-kD C1q binding protein). Rabbit polyclonal antibody 235 recognizes the intact cC1q-R (24). The IgG fraction from the rabbit antiserum was prepared using the ImmunoPure A/G IgG purification kit (Pierce) according to the manufacturer's directions.

Antibody against ICAM-1. This is a murine IgG_1 monoclonal antibody in the form of a tissue culture supernatant.

Preparation of BSA-rabbit anti-BSA IC, HigG, and HIS

ICs were prepared as described (4, 25). Briefly, 2 mg of BSA (Sigma Chemical Co., St. Louis, MO) were added to 5 ml (6 mg/ml) of rabbit IgG fraction to BSA (ICN/Cappel, Costa Mesa, CA). The mixture was incubated at 37°C for 2 h. The resulting IC were washed twice with PBS and then resuspended in PBS to a final concentration of ~5 mg/ml.

HIgG (Sigma Chemical Co.) was solubilized in 1 × PBS at a concentration of 20 mg/ml and then heated for 30 min in a water bath at 63°C.

HIS was prepared by heating normal human serum for 30 min in a water bath at 56°C.

C1q and IC dose-dependence

THP-1 cells were grown to a concentration of 10^6 cells/ml, and then all cells were rinsed twice with PBS and incubated for 3 h (37°C, 5% CO_2) in fresh medium under the following conditions: *a*) control conditions were media with 50% NHS with or without IC (0.48 mg/ml); *b*) media with no serum in the presence of a constant concentration of IC (0.48 mg/ml) and successively increasing concentrations of C1q (0, 10, 25, and 50 μg/ml; Quidel, San Diego, CA); *c*) media with no serum in the presence of a constant concentration of C1q (50 μg/ml) and successively increasing concentrations of IC (0, 0.0048, 0.048, and 0.48 mg/ml).

PBM in whole blood: incubation with IC

Two 10-cc aliquots of fresh whole blood were taken from each healthy human subject. IC were added to a final concentration of 0.48 mg/ml to one sample from each individual, and the other sample from that same individual was used as an untreated control. All aliquots were incubated for 3 h at 37°C. At the end of the incubation period, PBM were isolated from each sample using Nycoprep (GIBCO BRL), and mRNA was prepared from the PBM using the InVitrogen MicrofastTrack mRNA isolation kit according to the manufacturer's instructions.

Monocyte-derived macrophage preparation and incubation with IC-C1q

Human peripheral blood monocytes were isolated from a pooled human blood sample (kind gift of Dr. Joan Merrill) using ficoll gradient centrifugation followed by percoll gradient centrifugation (26). Cells were then plated at a density of 2 × 10^6/ml and treated with phorbol myristate acetate (250 ng/ml) for 3 days and then incubated for 3 h in the presence of 50% NHS with or without IC (0.48 mg/ml). RNA was isolated from the cells (Trizol) and reverse transcribed.

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Analysis of cholesterol 27-hydroxylase mRNA levels in THP-1 cells, HAEC, human monocytes, and monocyte-derived macrophages by RT-PCR

RT-PCR for cholesterol 27-hydroxylase was carried out as previously described (8, 27). Quantitative RT-PCR was performed using 5 μg of total RNA per condition for RT with oligo dT primers. Then, equal amounts of cDNA were taken from each RT reaction mixture for PCR amplification using both cholesterol 27-hydroxylase-specific primers and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control primers. The cholesterol 27-hydroxylase-specific primers (8) span a 311-base pair sequence encompassing nucleotides 491–802 of the human cholesterol 27-hydroxylase cDNA. The PCR products were loaded directly onto a 1% agarose gel and electrophoresed at 5 V/cm for 1.5 h. The GAPDH controls (10 μl/lane) were loaded at two concentrations, 0.5 and 0.25 μg/μl of cDNA. The GAPDH band intensities were linear, and all experimental results were normalized to the mean density of GAPDH. DNA was ethidium bromide stained and then visualized and photographed under ultraviolet light (320 nm).

Western blotting

Western blots were prepared as described preciously (9). Protein samples (20 μg/lane) were boiled for 5 min, loaded onto a 10% polyacrylamide gel, electrophoresed for 1.5 h at 100 V, then transferred to a nitrocellulose membrane in a semi-dry transblot apparatus for 1 h at 100 V. Membranes were stained with Ponceau red to verify uniformity of protein loading in each lane. The nitrocellulose membrane was blocked for 4 h at 4°C in blocking solution (3% nonfat dry milk dissolved in 1 × Tween 20 TBS [TTBS]) and then immersed in a 1:300 dilution of primary antibody (18.7 μg/ml) in blocking solution overnight at 4°C. The primary antibody is an affinity-purified rabbit polyclonal anti-peptide antibody raised against residues 15–28 of the cholesterol 27-hydroxylase protein (9, 28). The following day, the membrane was washed five times in TTBS for 5 min per wash and then incubated at room temperature in a 1:1000 dilution of an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody in blocking solution. The five washes in TTBS were repeated, and then the immunoreactive protein was visualized using the BCIP/NBT alkaline phosphatase substrate system (GIBCO BRL) according to the manufacturer's directions.

Before using our antibody in these experiments, we demonstrated that this anti-27-hydroxylase antibody specifically detects the appropriate molecular weight protein and that it can be used for quantitation by showing that band intensity after development of the immunoblot correlated with the quantity of 27-hydroxylase protein loaded onto the polyacrylamide gel (data not shown).

Band intensity measurements

Band intensities for both RT-PCR amplification products and Western blot protein samples were quantified using Kodak Digital Science 1D, version 2.0.3, after imaging with Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

Statistical analysis of experimental data

Statistical analysis was performed using SigmaStat, version 2.03 (SPSS, Inc.). Pairwise comparison was made between each treatment condition and control using Student's *t*-test.

