Interferon-γ Induces Downregulation of Tangier Disease Gene (ATP-Binding-Cassette Transporter 1) in Macrophage-Derived Foam Cells

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Abstract—Cholesterol efflux is a fundamental process that serves to mitigate cholesterol accumulation and macrophage foam cell formation. Recently, we reported that cholesterol efflux to high density lipoprotein subfraction 3 was reduced by interferon-γ (IFN-γ) and that this decrease was associated with an increase in acyl coenzyme A:cholesterol acyltransferase (ACAT) expression. In the present study, although treatment of murine peritoneal macrophages with IFN-γ resulted in a 2-fold decrease in HDL-mediated cholesterol efflux, efflux to lipid-free apolipoprotein A-I was reduced >4-fold and approached basal levels. This decrease was associated with a 3- to 4-fold reduction in ATP-binding-cassette transporter 1 (ABC1) mRNA content, the gene responsible for the defect in Tangier disease. Consistent with the reduction in cholesterol and phospholipid efflux in Tangier fibroblasts, downregulation of ABC1 expression by IFN-γ also resulted in reduced phosphatidylcholine and sphingomyelin efflux to apolipoprotein A-I. Whereas foam cells had a 3-fold increase in ABC1 mRNA, the decrease in ABC1 message levels by IFN-γ was observed in foam cells and control macrophages. This effect of IFN-γ was independent of general macrophage activation (inasmuch as similar changes were not detected with granulocyte-macrophage colony–stimulating factor) and was not observed with other ABC transporters (inasmuch as the expression of the transporter in antigen processing was upregulated 4-fold in these same cells). Therefore, by decreasing cholesterol efflux through pathways that include the upregulation of ACAT and the downregulation of ABC1, IFN-γ can shift the equilibrium between macrophages and foam cells and thus impact the progression of an atherosclerotic lesion. (Arterioscler Thromb Vasc Biol. 2000;20:1565-1571.)

Key Words: interferon-γ ■ cholesterol efflux ■ apolipoprotein A-I ■ ATP-binding-cassette transporter 1
expression of ABC1 by IFN-γ did not reflect a general effect on all ABC transporters because a related transporter associated with antigen presentation, transporter in antigen processing (TAP)-1, did, as expected, demonstrate an increase in message expression. The reduction of HDL 3 and apoA-I–mediated cholesterol efflux further supports a role for IFN-γ in promoting cholesteryl ester accumulation and foam cell formation within the arterial wall.

**Methods**

**Cell Culture**

Peritoneal macrophages were obtained from thioglycolate-elicited BALB/c mice by peritoneal lavage and maintained in culture in RPMI 1640 supplemented with 2% FCS (Hyclone Laboratories). Macrophages were converted to foam cells by incubation for 48 hours with 50 μg/mL acetylated LDL (Ac-LDL, Intracel Corp). Cells were washed with PBS and incubated for an additional 48 hours in RPMI 1640 with 1 mg/mL fatty acid–free BSA (Sigma Chemical Co) in the presence or absence of 100 U/mL IFN-γ (Biosource International) or 10 ng/mL granulocyte-macrophage colony–stimulating factor (GM-CSF, R&D Systems). This concentration of IFN-γ was determined to maximally inhibit cholesterol efflux. All animal studies were in compliance with institutional guidelines.

**Cholesterol Efflux**

Peritoneal macrophages were seeded in 24-well plates at 4×10⁵ cells per milliliter and converted to foam cells with Ac-LDL in the presence of [¹⁴C]cholesterol for 48 hours. Foam cells were washed with PBS and incubated in the absence or presence of 100 U/mL IFN-γ for 48 hours. A, Efflux to medium alone, BSA (100 μg/mL), HDL (100 μg/mL), and free apoA-I (50 μg/mL) was measured at 6 hours and expressed as percentage of total [¹⁴C]cholesterol. B, Cholesterol efflux was measured by increasing amounts of apoA-I as the acceptor species. Each value represents the mean±SD of triplicates. *P<0.01 for control vs IFN-γ–treated cells by 2-tailed unpaired Student t test.

