Role of Interferon-γ in Hypercholesterolemia-Induced Leukocyte–Endothelial Cell Adhesion

Karen Y. Stokes, BAMod; E. Chris Clanton; Kris P. Clements, BA; D. Neil Granger, PhD

Background—A T-cell–mediated inflammatory response occurs in the microcirculation during acute hypercholesterolemia. The objective of this study was to define the contribution of T-lymphocyte–derived interferon-γ (IFN-γ) to the leukocyte–endothelial cell adhesion induced by hypercholesterolemia.

Methods and Results—Intravital videomicroscopy was used to quantify the adhesion and emigration of leukocytes and oxidant stress (dihydrorhodamine [DHR] oxidation) in cremasteric venules. Wild-type (WT), IFN-γ−/−, and severe combined immunodeficiency (SCID) mice were placed on either a normal (ND) or high-cholesterol (HC) diet for 2 weeks. WT-HC mice exhibited exaggerated adhesion and emigration of leukocytes and enhanced DHR oxidation compared with WT-ND. The exaggerated adhesion responses and increased DHR oxidation were not seen in IFN-γ−/−–HC mice. SCID-HC mice also exhibited attenuated inflammatory responses compared with WT-HC. Reconstitution of either SCID-HC or IFN-γ−/−–HC mice with WT-HC splenocytes restored the inflammatory responses, whereas reconstitution of SCID-HC with IFN-γ−/−–HC splenocytes did not. The HC-induced oxidant stress was restored in IFN-γ−/−–HC mice reconstituted with WT-HC splenocytes.

Conclusions—These findings implicate IFN-γ as a cause of the inflammatory phenotype that is assumed by the microvasculature of hypercholesterolemic mice and suggest that T lymphocytes are a major source of this proinflammatory cytokine. (Circulation. 2003;107:2140-2145.)

Key Words: leukocytes • endothelium • hypercholesterolemia • lymphocytes

Hypercholesterolemia is a major risk factor for the development of cardiovascular diseases, including atherosclerosis. It is widely recognized that inflammatory cell recruitment and activation are important events in the pathological changes in large and microscopic blood vessels that are induced by hypercholesterolemia. Studies on large and small arteries have revealed contributions of different mediators and blood cell populations to the large and small arteries have revealed contributions of different mediators and blood cell populations to the pathological changes in large and microscopic blood vessels that are induced by hypercholesterolemia. The objective of this study was to define the contribution of T-lymphocyte–derived interferon-γ (IFN-γ) to the leukocyte–endothelial cell adhesion induced by hypercholesterolemia.

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These findings implicate IFN-γ as a cause of the inflammatory phenotype that is assumed by the microvasculature of hypercholesterolemic mice and suggest that T lymphocytes are a major source of this proinflammatory cytokine. (Circulation. 2003;107:2140-2145.)

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Methods

Animals
Wild-type C57BL/6J mice (WT), B6.CB17-Prkdcscid/SzJ mice (SCID), and B6.129S7-Ifngtm1Ts mice (IFN-γ−/−) were obtained from Jackson Laboratories, Bar Harbor, Me. Mice (5 to 6 weeks old) were placed on either a normal (ND) or high-cholesterol (HC) diet (Teklad 90221 containing 1.25% cholesterol, 0.125% choline chloride, 15.8% fat; Harlan Teklad) for 2 weeks (n = 5 to 6/group). IFN-γ−/−—HC mice were divided into 2 groups, IFN-γ−/−—HC and IFN-γ−/−—HC reconstituted with splenocytes from WT-HC mice (WT-HC→IFN-γ−/−—HC). SCID-HC mice were allocated into 3 groups, SCID-HC, SCID-HC reconstituted with splenocytes from WT-HC mice (WT-HC→SCID-HC) and SCID-HC reconstituted with splenocytes from IFN-γ−/−—HC mice (IFN-γ−/−—HC→SCID-HC).

Reconstitution Experiments
As described previously,7 spleens removed from donor mice after 9 days HC were scraped through a screen (E-C Apparatus Co) and 106 cells) at 9 days HC and allowed to recover for 5 days before intravital microscopy was performed at 2 weeks HC.

