The evolution of atherosclerotic lesions involves an interaction among immune cells. Since mice with a targeted disruption of the apoE gene (apoE 0 mice) develop profound atherosclerosis, we examined the role of IFN-γ in this process. First, the presence of CD4+ and CD8+ cells, which secrete lesional IFN-γ, was documented in apoE 0 atheromata. Then, the apoE 0 mice were crossed with IFN-γ receptor (IFNγR) 0 mice to generate apoE 0/IFNγR 0 mice. Compared to the apoE 0 mice, the compound knock-out mice exhibited a substantial reduction in atherosclerotic lesion size, a 60% reduction in lesion lipid accumulation, a decrease in lesion cellularity, but a marked increase in lesion collagen content. Evaluation of the plasma lipoproteins showed that the compound knock-out mice had a marked increase in potentially atheroprotective phospholipid/apoA-IV rich particles as well. This correlated with an induction of hepatic apoA-IV transcripts. These observations suggest that IFN-γ promotes and modifies atherosclerosis through both local effects in the arterial wall as well as a systemic effect on plasma lipoproteins. Therefore, therapeutic inhibition of IFN-γ signaling may lead to the formation of more lipid-poor and stable atheromata. (J. Clin. Invest. 1997; 99:2752–2761)  

Key words: IFN-γ • apolipoprotein-deficient mice • T cells • collagen

Introduction

The early colocalization of T cells and the potent immunostimulatory cytokine IFN-γ to atherosclerotic lesions suggests that the immune system contributes to atherogenesis. Since mice with a targeted disruption of the apoE gene (apoE 0 mice) develop profound atherosclerosis, we examined the role of IFN-γ in this process. First, the presence of CD4+ and CD8+ cells, which secrete lesional IFN-γ, was documented in apoE 0 atheromata. Then, the apoE 0 mice were crossed with IFN-γ receptor (IFNγR) 0 mice to generate apoE 0/IFNγR 0 mice. Compared to the apoE 0 mice, the compound knock-out mice exhibited a substantial reduction in atherosclerotic lesion size, a 60% reduction in lesion lipid accumulation, a decrease in lesion cellularity, but a marked increase in lesion collagen content. Evaluation of the plasma lipoproteins showed that the compound knock-out mice had a marked increase in potentially atheroprotective phospholipid/apoA-IV rich particles as well. This correlated with an induction of hepatic apoA-IV transcripts. These observations suggest that IFN-γ promotes and modifies atherosclerosis through both local effects in the arterial wall as well as a systemic effect on plasma lipoproteins. Therefore, therapeutic inhibition of IFN-γ signaling may lead to the formation of more lipid-poor and stable atheromata.

1. Abbreviations used in this paper: ECs, endothelial cells; IFNγR, IFNγ receptor; SMCs, smooth muscle cells.

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Methods

Mice. The IFNγR 0 mice, in a 129 background, were a generous gift from M. Aguet (Swiss Institute of Experimental Cancer Research,
Figure 1. Localization of CD4+ and CD8+ T cells to apoE−/− atherosclerotic lesions. Sections of proximal aorta from 17-wk-old apoE−/− mice, fed the Western type diet, were immunostained with CD4 (A and B) or CD8 (D and E) monoclonal antibodies. The sections were then counter-
Lausanne, Switzerland) (6). The apoE 0 mice, in a mixed 129/C57Bl/6J background, were a generous gift from J.L. Breslow (Rockefeller University, New York) (22). The apoE 0 and apoE 0/IFNγR 0 mice used in these studies were all derived from the offspring of a single cross between the original set of IFNγR 0 and apoE 0 mice. For many of the studies, the mice studied arose from one additional intercross between the apoE 0 and apoE 0/IFNγR 0 mice, in an effort to homogenize genetic backgrounds. All genotyping was initially carried out by a PCR-based assay and then confirmed with Southern blotting as described previously (6, 22). Northern blotting was done as described previously (25).

Once sufficient numbers of single and double knock-out mice were available, 5–10 age-matched (5 wk old) females were placed on a Western type diet (21% fat, 0.15% cholesterol; Harlan/Teklad, Madison, WI) for 3 mo. After this period the mice were killed and both plasma samples and hearts/proximal aorta preparations were collected.