RESULTS

Statistical data in this section are expressed as percentage of control value ± SEM.

Effects of cytokines on cholesterol 27-hydroxylase expression

Monocyte/macrophages play a crucial role in the atherosclerotic process (2, 29). To determine whether cholesterol 27-hydroxylase is regulated in macrophages by immune reactants, we studied expression of mRNA for the enzyme in THP-1 cells, a monocytoid cell line. IFN-γ down-regulated cholesterol 27-hydroxylase mRNA expres-

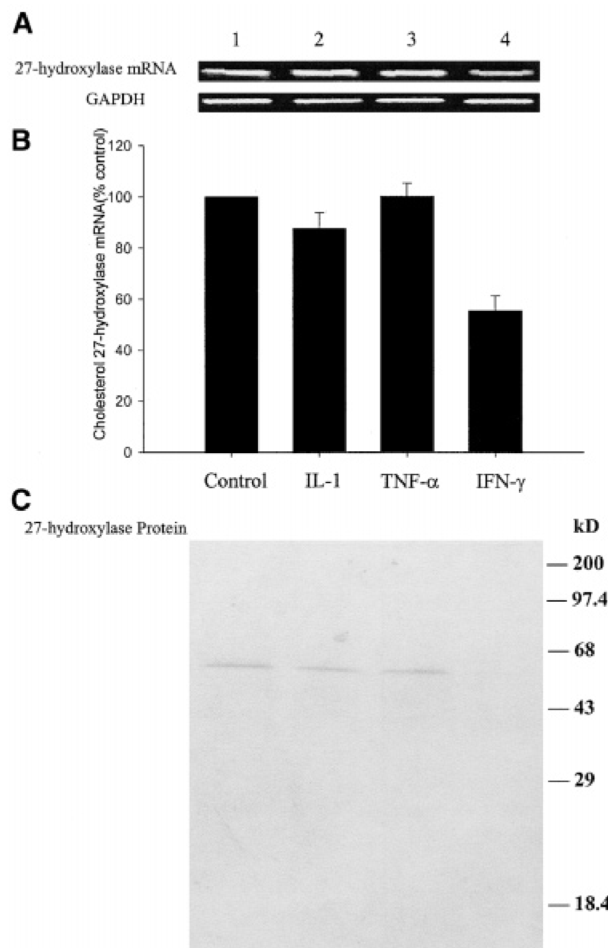

Fig. 1. IFN-γ down-regulates mRNA and protein for cholesterol 27-hydroxylase in THP-1 cells. THP-1 cells were incubated for 3 h before mRNA isolation and 6 h before protein isolation (37°C, 5% CO_2) in the presence of RPMI/50% normal human serum (NHS; control, lane 1), RPMI + interleukin-1 (IL-1; 10 U/ml, lane 2), RPMI +tumor necrosis factor (TNF)-α (100 U/ml, lane 3), and RPMI + IFN-γ (500 U/ml, lane 4). After isolation of total RNA (Trizol reagent), the RNA was reverse transcribed and the DNA was amplified by PCR, as described. Protein was also isolated with Trizol reagent. A: Photograph of ethidium bromide-stained PCR-amplified bands corresponding to message for cholesterol 27-hydroxylase. Directly beneath these is a photograph of ethidium bromide-stained PCR-amplified bands corresponding to message for GAPDH. B: Bar graph of the band intensities of the 27-hydroxylase message after normalization to GAPDH. C: Western immunoblot with bands corresponding to cholesterol 27-hydroxylase protein levels in THP-1 cell protein samples (20 μg per lane) obtained after incubation in conditions as described above. The entire Western immunoblot is shown here. Due to space considerations, only the relevant portions of subsequent immunoblots are shown.

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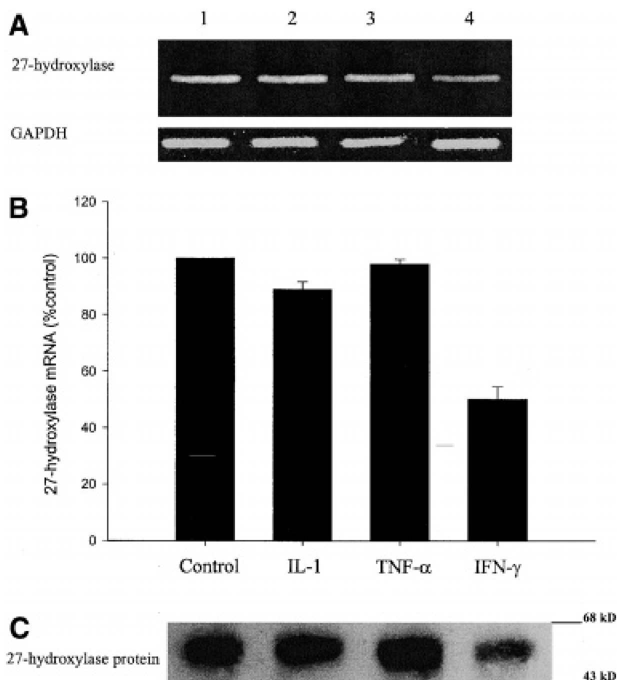

Fig. 2. IFN-γ down-regulates mRNA and protein for cholesterol 27-hydroxylase in human aortic endothelial cells (HAEC). HAEC were incubated for 3 h before mRNA isolation and 6 h before protein isolation (37°C, 5% CO_2) in the presence of endothelial growth medium (EGM; control, lane 1), EGM + IL-1 (10 U/ml, lane 2), EGM +TNF-α (100 U/ml, lane 3), and EGM + IFN-γ (500 U/ml, lane 4). After isolation of total RNA (Trizol reagent), the RNA was reverse transcribed and the DNA was amplified by PCR, as described. Protein was also isolated with Trizol reagent. A: Photograph of ethidium bromide-stained PCR-amplified bands corresponding to message for cholesterol 27-hydroxylase. Directly beneath these is a photograph of ethidium bromide-stained PCR-amplified bands corresponding to message for GAPDH. B: Bar graph of the band intensities of the 27-hydroxylase message after normalization to GAPDH. C: Western immunoblot with bands corresponding to cholesterol 27-hydroxylase protein levels in HAEC protein samples (20 μg per lane) obtained after incubation in conditions as described above.

sion by 44.6 ± 3.4% (n = 3, $P < 0.001$), whereas neither TNF-α nor IL-1, potent inflammatory cytokines, affected cholesterol 27-hydroxylase message (**Fig. 1A** and **B**). Similarly, IFN-γ, but not TNF-α or IL-1, diminished 27-hydroxylase protein levels in THP-1 cells (Fig. 1C).