**Phospholipid Efflux**

Foam cells were induced with IFN-γ and, during the last 18 hours, incubated with 0.4 μCi/mL of [¹⁴C]choline chloride to label the choline-containing phospholipids, phosphatidylcholine and sphingomyelin. Phospholipid efflux to either medium alone or 10 μg/mL apoA-I was measured at 6 hours. After incubation, cell monolayers were lysed with 0.2% SDS, and supernatants were clarified by centrifugation. Lipids from the cell lysate and the supernatant were first extracted according to the method of Bligh and Dyer and separated by thin-layer chromatography with the use of silica G plates developed in chloroform/methanol/ammonia (25% [wt/vol])/water (50:65:5:4 [vol/vol]). Phosphatidylcholine and sphingomyelin...
spots were visualized by I$_2$ vapors and identified by comigration with standards. Relative radioactivity was measured by Phosphoscreen and quantified by PhosphorImager (Molecular Dynamics Inc). Phospholipid efflux was expressed as percent counts in the supernatant versus total for each individual lipid.

**Northern Blot Analysis**

Total RNA followed by 1 round of poly(A)$^+$ RNA purification was performed by using RNA isolation kits (Qiagen). Poly(A)$^+$ RNA was resolved on 0.7% formaldehyde agarose gels and transferred to Nytran nylon membranes overnight by using the Turboblotter system (Schleicher and Schuell Inc). Membranes were prehybridized in hybridization buffer (200 mM NaCl, 50 mM Na$_2$HPO$_4$, 1 M EDTA, pH 8.0, 10% dextran sulfate, 1% SDS). Hybridization was performed relative to the hybridization intensity of the S29 signal. Membranes after hybridization were washed in 2x SSC and 0.1% SDS and exposed to Kodak Biomax-MS film or to PhosphorScreen. Quantification was performed with a PhosphorImager, and normalization was performed relative to the hybridization intensity of the S29 signal.

**Statistics**

Statistical analysis was performed by Student unpaired (2-tailed) t test. Values are reported as mean±SD. The 95% confidence limit ($P<0.05$) was taken as significant.

**Results**

The macrophage-activating factor INF-$\gamma$ has recently been demonstrated to inhibit cholesterol efflux in macrophage-derived foam cells when HDL$_3$ was used as the cholesterol acceptor species. These effects were consistent with an increase in ACAT activity and expression. However, to what extent the effects of INF-$\gamma$ on cholesterol efflux involved other processes associated with the intracellular regulation of cholesterol levels was unclear. In the present study, it was observed that the reduction of cholesterol efflux by INF-$\gamma$ was more significant when apoA-I rather than HDL$_3$, was used as the acceptor species [Figure 1A]. Although INF-$\gamma$ treatment resulted in an ~2-fold reduction in efflux when HDL$_3$ or total HDL was used as the acceptor particle, the decrease in efflux was 3- to 4-fold with apoA-I as the acceptor species. The residual efflux observed in INF-$\gamma$-stimulated foam cells was similar to that detected when the efflux was measured in the absence of cholesterol acceptor species with the use of BSA-supplemented media (Figure 1A). In control cells, the apoA-I–mediated cholesterol efflux was concentration dependent and reached maximum levels at 10 $\mu$g/mL (Figure 1B). However, cholesterol efflux in INF-$\gamma$–stimulated cells remained at basal levels even at apoA-I concentrations up to 40 $\mu$g/mL. This inhibition was specific to INF-$\gamma$ and was not related to differences in loading, because control and treated cells had equal amounts of radioactivity (data not shown). The more pronounced reduction in cholesterol efflux mediated by INF-$\gamma$ when apoA-I was used as the acceptor species suggested that regulation of cholesterol efflux by INF-$\gamma$ may occur by apoA-I–dependent pathways.

APO-A-I–mediated cholesterol efflux is defective in cells from patients with TD. The gene responsible for this phenotype was recently identified as the ABC1 transporter, and its expression is well correlated with conditions that affect apolipoprotein-mediated cholesterol efflux.

Therefore, Northern blot analysis was performed in control and macrophage-derived foam cells to determine the effect of INF-$\gamma$ stimulation on ABC1 expression. As demonstrated [Figure 2], cholesterol loading in macrophages resulted in a >3-fold induction in ABC1 expression. However, this cholesterol-dependent increase in ABC1 was completely inhibited by INF-$\gamma$-treatment, and even in nonfoam cells, INF-$\gamma$ significantly reduced ABC1 message expression. The magnitude in the reduction of ABC1 expression, between 3- and 4-fold, was consistent with the extent of cholesterol efflux inhibition when apoA-I was used as the acceptor species.