Surgical Protocol
Mice were anesthetized with ketamine hydrochloride (150 mg/kg body wt IP) and xylazine (7.5 mg/kg body wt IP). The right jugular vein was cannulated for administration of heparinized saline, and the left carotid artery was cannulated for systemic arterial pressure measurement. Core body temperature was maintained at 35 ± 0.5°C. Animal handling procedures were approved by the LSU Health Sciences Center Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

Intravital Microscopy
The cremaster muscle was prepared for intravital microscopy as described previously.6 Postcapillary venules were selected for observation. Leukocyte rolling velocity (Vrolling) and the number of rolling and adherent leukocytes were quantified in the cremaster muscle during playback of videotaped images. Rolling leukocytes were defined as white blood cells that move at a velocity less than 30 mm/s. A leukocyte was considered adherent if it remained stationary for at least 30 seconds and was measured throughout the observation period. Leukocyte emigration was measured online at the end of each 5-minute observation period. Emigrated leukocytes were expressed as the number of interstitial leukocytes per high-powered field of view adjacent to the segment under observation.

Experimental Protocol
Postcapillary venules (20 to 40 μm in diameter) with a wall shear rate (WSR) of ≥500/s were studied. This threshold was selected on the basis of previous reports describing a propensity for leukocytes to adhere in venules at low WSRs.8 The venule with the smallest number of adherent and emigrated leukocytes at the end of 30 minutes of stabilization was chosen for the study. Five-minute recordings were made of the first 100 μm of every 300 μm along the length of the unstimulated vessel, beginning as near to the source of the venule as possible. The mean value of each variable within a single venule was calculated, and comparisons were made between the experimental groups.

Dihydrorhodamine Oxidation
Separate groups of WT-ND, WT-HC, IFN-γ−/−—ND, IFN-γ−/−—HC, and WT-HC→IFN-γ−/−—HC animals were prepared for intravitral microscopy as described above. Background fluorescence (Ibackground) of the first 100 μm of every 300 μm was recorded along the length of the selected postcapillary venule with a xenon light source and a fluorescent camera and intensifier (Hamamatsu). Freshly prepared dihydorhodamine (DHR)-123 (1 mmol/L, a nonfluorescent dye that is oxidized to the fluorescent compound rhodamine-123) in bicarbonate-buffered saline (BBS) was superfused over the cremaster for 15 minutes. The tissue was then washed with BBS, and the fluorescent image of each section was recorded (Ifluorescent). Images were captured onto a computer, and an area 100 μm long and twice the vessel width was analyzed for each section by use of NIH Image 1.62 software, as previously described.10 The ratio of Ifluorescent/Ibackground was calculated for each section, the average ratio for each animal was determined, and comparisons were made between all groups.

Blood Leukocyte Counts
At the end of each experiment, blood was drawn from the heart, and 25 μL was mixed with 465 μL 3% acetic acid and 10 μL 1% crystal violet. Circulating blood leukocyte count was performed with the aid of a hemocytometer.

Serum Cholesterol Levels and Circulating IFN-γ Levels
Serum was frozen for subsequent measurement of cholesterol levels by use of a spectrophotometric assay (Sigma Chemical Co). Serum from WT-ND and WT-HC animals was frozen for estimation of IFN-γ levels using an ELISA (Biosource International).

Flow Cytometry
To confirm the absence of T cells in the SCID animals, flow cytometry was performed as described previously9 to assess the percentage of CD4+ and CD8+ blood leukocytes in all groups after 2 weeks ND or HC. Blood was labeled with allophycocyanin-conjugated anti-mouse CD4 or CD8 antibody (PharMingen Inc). In addition, the expression of 3 surface adhesion molecules, CD11b, CD18, and CD62L, was assessed. Fluorescein isothiocyanate–labeled anti-mouse CD11b, phycoerythrin-labeled anti-mouse CD18, and biotinylated anti-mouse CD62L antibodies were used (PharMingen). A secondary streptavidin-PerCP–labeled antibody was used with the CD62L primary antibody (PharMingen). Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

Statistical Analysis
All values are reported as mean ± SEM. ANOVA with Scheffé post hoc test was used for statistical comparison of experimental groups, with statistical significance set at P<0.05.

Results

Serum Cholesterol and Interferon-γ Levels
WT, IFN-γ−/−, and SCID mice maintained on HC diet for 2 weeks exhibited elevated serum cholesterol levels compared with mice on ND (Table 1). Cholesterol levels in WT-HC mice were more than double the concentrations in their ND counterparts (P<0.05). Both groups of IFN-γ−/− mice fed HC diet (IFN-γ−/−—HC and WT-HC→IFN-γ−/−—HC) were hypercholesterolemic, with cholesterol levels significantly higher than those of IFN-γ−/−—ND animals (P<0.05), and comparable to the other HC groups. Similarly, all SCID groups on HC diet, including reconstituted animals, exhibited significantly higher serum cholesterol levels compared with the SCID-ND group (P<0.05) but comparable to WT-HC.
mice. Serum IFN-γ levels rose from 0±0 pg/mL in WT mice to 1.6±0.58 pg/mL in WT-HC mice (P<0.05).