The arterial vascular endothelium also plays a role in peripheral cholesterol metabolism (8, 9, 12). We therefore determined whether cytokines regulate arterial endothelial expression of cholesterol 27-hydroxylase. As in THP-1 cells, IFN-γ, but not IL-1 or TNF-α, suppressed expression of cholesterol 27-hydroxylase message by 50.2 ± 4.3% (n = 3, $P < 0.018$) and protein (**Fig. 2**).

Effect of IC on cholesterol 27-hydroxylase expression

Previous studies have suggested that IC play a role in the pathogenesis of atherosclerosis (4, 30). IC may interact either with receptors for the Fc portion of IgG or, after fixation of complement, with one of several complement binding proteins on the surface of macrophages. We therefore tested the effect of IC in the presence of serum containing active complement (NHS) or serum in which C′ was inactivated (HIS) on expression of cholesterol 27-hydroxylase message and protein in THP-1 cells. In the presence of NHS, but not HIS, both BSA-rabbit anti-BSA and HIgG down-regulated cholesterol 27-hydroxylase message by 49.3 ± 5.1%, n = 3; and 60.3 ± 4.7%, n = 3, respectively ($P < 0.002$ for both) (**Fig. 3A** and **B**). Reductions in cholesterol 27-hydroxylase protein levels in THP-1 cells were observed after 6 h of incubation with HIgG in the presence of NHS, but not HIS (by 52.3 ± 10.0%, n = 3, $P < 0.012$) (Fig. 3C).

Unlike macrophages, endothelial cells do not express Fc receptors under resting conditions (31), but they do express receptors for C1q (6, 32). When we tested the effect of IC on cholesterol 27-hydroxylase expression in HAEC, we observed reduced expression only when NHS was present. Both BSA-rabbit anti-BSA and HIgG diminished levels of mRNA for cholesterol 27-hydroxylase equally (by 60.0 ± 2.3%, n = 4, and 49.0 ± 1.0%, n = 3,

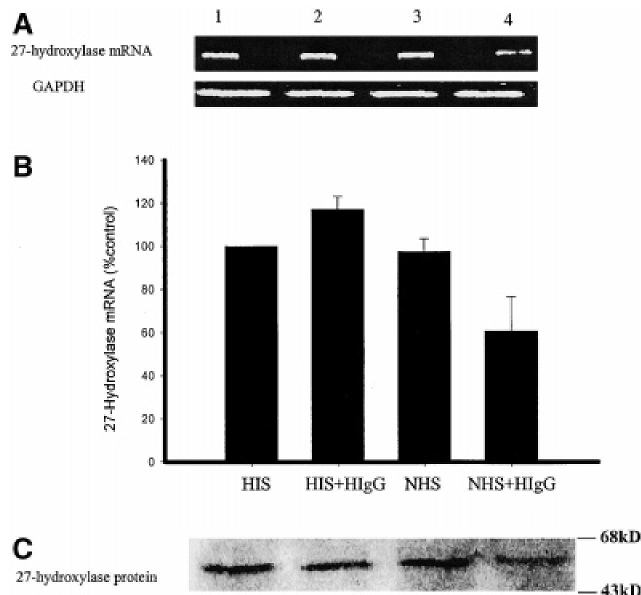

Fig. 3. Immune complexes (IC) down-regulate cholesterol 27-hydroxylase mRNA and protein in THP-1 cells in the presence of NHS, but not heat-inactivated human serum (HIS). THP-1 cells were incubated for 3 h before RNA isolation and 6 h before protein isolation (37°C, 5% CO_2) under the following conditions: lane 1, RPMI/50% HIS; lane 2, RPMI/50% HIS + heat aggregatead human IgG (HigG; 900 μg/ml); lane 3, RPMI/50% NHS (control); and lane 4, RPMI/50% NHS + HIgG (900 μg/ml). After isolation of total RNA (Trizol reagent), the RNA was reverse transcribed and the cDNA was amplified by PCR, as described. Protein was also isolated with Trizol reagent. A: Photograph of ethidium bromide-stained PCR-amplified bands corresponding to message for cholesterol 27-hydroxylase. Directly beneath these is a photograph of ethidium bromide-stained PCR-amplified bands corresponding to message for GAPDH. B: Bar graph of the band intensities of the cholesterol 27-hydroxylase message after normalization to GAPDH. C: Western immunoblot with bands corresponding to cholesterol 27-hydroxylase protein levels in THP-1 lysates (20 μg per lane) obtained after incubation in conditions as described above.