**Histochemistry.** Hearts were perfused with phosphate-buffered saline, embedded in O.C.T. compound (Tissue-Tek; Miles Laboratories, Elkhart, IN) and snap-frozen. 10-μm-thick transverse sections were collected from the proximal aorta, fixed in cold acetone, and stained with CD4 or CD8 monoclonal antibodies, as recommended by the manufacturer (GIBCO BRL, Gaithersburg, MD). Bound antibodies were detected with a biotin coupled alkaline phosphatase kit (Vectastain ABC-AP; Vector Laboratories Inc., Burlingame, CA) after treating the specimens with levamisol to reduce background.

Specimens were then visualized with a Nikon Optiphot microscope with ×4, ×10, and ×40 objectives.

For quantitation, 10-μm-thick transverse sections of atherosclerotic lesions (covering a length of 350–400 μm) from the proximal aorta were stained with oil red-O, hematoxylin and eosin, or trichrome after fixation in 4% formaldehyde. For the samples stained with oil red-O, every eighth section, for a total of eight sections, were quantitated for accumulation of intimal lipid by video microscopy as described previously (22, 26, 27), and a cumulative value was determined for each mouse.

**Lipid studies.** Plasma samples, collected at the time of killing, were pooled as indicated and evaluated for phospholipid, cholesteryl ester, and free cholesterol by a 4-aminoantipyrine–based enzymatic assay (Wako Bioproducts, Richmond, VA). Samples were also fractionated by FPLC (Superose 6; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) or by sequential buoyant ultracentrifugation and SDS/PAGE gel as described previously (22, 28).

**Results**

**CD4+ and CD8+ cells colocalize to atherosclerotic lesions in apoE 0 mice.** Since studies on the role of lesional IFN-γ on atherosclerosis are predicated on the presence of T cells within lesions, the apoE 0 lesions were evaluated for the presence of CD4+ and CD8+ cells. Frozen sections were prepared from the
bands of the IFNγR knock-out allele and the 2.9-kb band of the apoE knock-out allele (Fig. 2, M1–M5). The final population of apoE 0 mice was only homozygous for the 2.9-kb band of the apoE knock-out allele (data not shown).

**Atherosclerosis in apoE/IFNγR compound knockout mice.**

To evaluate atherosclerotic lesion development, a matched set of apoE 0 and apoE 0/IFNγR 0 mice was placed on a Western type diet (22). After 3 mo, the mice were killed and the atherosclerotic lesions in the proximal aorta were evaluated. Standard histology indicated that the lesions in the apoE 0/IFNγR 0 mice were substantially smaller than those in the apoE 0 mice (see Fig. 4). As other murine studies had validated assessment of subintimal lipid accumulation as an effective method for the quantitation of murine atherosclerotic lesions (22, 26, 27), such an analysis was undertaken with these mice. Evaluation of 6 apoE 0 and 10 apoE 0/IFNγR 0 mice, matched for age and sex, determined a significant 59% (P < 0.0001) reduction in lesionary lipid content in the compound knock-out mice (Fig. 3). Consistent with these observations, evaluation of a temporally distinct set of age- and sex-matched apoE 0 (n = 5) and apoE 0/IFNγR 0 (n = 6) mice demonstrated a 58% reduction (P < 0.002) in subintimal lipid accumulation (data not shown). Not only did these observations support our initial impression that lesions in apoE 0/IFNγR 0 mice are smaller than those in apoE 0 mice, but the fact that our P values were low in the setting of a relatively large sample size indicates that individual genetic variation had little effect on these results.

To determine whether there were any qualitative differences in lesions in addition to those of lipid content, a careful histological analysis was carried out. Hematoxylin and eosin sections, prepared from three mice from the control (apoE 0) and study groups (apoE 0/IFNγR 0), were evaluated blindly. In each case, lesions from the apoE 0 mice were larger and more cellular (Fig. 4). Consistent with what has been reported previously, the structural features observed in these sections, as well as those determined with specialized stains (e.g., oil red-O staining, Fig. 3; CD4 and CD8 immunohistochemistry, Fig. 1; and a trichrome stain, Fig. 5), suggested that foam cells, SMCs, and lymphocytes were present in the apoE 0 lesions (22, 24, 29, 30). In contrast, the apoE 0/IFNγR 0 lesions were strikingly less cellular (Fig. 4), but still contained many of the same cell types, including CD4 and CD8 positive cells (data not shown). To evaluate the possibility that the decreased cellularity of the compound knock-out lesions might reflect an increase in extracellular matrix, additional sections were evaluated with a trichrome stain. These sections demonstrated a marked increase in blue staining collagen, spanning the entire thickness of the lesion. This pattern was distinct from the apoE