**Venular WSRs**
Measurement of venular WSR (Table 1) revealed no statistically significant difference between any of the experimental groups, ie, WT, IFN-γ−/−, SCID, ND, and HC.

**Peripheral Blood Leukocyte Counts**
Despite small increases in the numbers of circulating neutrophils and monocytes, WT mice exhibited a slight decrease in the number of blood leukocytes after 2 weeks HC (Table 2) because of a parallel decrease (not significant) in lymphocyte levels (data not shown). No significant alterations in the number of circulating blood leukocytes accompanied the elevation of cholesterol levels in IFN-γ−/− animals (Table 2). Not surprisingly, there were significantly fewer circulating leukocytes in SCID mice than in either WT or IFN-γ−/− mice on ND (P<0.001) or HC (P<0.05). The transfer of WT-HC or IFN-γ−/−–HC splenocytes to SCID-HC mice failed to restore circulating blood leukocyte counts to levels observed in WT-HC mice. Furthermore, injection of splenocytes in the WT-IIC→IFN-γ−/−–HC group did not enhance blood leukocyte counts beyond those found in the IFN-γ−/−–HC mice.

**Flow Cytometry**
The WT-HC group exhibited lower levels of CD4+ blood leukocytes and a significantly decreased percentage of CD8+ blood leukocytes compared with their ND counterparts (P<0.05) (Table 2), most likely reflecting the small changes in the numbers of all leukocyte types. The percentages of CD4+ and CD8+ blood leukocytes in the IFN-γ−/−–ND and IFN-γ−/−–HC animals were similar to those of the WT-ND group. IFN-γ−/−–HC mice exhibited significantly higher levels of CD8+ leukocytes than WT-HC mice. CD4+ and CD8+ leukocytes were largely absent in the SCID-ND and SCID-HC groups. SCID-HC mice that received splenocytes from WT-HC or IFN-γ−/−–HC mice similarly demonstrated almost complete absence of CD4+ and CD8+ blood leukocytes. The administration of WT-HC splenocytes to IFN-γ−/−–HC mice did not alter their populations of CD4+ or CD8+ leukocytes. Flow cytometric analysis also revealed that the surface expression of CD11b, CD18, and CD62L on peripheral blood leukocytes did not differ significantly between any group or dietary regimen.

**Leukocyte–Endothelial Cell Interactions**
Leukocyte rolling flux and Vwbc were unaltered by HC in WT mice (data not shown). IFN-γ−/− mice exhibited similar leukocyte rolling flux and Vwbc compared with their WT counterparts regardless of diet. In contrast, leukocyte adhe-

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<th>TABLE 1. Serum Cholesterol Concentration and Venular WSR in WT, IFN-γ−/−, and SCID Mice Maintained on ND or HC Diet for 2 Weeks</th>
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Separate SCID-HC and IFN-γ−/−–HC groups received splenocytes from donors after 9 days HC (donor→recipient).

*P<0.05 vs WT-ND group.
†P<0.05 vs IFN-γ−/−–ND group.
‡P<0.05 vs SCID-HC group.
§P<0.001 vs IFN-γ−/−–HC group.

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<th>TABLE 2. Blood Leukocyte Count and Percentages of CD4⁺ and CD8⁺ Populations in Circulating Blood in WT, IFN-γ−/−, and SCID Mice Maintained on ND or HC Diet for 2 Weeks</th>
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Separate SCID and IFN-γ−/−–HC groups received splenocytes from donors at day 9 of diet (donor→recipient).

*P<0.05 vs WT-ND and WT-HC groups.
†P<0.001 vs IFN-γ−/−–HC groups.
‡P<0.001 vs all IFN-γ−/− groups.
§P<0.001 vs all IFN-γ−/− and WT-HC groups.
||P<0.05 vs WT-HC.
Effects of hypercholesterolemia on number of adherent (A) and emigrated (B) leukocytes in normal (WT) and interferon-γ knockout (IFN-γ −/−) mice placed on a normal (ND) or high cholesterol (HC) diet for 2 weeks. Leukocyte adherence and emigration were significantly higher in WT-HC group than WT-ND animals (P<0.005). These increases were prevented in WT-ND animals (*P<0.005) (Figure 1A) and emigration (P<0.0005) (Figure 1B) were significantly elevated in WT-HC mice compared with WT-ND animals. The number of adherent leukocytes in IFN-γ −/−–ND mice were similar to the WT-ND group (Figure 1A). However, leukocyte adhesion was significantly reduced in IFN-γ −/−–HC mice compared with WT-HC animals (P<0.005), to values obtained in WT-ND and IFN-γ −/−–ND groups. Leukocyte emigration showed a similar pattern of responses. IFN-γ −/−–ND mice demonstrated leukocyte emigration comparable to that in WT-ND mice. IFN-γ −/−–HC mice exhibited leukocyte emigration that was significantly lower than that in WT-HC mice (P<0.0001) but similar to that in WT-ND and IFN-γ −/−–ND animals (Figure 1B). These data strongly support a role for IFN-γ in hypercholesterolemia-induced leukocyte–endothelial cell interactions.