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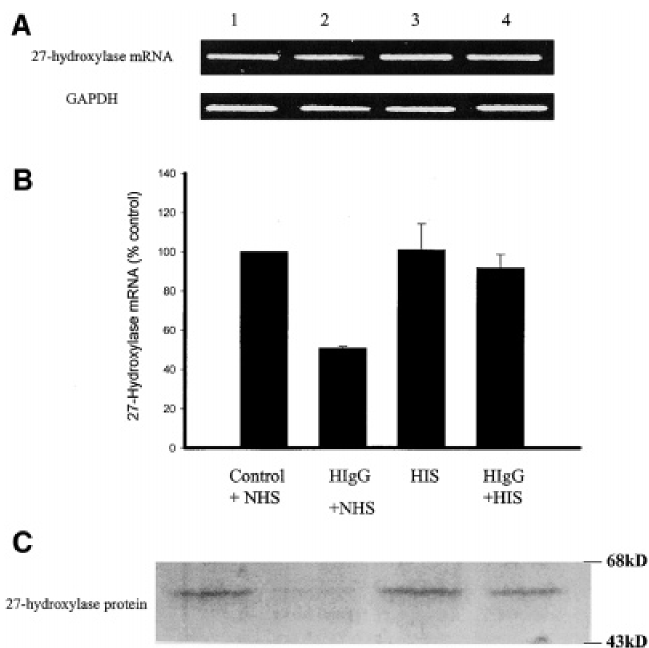

Fig. 4. IC down-regulate cholesterol 27-hydroxylase mRNA and protein in HAEC in the presence of NHS, but not HIS. HAEC cells were incubated for 3 h before RNA isolation or 6 h before protein isolation (37°C, 5% CO_2) under the following conditions: lane 1, EGM/50% NHS (control); lane 2, EGM/50% NHS + HIgG (900 μg/ml); lane 3, EGM/50% HIS; and lane 4, EGM/50% HIS + HIgG (900 μg/ml). After isolation of total RNA (Trizol reagent), the RNA was reverse transcribed and the cDNA was amplified by PCR, as described. Protein was also isolated with Trizol reagent. A: Photograph of ethidium bromide-stained PCR-amplified bands corresponding to message for cholesterol 27-hydroxylase. Directly beneath these is a photograph of ethidium bromide-stained PCR-amplified bands corresponding to message for GAPDH. B: Bar graph of the band intensities of the cholesterol 27-hydroxylase message after normalization to GAPDH. C: Western immunoblot with bands corresponding to cholesterol 27-hydroxylase protein levels in HAEC protein samples (20 μg per lane) obtained after incubation in conditions as described above.

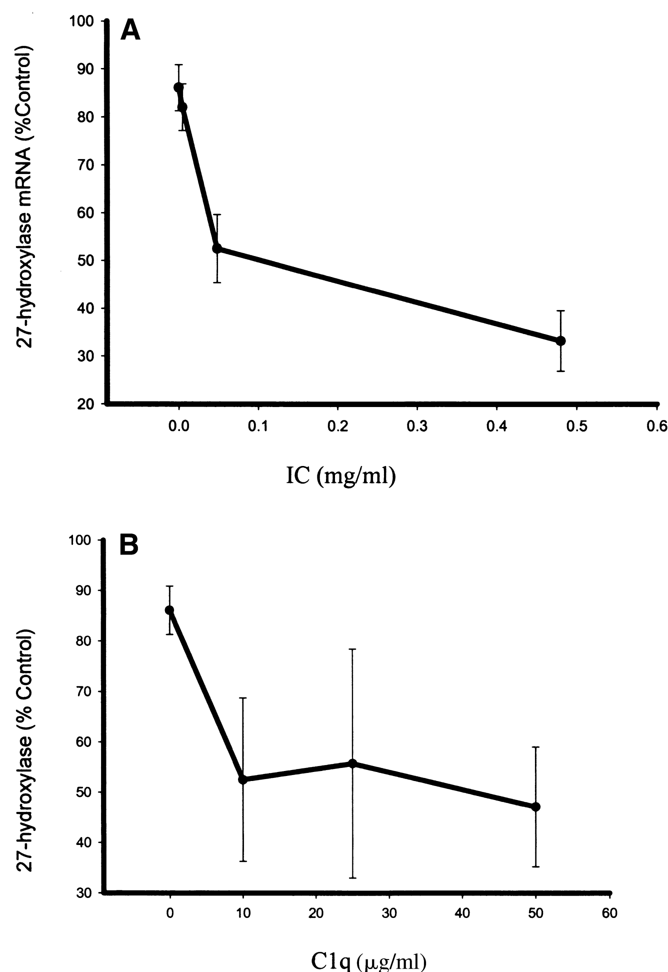

Fig. 5. IC and C1q down-regulate cholesterol 27-hydroxylase message in THP-1 cells. A: THP-1 cells were incubated for 3 h (37°C, 5% CO_2) with RPMI + C1q (50 μg/ml) or RPMI + C1q (50 μg/ml) + BSA-anti-BSA IC (0.0048–0.48 mg/ml). After isolation of total RNA (Trizol reagent), the RNA was reverse transcribed and the cDNA was amplified by PCR, as described. B: THP-1 cells were incubated for 3 h (37°C, 5% CO_2) with RPMI + IC (0.48 mg/ml) or RPMI + IC (0.48 mg/ml) + C1q (10–50 μg/ml). After isolation of total RNA (Trizol reagent), the RNA was reverse transcribed and the cDNA was amplified by PCR, as described.

respectively, $P < 0.001$ vs. control for both) (**Fig. 4A** and **B**). After incubation with HIgG, cholesterol 27-hydroxylase protein was diminished by 60.3 ± 11.0% (n = 3, $P < 0.018$) in the presence of NHS, but not HIS (Fig. 4C).

C1q is necessary and sufficient for IC-mediated down-regulation of cholesterol 27-hydroxylase

To determine whether interaction of C1q with IC are sufficient to support IC-mediated down-regulation of cholesterol 27-hydroxylase, we examined the effect of C1q on IC-mediated down-regulation of 27-hydroxylase mRNA in the absence of serum. Neither IC nor C1q alone affects expression of cholesterol 27-hydroxylase. However, even in the presence of subphysiologic concentrations of C1q, IC down-regulate 27-hydroxylase mRNA in a dose-dependent manner (**Fig. 5A**). In the absence of serum, with no exogenous C1q added, IC alone do not down-regulate cholesterol 27-hydroxylase message. In contrast, in the presence of concentrations of C1q ranging from 10 to 50 μg/ml, IC down-regulate 27-hydroxylase mRNA expression in THP-1 cells (**Fig. 5B**). Thus, C1q is necessary and sufficient for IC-mediated down-regulation of 27-hydroxylase message in THP-1 cells.