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**Table I. Plasma Lipid Concentration (Milligrams per Deciliter)**

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Phospholipid</th>
<th>Cholesteryl ester</th>
<th>Free cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE 0</td>
<td>227±31</td>
<td>66±5</td>
<td></td>
<td>11±0.4</td>
</tr>
<tr>
<td>IFNγR 0</td>
<td>216±22</td>
<td>57±11</td>
<td></td>
<td>10±1.7</td>
</tr>
<tr>
<td>apoE 0</td>
<td>320±20</td>
<td>448±66</td>
<td></td>
<td>281±42</td>
</tr>
<tr>
<td>apoE 0/IFNγR 0</td>
<td>487±62*</td>
<td>501±52</td>
<td></td>
<td>342±39</td>
</tr>
</tbody>
</table>

Pooled plasma from apoE 0 and apoE 0/IFNγR 0 mice (Fig. 2) was evaluated for total phospholipid, cholesteryl ester, and free cholesterol ester content. *P < 0.001 when compared to apoE 0; ‡P < 0.01 when compared to apoE 0.
0 mice, where the majority of the collagen staining was evident at the adluminal surface of the lesion. These studies indicate that a loss in the ability to signal through IFN-γ leads to substantial changes in lesion size and structure.

Figure 4. Hematoxylin and eosin stain of atherosclerotic lesions from apoE 0 and apoE 0/IFNγR 0 mice. Sections prepared from apoE 0 (A and EC) and apoE 0/IFNγR 0 (B and D) mice, as described in Fig. 3, were stained with hematoxylin and eosin and examined with a Nikon Optiphot microscope at ×100 (A and B) and ×400 (C and D). The areas magnified at ×400 are indicated by white boxes in A and B. EC and vessel wall (VW) are indicated.

Serum lipoprotein profiles in IFNγR/ApoE compound knock-out mice. Next, the plasma lipoprotein levels in apoE 0 and apoE 0/IFNγR 0 mice were examined. First, pooled plasma samples were fractionated by FPLC. The apoE 0 mice...
demonstrated the anticipated large VLDL peak eluting in the void volume (Fig. 6A) (22, 24). Intriguingly, the FPLC profiles of the apoE 0/IFNγR 0 mice exhibited an increase in cholesterol rich lipoproteins between fractions 15 and 22 (i.e., a shoulder to the right of the VLDL peak; Fig. 6A, arrow). Furthermore, there was a marked increase in phospholipids eluting in the VLDL region and extending into the LDL region (Fig. 6B). These observations were confirmed by an evaluation of the second set of matched apoE 0 and apoE 0/IFNγR 0 mice (data not shown). Control studies on plasma prepared from IFNγR 0 and 129 mice revealed only small LDL peaks, typical of wild-type mice (data not shown). Phospholipid and cholesterol levels of unfractionated plasma were also consistent with these studies, demonstrating an increase in the relative levels of phospholipid and free cholesterol in the apoE 0/IFNγR 0 mice (Table I). Plasma triglycerides were similar in both groups. Thus, the loss in IFN-γ signaling led to an increase in free cholesterol and phospholipid rich lipoprotein particles in the apoE 0 background.

To evaluate changes in plasma lipoproteins in more detail, pooled plasma samples from both sets of apoE 0 and apoE 0/IFNγR 0 mice were fractionated sequentially by buoyant density ultracentrifugation and analyzed by SDS/PAGE in several independent experiments (28). Results from three studies with chow-fed mice determined a 9.5-fold increase ($P < 0.00001$) in apoA-IV levels in the VLDL and/or LDL plasma fractions.
from compound knock-out mice in comparison to apoE 0 mice. Similarly, three studies on mice fed a Western type diet identified a comparative 7.6-fold increase ($P < 0.001$) in compound knock-out plasma apoA-IV levels. The results of one of these studies, where chow and Western type diets are compared directly, are shown in Fig. 7. In this experiment there was also a small decrease in apoA-I in the HDL fractions and a corresponding increase in the LDL fractions, which is consistent with a redistribution of HDL particles to lower density secondary to the increased phospholipid content (31). In contrast to the results from the apoE 0 and apoE 0/IFN$\gamma$R 0 mice, evaluation of the control 129 mice did not reveal any increase in the apoA-IV in the VLDL and LDL plasma fractions.