In SCID mice, leukocyte rolling parameters were not significantly different between ND and HC and WT animals. Both SCID-ND and SCID-HC exhibited levels of leukocyte adhesion (P<0.005 versus WT-HC) (Figure 2A) and emigration (P<0.0001 versus WT-HC) (Figure 2B) that were comparable to those in WT-ND animals. The HC-induced adhesion and emigration were restored to WT-HC levels by reconstitution of SCID-HC with WT-HC splenocytes 5 days before the experiment (P<0.001 and P<0.0001 versus SCID-HC, respectively). Similarly, leukocyte adhesion was restored to WT-HC levels in IFN-γ −/−–HC mice by administration of splenocytes from WT-HC animals (WT-HC→IFN-γ −/−–HC) (P<0.005 versus IFN-γ −/−–HC) (Figure 2A). In contrast, the reconstitution of SCID-HC mice with IFN-γ −/−–HC splenocytes did not restore the adhesion responses; instead, leukocyte adhesion was comparable to WT-ND levels (P<0.01 versus WT-HC). A similar pattern of responses was noted for leukocyte emigration, ie, leukocyte emigration was restored in the WT-HC→IFN-γ −/−–HC mice but not in SCID-HC mice that received splenocytes from IFN-γ −/−–HC mice (Figure 2B). These findings suggest that lymphocytes are an important source of IFN-γ that mediates leukocyte–endothelial cell interactions during hypercholesterolemia.
molecules on endothelial cells, an enhanced adherence effects of ischemia-reperfusion and proinflammatory mediators-induced inflammatory phenotype is that the microvasculature is rendered more susceptible to the deleterious oxidative stress. This response was abolished in mice deficient in IFN-γ (IFN-γ−/−−HC) (P<0.05 vs WT-HC). However, hypercholesterolemia-induced oxidant stress was restored to WT-HC levels in IFN-γ−/−−HC mice by administration of WT-HC splenocytes 5 days before measurement (WT-HC→IFN-γ−/−−HC) (P<0.005 vs IFN-γ−/−−HC).

**Oxidative Stress**

WT-HC animals, compared with ND counterparts, exhibited significantly (P<0.005) higher DHR oxidation, an index of oxidative stress (Figure 3). In IFN-γ−/−−ND mice, oxidative stress was comparable to WT-ND levels. However, IFN-γ−/−−HC demonstrated DHR oxidation that was significantly lower versus WT-HC animals (P=0.001) but similar to ND values. Reconstitution of IFN-γ−/−−HC mice with WT-HC splenocytes (WT-HC→IFN-γ−/−−HC) restored the DHR oxidation response to WT-HC levels, significantly higher than the DHR oxidation detected in the IFN-γ−/−−HC group (P<0.005).

**Discussion**

Previous studies in our laboratory and by others indicate that 2 weeks of diet-induced hypercholesterolemia in otherwise normal animals is associated with phenotypic changes in postcapillary venules that are consistent with a proinflammatory state. These changes include increased production of reactive oxygen species, elevated expression of adhesion molecules on endothelial cells, and an enhanced adherence and transendothelial migration (emigration) of leukocytes, primarily granulocytes. A consequence of this hypercholesterolemia-induced inflammatory phenotype is that the microvasculature is rendered more susceptible to the deleterious effects of ischemia-reperfusion and proinflammatory mediators. Studies addressing the mechanisms that underlie the microvascular inflammation associated with hypercholesterolemia have revealed a role for reactive oxygen species and both CD4+ and CD8+ T lymphocytes. These studies raise the possibility that T-cell-derived products, such as cytokines, provide a link between the leukocyte–endothelial cell adhesion and enhanced oxidant production induced in postcapillary venules by hypercholesterolemia. In the present study, we assessed the potential contribution of IFN-γ to the inflammatory responses elicited by hypercholesterolemia. This cytokine was chosen for evaluation because (1) it is produced by all CD8+ T cells and the Th1 subset of CD4+ T cells; (2) it has been shown to promote oxidative stress by activating enzymes such as NAD(P)H oxidase; (3) it seems to participate in atherosclerotic plaque development as evidenced by its colocalization with activated MHC-II–expressing macrophages in atherosclerotic lesions, and (4) mice lacking IFN-γ receptor are protected against atherosclerotic plaque development, whereas exogenous IFN-γ potentiates lesion formation in hypercholesterolemic mice. Our findings indicate that IFN-γ−/−− mice placed on a cholesterol-enriched diet do not exhibit the substantial adherence and emigration of leukocytes that is seen in postcapillary venules of WT mice placed on the same diet. Furthermore, the hypercholesterolemic IFN-γ−/−− mice showed a significantly blunted oxidative stress in postcapillary venules compared with their WT counterparts. These observations provide strong support for IFN-γ as an important mediator of the inflammatory response elicited by hypercholesterolemia.