IC-mediated down-regulation of cholesterol 27-hydroxylase in THP-1 cells and HAEC occurs via the 126-kD C1qRp protein

Three binding proteins for C1q have been described (23, 33, 34). To determine which of the three major C1q binding proteins mediate down-regulation of cholesterol 27-hydroxylase in THP-1 cells, we pretreated these cells with antibodies to each of three known C1q binding proteins [R139, 126-kD C1qRp protein (22); anti-gC1qR, 33-kD binding protein (23); and anti-cC1qR, 67-kD binding protein (24)] or a common cell surface determinant (ICAM-1). None of the antibodies studied altered basal

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TABLE 1. Cholesterol 27-hydroxylase mRNA down-regulation in THP-1 cells by immune complexes (IC) in the presence or absence of C1q receptor blocking antibodies

	No Antibody	R139	α-cC1qR	α-gC1qR	α-ICAM
			% control ± SEM		
Control	100% (n = 6)	86 ± 11% (n = 6)	96 ± 7% (n = 3)	92 ± 4% (n = 3)	93 ± 6% (n = 6)
BSA–α-BSA immune complexes	59 ± 6% (n = 6)	94 ± 10% (n = 6)	56 ± 7% (n = 3)	69 ± 7% (n = 3)	49 ± 6% (n = 6)
P value versus control[a]	<0.012	Not significant	<0.050	<0.050	<0.008

[a] Statistical analysis was performed using SigmaStat V.2.03 (SPSS, ANOVA).

expression of message for cholesterol 27-hydroxylase. In THP-1 cells, IC significantly down-regulated 27-hydroxylase mRNA expression after pretreatment with medium alone (59.0 ± 6, n = 6, $P < 0.002$) or medium containing anti-gC1qR (69.0 ± 7.7%, n = 6, $P < 0.002$) or anti-cC1qR antibodies (56.0 ± 7.0%, n = 6, $P < 0.002$). IC also down-regulated 27-hydroxylase mRNA expression after pretreatment with antibodies to the cell surface determinant ICAM-1 in THP-1 cells (49.0 ± 6.1%, n = 6, $P < 0.002$). In contrast, pretreatment with antibody against the 126-kD C1qRp protein R139 completely abrogated the IC-mediated down-regulation of 27-hydroxylase message (P = not significant vs. control, n = 3) (**Table 1**).

Western blot analysis confirmed that down-regulation of cholesterol 27-hydroxylase protein in THP-1 cells is mediated by occupancy of the 126-kD C1qRp protein (**Fig. 6A** and **B**). R139 pretreatment of THP-1 cells blocked down-regulation of 27-hydroxylase protein by HIgG so that the level of the cholesterol 27-hydroxylase protein remained at 95% of the control level. Pretreatment for 1 h with an antibody to ICAM-1 did not interfere with down-regulation of 27-hydroxylase protein by HIgG in the presence of NHS. After pretreatment with anti-ICAM-1, HIgG, in the presence of NHS, reduced 27-hydroxylase protein to 48% of control, whereas HIgG exposure reduced 27-hydroxylase protein to 52% of control after pretreatment with medium alone (Fig. 6A and B).

Either message or protein for all three C1q binding proteins (126-kD C1qRp protein, gC1qR, and cC1qR) have been demonstrated in endothelial cells (6, 32). To further determine which of the endothelial C1q binding proteins are involved in this process, we pretreated endothelial cells with the three anti-C1q binding protein antibodies or an antibody to an endothelial cell surface determinant (ICAM-1). We found that in HAEC, like THP-1 cells, only the antibody against the 126-kD C1qRp protein, R139, reversed the effect of IC on expression of both cholesterol 27-hydroxylase message (**Table 2**) and protein (**Fig. 7A** and **B**). Also like THP-1 cells, IC significantly down-regulated 27-hydroxylase message in HAEC after pretreatment with medium alone (64.0 ± 5.6%, n = 3, $P < 0.001$) or pretreatment with anti-C1q binding protein antibodies anti-gC1qR (67.0 ± 5.8%, n = 3, $P < 0.001$) and anti-cC1qR (66.0 ± 4.6%, n = 3, $P < 0.001$) and anti-ICAM-1 antibodies (42.0 ± 9.6%, n = 3, $P < 0.001$) (Table 2). Again, parallel to our observations in THP-1 cells, pretreatment with antibody against the 126-kD C1qRp protein R139 completely abrogated the IC-mediated down-regulation of cholesterol 27-hydroxylase message (P = not significant vs. control, n = 3) in HAEC (Table 2).

Immunoblotting studies demonstrated that, as with the message, only R139, the antibody to the 126-kD C1qRp protein, abrogated IC-mediated down-regulation of cholesterol 27-hydroxylase protein in HAEC (Fig. 7A and B).