The large rise in apoA-IV levels observed in the compound knock-out mice suggested that IFN$\gamma$ regulates apoA-IV levels. To investigate the potential mechanism behind this regulation, apoA-IV gene expression patterns were examined in several candidate tissues. As hepatic expression was most robust, the comparison between expression in apoE 0, apoE 0/IFN$\gamma$R 0 mice, and the parental strains (i.e., 129 and C57Bl/6J) was undertaken in this tissue. Although we did observe a good level of expression in the 129 mice (Fig. 8), the difference with respect to C57Bl/6J was more modest than had been reported previously (32). In contrast, the apoE 0/IFN$\gamma$R 0 mice exhibited a more marked (i.e., greater than or equal to threefold, $P < 0.001$) increase in apoA-IV expression (Fig. 8). This increase was even more striking when one considers that apoA-IV expression in 129-C57Bl/6J interbred mice has been reported to be less than that of 129 mice (32). This provides compelling evidence that in the setting of hypercholesterolemia IFN$\gamma$ exerts an important level of regulation on apoA-IV expression. These studies suggest that the ability of IFN$\gamma$ to downregulate the expression of an atheroprotective (33, 34) apolipoprotein apoA-IV contributes to atherogenesis in apoE 0 mice.

**Discussion**

The early colocalization of T cells and macrophages to atherosclerotic lesions supports a role for the immune system in the
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Although the nature of the involved immune response is not known, immunohistochemical studies have identified several potentially important components. Both major classes of T cells (i.e., CD4+ and CD8+), and the potent immunomodulatory cytokine they secrete, IFN-γ, have been colocalized to lesions (2, 3). Moreover, IFN-γ has been shown to have important effects on the cellular components of atherosclerotic lesions. In addition to being a potent coactivator of macrophages (17), IFN-γ has been shown to downregulate the expression of the scavenger receptor A, the low density lipoprotein related receptor, and lipoprotein lipase (LPL) in some cell types (12, 13, 37, 38), begetting the question of whether this potent cytokine is a protagonist or antagonist in the pathogenesis of atherosclerotic lesions. The observation that IFN-γ blocks the ability of SMC to proliferate and synthesize collagen (14, 15), both hallmarks of atherogenesis (1, 14), has further fueled this controversy. These latter observations have led to a proposal that IFN-γ does not play an important role in plaque growth, but rather contributes to plaque rupture by promoting a thinning and destabilization of the fibrous cap at the plaque’s shoulders (14, 36). Consistent with this model, T cells appear to localize to the shoulder region, and IFN-γ has recently been shown to stimulate the secretion of cathepsin-S, a neutral cysteine protease, from macrophages (14).

In an effort to clarify the role of IFN-γ in atherosclerosis, we have examined the development of lesions in IFNγR0 mice crossed with the hypercholesterolemia and atherosclerosis-prone apoE0 mice. When compared to apoE0 mice, lesions in the apoE0/IFNγR0 mice were significantly smaller, with a 60% decrease in lipid content. These observations support a model where IFN-γ potentiates an immune response, which in turn promotes atherogenesis. Likewise, detailed studies on how T cells and macrophages interact to mediate an immune response in other systems have delimited a pivotal role of IFN-γ (4–6). These studies indicate that an immune response is initiated by a pathogen-mediated macrophage stress (5, 17), which may be functionally analogous to an initiating stress in atherosclerosis (e.g., oxidant stress in the setting of elevated lipids, or LDL aggregation). This initiating stress may also be the stimulus that draws both T cells and macrophages into early lesions. Subsequently, T cells (i.e., the Th1 subset) are stimulated to secrete IFN-γ (5, 17), and potentially other growth factors (39, 40), in an antigen-dependent process. Although the nature of the stimulating antigen has not been determined for atherosclerosis, recent studies have implicated oxidized LDL, providing another potential link to an established risk factor of atherogenesis (41). The secreted IFN-γ is likely to have potent activity on SMCs (14), as well as macrophages, where it acts to prime these cells to destroy offending agents, in part through the generation of reactive oxygen species (5, 17, 42). A secondary stimulus, required by mac-