The possibility that T lymphocytes are an important source of the IFN-γ during hypercholesterolemia was assessed in SCID mice, which lack both T and B lymphocytes. The present study confirms a previously reported observation that SCID mice exhibit an attenuated leukocyte adhesion and emigration response to hypercholesterolemia. Furthermore, CD4+ and CD8+ T cells, both of which produce IFN-γ, have been shown to be important in this response. We show here that reconstitution of hypercholesterolemic SCID mice with splenocytes from hypercholesterolemic WT mice restores the leukocyte–endothelial cell adhesion responses to WT-HC levels. This occurred in the absence of reconstituted peripheral blood lymphocyte populations. Because all other leukocyte populations are normal in SCID mice, this suggests that the injected lymphocytes are mediating the injury by exerting their proinflammatory effects through the production of one or more soluble factors, such as IFN-γ. The importance of IFN-γ as a contributor to the lymphocyte-mediated inflammatory responses was demonstrated in 2 ways: (1) reconstitution of SCID-HC mice with splenocytes from IFN-γ−/−−HC mice did not restore the leukocyte adhesion response, and (2) administration of splenocytes from WT-HC mice into IFN-γ−/−−HC mice restored the leukocyte adhesion responses to levels seen in WT-HC mice. In both instances, the findings are consistent with lymphocytes as an important source of IFN-γ. Because both T and B lymphocytes are absent in SCID mice and because B cells do not produce IFN-γ, it seems likely that T cells are the primary source of IFN-γ in our model.

We have previously demonstrated that superoxide, generated at least in part from NAD(P)H oxidase in leukocytes and the vessel wall, contributes to the microvascular inflammatory responses induced by hypercholesterolemia. Our present findings with DHR oxidation are consistent with the view that hypercholesterolemia induces an oxidant stress in postcapillary venules. Furthermore, the observations that a hypercholesterolemia-induced oxidant stress is absent in venules of IFN-γ−/−−HC mice and that the oxidant stress can be restored by administration of WT-HC splenocytes into IFN-γ−/−−HC.
mice are consistent with a role for lymphocyte-derived IFN-γ in the oxidant component of the inflammatory response. Although the relationship between the early inflammatory responses (leukocyte adhesion/emigration, oxidative stress) in venules and the later development of atherosclerotic lesions remains unclear, an improved understanding of the hypercholesterolemia-induced inflammatory phenotype in venules may provide a mechanistic insight into the process of atherogenesis. It is possible that the rapid and prolonged inflammatory response elicited in the microcirculation by hypercholesterolemia may predispose large arteries to an inflammatory response and subsequent lesion formation. Furthermore, our previous work on ischemia-reperfusion injury clearly indicates that hypercholesterolemia renders the microcirculation more vulnerable to the endothelial cell dysfunction caused by ischemia-reperfusion.

The exact nature of the relationship between hypercholesterolemia, the production of lymphocyte-derived IFN-γ, oxidant stress, and leukocyte–endothelial cell adhesion in our model remains unclear. It is known that (1) oxLDL stimulates IFN-γ production by T lymphocytes in vitro, (2) IFN-γ is capable of promoting LDL oxidation under certain conditions, and (3) IFN-γ is a strong activator of NAD(P)H oxidase. Consequently, hypercholesterolemia could be linked to the different inflammatory responses by a variety of scenarios. Additional work is needed to more fully define the molecular and cellular basis for the observed relationship between hypercholesterolemia, the production of lymphocyte-derived IFN-γ, oxidant stress, and leukocyte–endothelial cell adhesion. Such an effort should help to explain why the microvasculature of hypercholesterolemic animals is more vulnerable to the deleterious effects of ischemia-reperfusion, and it may provide insights into the role of inflammation in the pathogenesis of atherosclerosis.

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References
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