Effects of IC on cholesterol 27-hydroxylase expression in PBM and monocyte-derived macrophages

We have previously demonstrated that cholesterol 27-hydroxylase mRNA is present in the PBM of normocholesterolemic healthy human subjects as well as in their

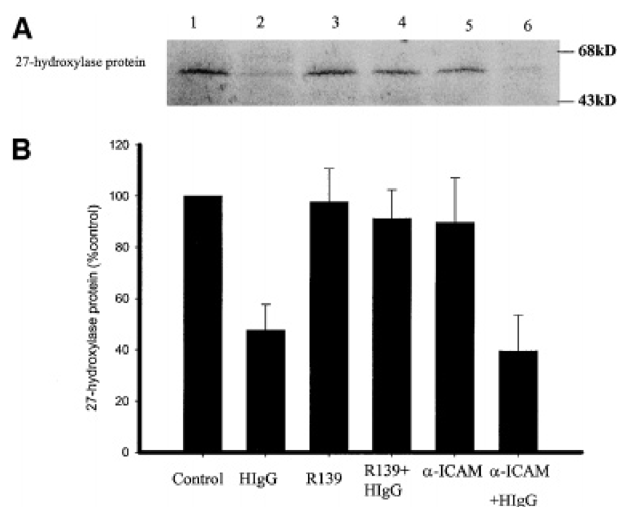

Fig. 6. HIgG down-regulates cholesterol 27-hydroxylase protein in THP-1 cells. THP-1 cells were incubated for 6 h (37°C, 5% CO_2) in the presence of RPMI/50% NHS (control, lane 1), RPMI/50% NHS + HIgG (900 μg/ml, lane 2) or pretreated with antibody to the 126-kD C1qRp protein (R139, 6 μg/ml) for 1 h followed by a 6-h incubation with either RPMI/50% NHS (lane 3) or RPMI/50% NHS + HIgG (900 μg/ml, lane 4). Alternatively, cells were pretreated with α-ICAM-1 antibody (28 μg/ml) for 1 h followed by a 6-h incubation with either RPMI/50% NHS (lane 5) or EGM/50% NHS + HIgG (900 μg/ml, lane 6). THP-1 protein samples (20 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to immunostaining, as described. A: Western immunoblot with bands corresponding to cholesterol 27-hydroxylase protein levels in THP-1 protein samples (20 μg per lane) obtained after incubation in conditions as described above. B: Bar graph of the average intensity of the cholesterol 27-hydroxylase protein band on Western immunoblot under conditions as described above (n = 3 for each condition).

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TABLE 2. Cholesterol 27-hydroxylase mRNA down-regulation in human aortic endothelial cells (HAEC) by IC in the presence or absence of C1q receptor blocking antibodies

	No Antibody	R139	α-cC1qR	α-gC1qR	α-ICAM
			% control ± SEM		
Control	100% (n = 6)	97 ± 7% (n = 6)	96 ± 8% (n = 3)	97 ± 6% (n = 3)	95 ± 6% (n = 6)
BSA–α-BSA immune complexes	64 ± 6% (n = 6)	98 ± 4% (n = 6)	66 ± 4% (n = 3)	67 ± 5% (n = 3)	42 ± 9% (n = 6)
P value versus control[a]	<0.001	Not significant	<0.050	<0.050	<0.001

[a] Statistical analysis was performed using SigmaStat V.2.03 (SPSS, ANOVA).

monocyte-derived macrophages (35, 36). Human lung macrophages also express high levels of cholesterol 27-hydroxylase (10). When PBM in whole blood (containing normal concentrations of C1q) taken from healthy human volunteers were exposed to IC for 3 h, 27-hydroxylase mRNA levels were reduced dramatically to ~20% of control (**Fig. 8A**). Human monocyte-derived macrophages express levels of cholesterol 27-hydroxylase mRNA comparable to those of PBM. Like PBM and THP-1 cells, monocyte-derived macrophages down-regulate cholesterol 27-hydroxylase message levels after exposure to IC-C1q (53.0 ±13% down-regulation vs. control, n = 4, $P < 0.002$) (Fig. 8B).

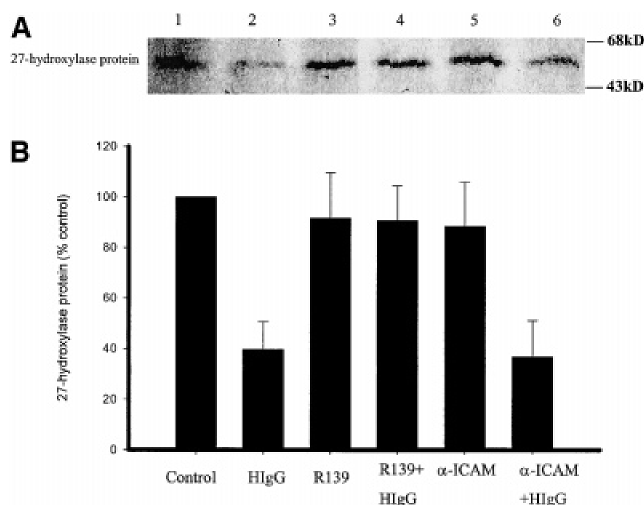

Fig. 7. HIgG down-regulates cholesterol 27-hydroxylase protein in HAEC. HAEC were incubated for 6 h (37°C, 5% CO_2) in the presence of EGM/50% NHS (control, lane 1), EGM/50% NHS + HIgG (900 μg/ml, lane 2), or pretreated with antibody to the 126-kD C1qRp protein (R139, 6 μg/ml) for 1 h followed by a 6-h incubation with either EGM/50% NHS (lane 3) or EGM/50% NHS + HIgG (900 μg/ml, lane 4). Alternatively, cells were pretreated with α-ICAM-1 antibody (0.005μg/ml) for 1 h followed by a 6-h incubation with either EGM/50% NHS (lane 5) or EGM/50% NHS + HIgG (900 μg/ml, lane 6). HAEC protein samples (20 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to immunostaining, as described. A: Western immunoblot with bands corresponding to cholesterol 27-hydroxylase protein levels in HAEC protein samples (20 μg per lane) obtained after incubation in conditions as described above. B: Bar graph of the average intensity of the cholesterol 27-hydroxylase protein band on Western immunoblot under conditions as described above (n = 3 for each condition).