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**Figure 8.** Pattern of hepatic apoA-IV expression varies with the strain of different strains of mice. (A) 20 μg of total hepatic RNA, from 3–6-mo old chow fed mice, was evaluated after fractionation in a formaldehyde gel by hybridization with a 427-bp Afl III/Pst I fragment of the murine apoA-IV cDNA (53), or a 245-bp KpnI/XbaI murine β-actin probe (Ambion Inc., Austin, TX) as described previously (25). Each strain including 129, C57Bl/6J, apoE0 (mixed 129/C57Bl/6J), apoE0/IFNγR0 (mixed 129/C57Bl/6J) was evaluated in duplicate as shown. (B) Hybridization intensity of apoA-IV bands from four samples (two from A and two additional samples) of knock-out mice (i.e., apoE0, IFNγR0, and apoE0/IFNγR0) was determined (by Scanner; Molecular Devices, Sunnyvale, CA), compiled, and represented graphically.
rophages to attain their full inflammatory potential (5, 17), is likely to be provided by other factors that have been localized to atherosclerotic lesions as well (e.g., IL-1, TNF, or oxidative stress) (1, 14, 43–45). Consistent with this model, recent studies with macrophage-deficient apoE0 mice demonstrate that macrophages play an important role in atherogenesis (46). Moreover, our studies on murine diet induced atherosclerosis, and other studies on murine transplant atherosclerosis, have directly implicated IFN-γ in the pathogenesis of atherosclerotic lesions (47, 48). Furthermore, we find that lesions in the apoE0/IFNγR0 mice are not only smaller, but significantly less cellular, with a concomitant increase in extracellular collagen. Although our studies do not discriminate between a potential effect of IFN-γ on SMC collagen synthesis (i.e., down-regulation) (14) or collagenase activity (14), they strongly implicate IFN-γ in the regulation of the level of lesional collagen. Intriguingly, extracellular fibrillar collagen has been shown recently to block SMC proliferation, potentially accounting for some of this decrease in cellularity (49), and highlighting the potential importance of IFN-γ’s regulation of lesional collagen content. As decreases in lesional collagen content correlate with plaque instability (14, 36), IFN-γ antagonists may serve to stabilize plaques.

In addition to the intracellular effects, IFN-γ was found to affect lipoprotein metabolism as well. Plasma lipoproteins from apoE0/IFNγR0 mice exhibited a significant increase in a distinct population of lipoprotein particles that are rich in apoA-IV, phospholipid, and free cholesterol. As both an increase in expression of transgenic apoA-IV (33, 34) and phospholipid infusions (50, 51) have been shown to be antiatherogenic, it is very likely that the apoA-IV/phospholipid particles observed in the compound knock-out mice contributed to the observed reduction in lesion size, perhaps by decreasing subintimal lipid accumulation. Notably, expression of human apoA-IV transgenes in mice does not lead to an increase in plasma phospholipid levels, suggesting that the increase in phospholipids observed in the compound knock-out mice may be an independent property of that background (Jiang, X., and A. Tall, unpublished observation). However, the threefold increase in hepatic apoA-IV expression in compound knock-out mice directly implicates the loss in IFN-γ signaling to the increase in plasma apoA-IV levels. Consistent with the emerging concept that the liver may be a physiologically important target tissue of cytokines released into the portal circulation (52), these studies provide evidence that the liver is an important target of IFN-γ. Moreover, IFN-γ, or its receptor, may be one of the previously alluded to trans-acting factors that contribute to species-specific patterns of apoA-IV expression (32). In summary, our studies demonstrate that IFN-γ has important proatherogenic effects that are mediated by both intraleisonal and extraleisonal targets. The reduced size and lipid content of apoE0/IFNγR0 atheromata, together with a striking increase in lesional collagen, suggests that inhibition of IFN-γ activity could be a therapeutic strategy to stabilize human atherosclerotic plaques.

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References