To determine whether the downregulation of ABC1 was specific to INF-$\gamma$ and not a general response associated with macrophage activation, Northern blot analysis was performed on control cells or macrophages stimulated with INF-$\gamma$ or GM-CSF. As demonstrated [Figure 3], a 4-fold reduction in ABC1 mRNA levels was detected on INF-$\gamma$ treatment, whereas similar effects were not observed with GM-CSF. To confirm that the effect of INF-$\gamma$ was specific to ABC1 and did not reflect a general decrease in all ABC transporters, the effect of INF-$\gamma$ on TAP-1, a transporter that also belongs to the ABC family, was evaluated. As observed [Figure 3B], the expression of TAP-1 was induced >4-fold, whereas again, no changes were observed with GM-CSF treatment. Finally,
consistent with a previous report from this laboratory, a 75% increase in ACAT1 expression was detected with IFN-γ, whereas GM-CSF effects did not achieve statistical significance. These results suggest that the inhibition of ABC1 expression is specific to IFN-γ and that the upregulation of ACAT and the downregulation of ABC1 contribute to the reduction of cholesterol efflux by IFN-γ.

Although the downregulation of ABC1 was associated with a significant reduction in cholesterol efflux, its impact on apoA-I to acquire phospholipid from the plasma membrane was unknown. The defect in lipidation of apoA-I has been suggested to be a major cause of the impaired ability of apoA-I to stimulate cholesterol efflux from Tangier cells. To confirm that this process was also downregulated by IFN-γ, foam cells were incubated with [14C]choline chloride to label the choline-containing phospholipids, phosphatidylcholine and sphingomyelin. As demonstrated (Figure 4A), >80% of the label was incorporated into phosphatidylcholine compared with sphingomyelin. IFN-γ inhibited the synthesis of new phosphatidylcholine and sphingomyelin because less [14C]choline incorporation was detected with IFN-γ, an observation also reported in Tangier fibroblasts. Therefore, phospholipid efflux was expressed as the percent radioactivity recovered in the medium versus cells and medium combined. As shown (Figure 4B), IFN-γ resulted in a >4-fold reduction of apoA-I–mediated phosphatidylcholine and sphingomyelin efflux. Therefore, these data suggest that the downregulation of ABC1 expression by IFN-γ inhibits the ability of apoA-I to extract phospholipids and cholesterol from macrophage-derived foam cells.

Discussion

IFN-γ has been demonstrated to be present within human atherosclerotic lesions after the infiltration of CD4+ Th1 cells and may contribute to the pathology associated with such lesions. The 2 major cell types that accumulate lipid and convert to foam cells within the atherosclerotic plaque, macrophages and smooth muscle cells (SMCs), express IFN-γ receptors. However, the role of IFN-γ in atherogenesis remains controversial because IFN-γ receptor knockout mice crossed with apoE knockout mice showed reduced lesions, whereas immune-deficient RAG-2 knockout mice crossed with apoE knockout mice did not exhibit a comparable effect. IFN-γ treatment in vitro has been reported to decrease the expression of type A scavenger receptors, LDL receptor–related protein, and lipoprotein lipase in macrophages, suggesting antiatherogenic properties, and yet to stimulate the expression of vascular cell adhesion molecule-1 on endothelial cells, class II major histocompatibility antigens in macrophages and SMCs, and type A scavenger receptors on SMCs, effects consistent with proatherogenic changes.

Previous studies from this laboratory have reported that treatment of murine macrophage-derived foam cells with IFN-γ resulted in a reduction in HDL3-mediated cholesterol efflux, an increase in ACAT message and activity, and an
increase in cholesteryl ester accumulation, with the latter observation also reported by Whitman et al.36 In the present study, macrophage activation by IFN-γ resulted in a significant decrease in ABC1 mRNA levels. Consistent with this downregulation, apoA-I–mediated efflux of cholesteryl- and choline-containing phospholipids was significantly reduced. Furthermore, the downregulation of ABC1 was specific for IFN-γ and was not a general property of ABC transporters or activated macrophages.