DISCUSSION

Human arterial endothelium, monocyte/macrophages, and THP-1 monocytoid cells express high levels of cholesterol 27-hydroxylase (8, 10, 36). In the present studies, we demonstrate that IFN-γ, but not other cytokines such as IL-1 and TNF-α, down-regulates cholesterol 27-hydroxyase mRNA and protein. Similarly, IC that have fixed C1q interact with C1q binding proteins on THP-1 cells, HAEC, and, most likely, peripheral blood monocytes and monocyte-derived macrophages. To our knowledge, this is the first demonstration that expression of cholesterol 27-hydroxylase is regulated in extrahepatic tissues.

IFN-γ, a cytokine produced by certain subsets of T-cells (37), plays a critical role in the pathogenesis of atherosclerosis in murine models, contributes to the rapid onset of coronary atherosclerosis in transplanted hearts (38), and potentiates the development of atherosclerosis in apolipoprotein E knockout mice (3). The observations reported here are consistent with the role of IFN- γ in the pathogenesis of atherosclerosis and suggest a mechanism whereby IFN-γ can influence this process; IFN-γ down-regulates message and protein for a critical enzyme required for extrahepatic cholesterol metabolism.

Cell-associated C1q-binding proteins include those for collagen stalks (cC1qR) (39, 40) and globular heads (gC1qR) (23) and the 126-kD polypeptide present on macrophages and neutrophils that is a component of the monocyte C1qR that modulates phagocytosis (C1qRp, receptor for C1q, regulating phagocytosis) (22). Our data show that R139, an antibody directed against the 126-kD C1qRp molecule, blocks down-regulation of cholesterol 27-hydroxylase by IC-C1q in both HAEC and THP-1 cells in culture. Although the most obvious interpretation is that this is due to blockade by R139 of C1q binding to the 126-kD C1qRp protein with consequent disruption of downstream signaling events, other explanations that also fit the data must be considered. For example, the 126-kD C1qRp protein may function as a facilitator, increasing the attachment of IC-C1q complexes to the cell surface, where these complexes then bind to other cell surface signaling proteins. Alternatively, a dominant signal to block down-regulation of cholesterol 27-hydroxylase may be mediated by cross-linking of the 126-kD C1qRp protein (41), and this cross-linking may be inhibited by R139 binding. Finally, the 126-kD C1qRp protein may be part of a C1q receptor complex whose formation is prevented by R139. Although it is controversial whether gC1qR (23, 42, 43) or

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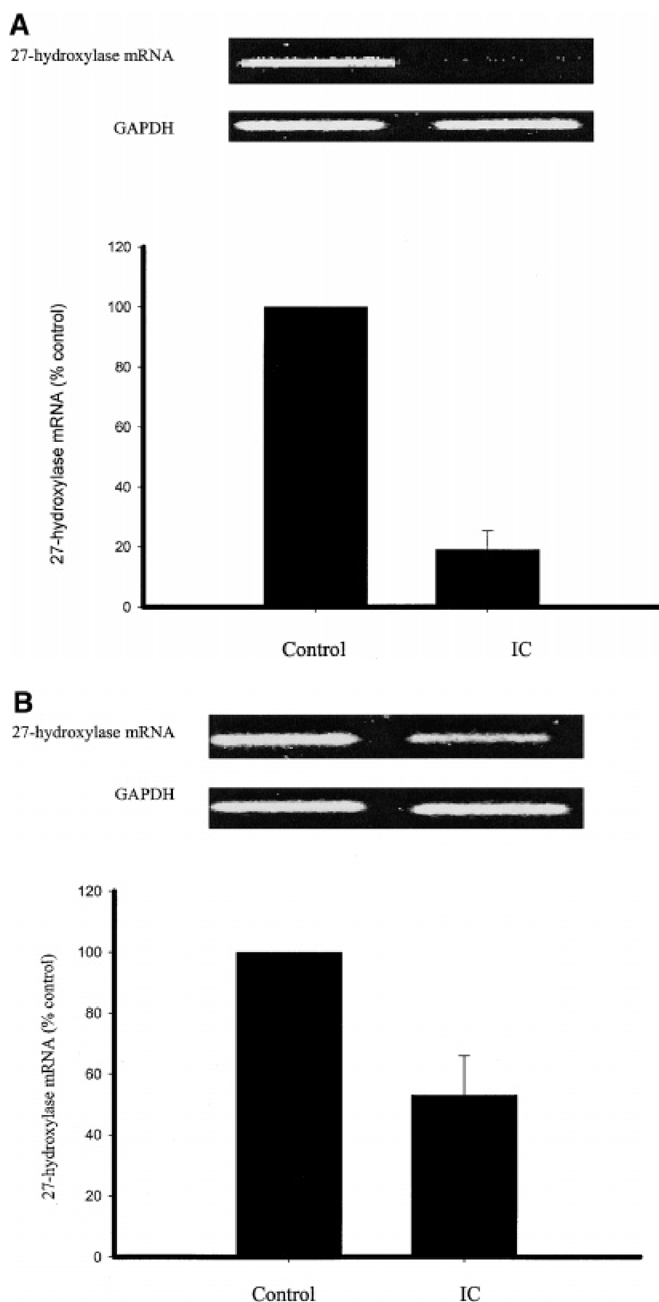

Fig. 8. IC-C1q down-regulates message for cholesterol 27-hydroxylase in human peripheral blood mononuclear cells (PBM) and monocyte-derived macrophages. A: Fresh whole blood (containing normal concentrations of C1q) was incubated for 3 h (37°C, 5% CO_2) alone or in the presence of IC (0.48 mg/ml). After separation of PBM from whole blood, PBM mRNA was isolated and then reverse transcribed, and the cDNA was amplified by PCR, as described. B: Human PBM isolated from a pooled human blood sample (kind gift of Dr. Joan Merrill) were treated with phorbol myristate acetate (250 ng/ml) for 3 days and then incubated for 3 h in the presence of 50%NHS with or without IC (0.48 mg/ml). RNA was isolated from the cells (Trizol) and then reverse transcribed, and the cDNA was amplified by PCR, as described.

cC1qR (33, 44) are expressed on the cell surface, our results provide no evidence that they play any role, direct or indirect, in regulation of cholesterol 27-hydroxylase expression.

Little is known about the regulation of cholesterol 27-hydroxylase. In the liver, 27-hydroxylase mRNA expression is negatively regulated in hepatocytes by insulin (45). The results of our experiments provide the first demonstration that human cholesterol 27-hydroxylase is regulated in extrahepatic tissues. In prior work by Babiker et al. (12), attempts to up-regulate 27-hydroxylase enzyme activity in macrophages by adding a variety of hormones were unsuccessful.

Extrahepatic cells such as HAEC and monocytes/macrophages can eliminate intracellular cholesterol in a lipoprotein-dependent manner and also by an alternative reverse cholesterol transport mechanism involving the cholesterol 27-hydroxylase enzyme (8, 10, 12). The alternative cholesterol removal pathway involves conversion of cholesterol to oxygenated products such as 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid by cholesterol 27-hydroxylase followed by excretion of these oxygenated products from the cell and transport to the liver for uptake and metabolism to bile acids. Up to 4% of total bile acid formation in humans occurs by a pathway commencing with extrahepatic 27-hydroxylation of cholesterol (11), and this extrahepatic pathway for metabolism of cholesterol contributes an important defense against the development of atherosclerosis.

The metabolic product of the cholesterol 27-hydroxylase is 27-hydroxycholesterol, an oxysterol compound that plays an important role in cholesterol homeostasis. 27-Hydroxycholesterol is a powerful inhibitor of the activity of the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase (15). Moreover, oxysterols, including 27-hydroxycholesterol, suppress transcription of the message for HMG-CoA reductase, HMG-CoA synthase, and the LDL receptor in vitro (16) and inhibit translation of HMG-CoA reductase mRNA (17). 27-Hydroxycholesterol suppresses receptor-mediated LDL uptake and degradation in cultured human endothelial cells and hepatoma cells at concentrations similar to those found in human serum (18). This suppression of LDL uptake may provide a defense against cholesterol overload in the periphery. Oxysterols have been shown to induce ATP binding cassette transporter 1 (ABCA1) protein mRNA (46). ABCA1 is a protein that was recently found to play a crucial role in facilitating the efflux of cellular cholesterol to extracellular apolipoprotein A-I or HDL. It mediates the loading with phospholipids and cholesterol of nascent apolipoprotein particles (47).

Oxysterols also inhibit vascular smooth muscle proliferation (17, 18). Our laboratory has demonstrated a dose-dependent inhibition by 27-hydroxycholesterol of rabbit aortic vascular smooth muscle cell proliferation (19). Proliferation of smooth muscle cells within the vessel wall is a prerequisite to vascular occlusion in atherosclerosis. Although many substances have been found that promote smooth muscle proliferation in culture, few are known to inhibit proliferation. Down-regulation of cholesterol biosynthetic enzymes, peripheral suppression of LDL uptake, and inhibition of smooth muscle proliferation are all anti-atherogenic effects, and diminished 27-hydroxylase enzyme levels in the arterial wall may contribute to the development of atherosclerosis.

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The importance of cholesterol 27-hydroxylase as a defense against atherosclerosis is underscored in patients lacking this enzyme. Patients with genetic deficiency of cholesterol 27-hydroxylase suffer from premature atherosclerotic cardiovascular disease despite the presence of normal serum lipids. Premature atherosclerotic cardiovascular disease is thought to occur in these individuals as a consequence of both the loss of the direct anti-atherogenic effects of 27-hydroxycholesterol and the loss of a pathway for cellular cholesterol efflux (20, 21).

We previously demonstrated that IC that have fixed complement (IC-C1q) stimulate vascular endothelial cells to express adhesion molecules including VCAM-1 via endothelial C1q receptors (4). Early atherosclerotic lesions begin as local infiltrates of monocyte-derived macrophages, T lymphocytes, and lipoproteins in the arterial wall (2, 29). Endothelial expression of VCAM-1 plays a role in the pathogenesis of atherosclerosis by recruiting monocytes to the arterial wall (48). Serum concentrations of circulating adhesion molecules, particularly VCAM-1, are elevated in patients with systemic lupus erythematosus (SLE), an inflammatory systemic disorder in which premature atherosclerosis is a common sequela (49). Circulating IC are also commonly present in the serum of patients with SLE and account for many of its acute inflammatory manifestations. Our observation that cholesterol 27-hydroxylase mRNA and protein are dramatically diminished in endothelial cells and macrophages exposed to IC-C1q lend further support to the hypothesis that IC-C1q contribute to the development of atherosclerosis and may influence critical metabolic events in the vessel wall. Circulating IC thus exert a pro-atherogenic effect by two mechanisms: 1) promoting macrophage recruitment to the vessel wall and 2) diminishing the capacity of endothelial cells and macrophages to perform reverse cholesterol transport by lowering intracellular cholesterol 27-hydroxylase levels.

IC and IFN-γ may play multiple roles in the pathogenesis of atherosclerosis (30, 50). We have previously demonstrated that IC that have fixed C1q stimulate endothelial cells to express VCAM-1, an adhesion molecule that participates in the recruitment of monocytes to the arterial wall (4). Oxidized LDL is immunogenic, and both serum and atherosclerotic lesions contain antibodies to oxidized LDL and lipoproteins (51, 52). Thus, IC are present in atherosclerotic lesions where they can not only regulate recruitment of monocytes from peripheral blood but also interfere with cholesterol homeostasis within the arterial wall. In addition to its other effects on the cells of the arterial wall, our data suggest that IFN-γ also interferes with cholesterol homeostasis, an effect which will further add to its atherogenic properties.

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