Cholesterol efflux can be mediated by 2 mechanisms, passive diffusion or an active apolipoprotein-dependent process.5–9 This latter process is defective in TD, in which the macrophages are characterized by significant intracellular accumulation of lipids and cholesteryl esters.10,11 The high degree of ABC1 expression in macrophages is consistent with their ability to efflux cholesterol through an apolipoprotein-mediated pathway compared with other cells, such as SMCs.37–40 However, the exact role that ABC1 plays in this process remains unclear. In a proposed model, ABC1 is a cholesteryl and/or phospholipid transporter, similar to MDR2, an ABC transporter that is implicated in the secretion of phospholipid and cholesterol into bile.41–43 In this model, apoA-I interacts with ABC1 on the cell surface, followed by endocytosis and lipiddation of apoA-I with cholesterol and phospholipid to form nascent HDL particles, which are then secreted. These particles can then interact with HDL receptors, such as SR-B1 and CD36, and further stimulate cholesterol efflux. Consistent with this model, it has been demonstrated that ABC1 is expressed on the plasma membrane and that cAMP elevation, which increases ABC1 expression, also resulted in the formation of a complex between apoA-I and a cell surface protein with a molecular weight similar to ABC1. Furthermore, a cAMP-inducible receptor has been reported to promote apoA-I–mediated cholesterol efflux after receptor-mediated endocytosis and by recrification of nascent lipoprotein particles.18,44,45 This model would predict a reduction in phospholipid and cholesterol efflux by stimuli that decrease ABC1 expression or by loss of function mutations. The present study supports ABC1 involvement in the lipiddation of apoA-I, because a 4-fold reduction in phosphatidylincholine and sphingomyelin efflux was observed in IFN-γ–stimulated macrophages.

In the present study, treatment of macrophage-derived foam cells with IFN-γ resulted in a 2-fold reduction in HDL-mediated cholesterol efflux, whereas the apoA-I–mediated efflux was almost completely inhibited. Cholesterol efflux to HDL was almost double that of apoA-I, presumably because HDL is already lipidated and can accept cholesterol by passive diffusion as well as through the ABC1-mediated process. The mechanism that underlies the downregulation of apoA-I–mediated cholesterol efflux by IFN-γ appears to be due primarily to the significant reduction in ABC1 message. In contrast, the mechanism by which IFN-γ reduces cholesterol efflux to HDL appears to be multifactorial. Previous studies from this laboratory have demonstrated that IFN-γ decreases HDL binding and is associated with the downregulation of CD36 (S.H. Zuckerman and C.G. Panousis, unpublished data, 2000). In addition, by increasing ACAT activity and expression, IFN-γ stimulates cholesterol esterification, thereby reducing the free cholesterol pool available for efflux.19

ABC1 is upregulated by sterols and during macrophage differentiation. Increases in intracellular cholesterol induce ABC1 message levels, and this effect can be reversed by depletion of the intracellular cholesterol pool through stimulation of cholesterol efflux by HDL.17 Similarly, we observed a >3-fold induction of ABC1 message on the incubation of macrophages with Ac-LDL. The mechanism by which ABC1 regulation is linked to the functional status of the macrophage remains unclear. Although the pathway by which IFN-γ decreases ABC1 expression is uncertain, its possible linkage to the Janus kinase/signal transducer(s) and activator(s) of transcription (Stat) pathway requires further consideration. IFN-γ preferentially activates Stat1, and yet Stat1 activity is inhibited by cAMP, an agent that has been reported to induce ABC1 expression.46 Future studies will focus on this pathway and its relevance to IFN-γ–mediated...
regulation of ABC1 expression. Interestingly and in accordance with the possible role of Stat1 in foam cell formation, Grewal et al. reported a case of 2 siblings without hyperlipidemia but with increased macrophage foam cell formation and clinical evidence of extensive xanthomatosis. These patients had increased expression of the IFN-γ-induced genes Stat1α and inducible protein-10 compared with their nonaffected siblings, further supporting the importance of IFN-γ and Stat1 in cholesterol trafficking.

The present study, in agreement with previous work, demonstrates that IFN-γ inhibits cholesterol efflux in macrophages, a process that is important for the prevention of lipid accumulation and conversion to foam cells. The inhibition appears to affect passive and active cholesterol efflux and is mediated through the downregulation of ABC1 as well as the statin/sterol pool. Therefore, IFN-γ has the potential to induce foam cell formation within the atherosclerotic plaque, consistent with its proatherogenic properties. Elucidation and inhibition of the mechanism by which IFN-γ exerts its effects, and specifically the pathway that downregulates ABC1, could lead to development of therapeutic agents that impact vascular lesion progression without necessarily lowering serum cholesterol levels.

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