Direct Anti-Inflammatory Mechanisms Contribute to Attenuation of Experimental Allograft Arteriosclerosis by Statins

Koichi Shimizu, MD, PhD; Masanori Aikawa, MD, PhD; Kiyoshi Takayama, PhD; Peter Libby, MD; Richard N. Mitchell, MD, PhD

Background—Despite the development of effective immunosuppressive therapy, transplant graft arterial disease (GAD) remains the major limitation to long-term graft survival. The interplay between host inflammatory cells and donor vascular wall cells results in an intimal hyperplastic lesion, which leads to ischemia and graft failure. HMG-CoA reductase inhibitors (statins) reduce GAD in human cardiac allografts, although it is unclear whether this is secondary to cholesterol lowering or other mechanisms. This study tested the hypothesis that statins can suppress GAD by cholesterol-independent pathways.

Methods and Results—We performed heterotopic murine cardiac transplants in total allogeneic or major histocompatibility complex class II–mismatched combinations. Transplanted animals received either control chow, chow containing 25 ppm cerivastatin (low dose), or chow containing 125 ppm cerivastatin (high dose). Mean plasma cerivastatin concentrations were 0.0 (control), 10.1 (low dose), and 21.9 (high dose) nmol/L, respectively. Plasma cholesterol levels were the same in all groups. GAD scores decreased in low-dose (P<0.05) and high-dose (P<0.0001) cerivastatin groups compared with controls, with concomitant reduction in graft-infiltrating cells and significantly decreased intragraft RANTES and monocyte chemotactic protein-1 mRNA expression. Cerivastatin, as well as other statins, also reduced RANTES and monocyte chemotactic protein-1 production in mouse endothelial cells stimulated with interferon-γ and tumor necrosis factor-α in vitro.

Conclusions—Clinically achievable levels of an HMG-CoA reductase inhibitor attenuate GAD in murine heart transplants, diminish host inflammatory cell recruitment, and do not alter cholesterol levels. These results indicate that statins can affect arterial biology and inflammation independently of their effects on cholesterol metabolism. (Circulation. 2003;108:2113-2120.)

Key Words: statins ■ inflammation ■ arteriosclerosis ■ endothelium ■ transplantation

Because immunosuppressive therapy has effectively limited most allograft parenchymal rejection (PR), graft arterial disease (GAD) has become the major limitation to long-term graft survival. GAD involves an allogeneic immune response; host atrial remnants in human heart transplants are not affected, nor does it typically occur in animal syngeneic grafts. GAD likely develops from an intimal perivascular recruitment and activation of macrophages and T cells. Recognition of foreign tissue by T-cell interaction with donor major histocompatibility complex (MHC) promotes clonal expansion of the T cells; activated T cells then secrete a variety of cytokines, including interleukin-2, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). These ultimately drive the smooth muscle cell recruitment that forms the intimal hyperplastic lesion of GAD; the end result is graft ischemia and failure.

Accumulating evidence suggests that HMG-CoA reductase inhibitors (statins) may reduce cardiovascular events in part independently of their cholesterol-lowering abilities. For example, statins have direct anti-inflammatory properties; we previously demonstrated that clinically achievable doses of cerivastatin reduce macrophage proliferation and activation in vitro. Statins may also indirectly influence immune responses. For example, atorvastatin diminished MHC class II expression and reduced mixed lymphocyte responses in vitro. Pravastatin treatment in human kidney transplant recipients resulted in reduced rejection scores and natural killer cell cytotoxicity.

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The basis of these observations is largely unknown, particularly regarding the putatively hepatic-selective statins such as pravastatin. Animal studies demonstrate that cerivastatin is the most pharmacologically potent statin, at least with regard to HMG-CoA reductase inhibition. Cerivastatin is more cell permeant (lipophilic) than pravastatin (hydrophilic) and may have extrahepatic activities, with possibly greater efficacy regarding GAD development.

To explore the mechanisms by which statins affect organ transplant outcomes without confounding effects attributable to lipid lowering, we studied heart transplants in normocholesterolemic mice. We conducted the in vivo studies with cerivastatin because of its potency and high degree of permeability into nonhepatocytes. Specifically, we tested the hypothesis that a cell-permeant statin can suppress host inflammatory cell recruitment and activation and thereby modulate the downstream effector mechanisms that lead to GAD progression.

Methods

Animals

C57Bl/6 (B6; H-2b), B6.C-H2<bm12>; KhEg (bm12; H-2<sup>bm12</sup>), or BALB/c (B/c; H-2<sup>c</sup>) mice were obtained from the Taconic Farm (Germantown, NY) or the Jackson Laboratory (Bar Harbor, ME). Mice were maintained on acidified water in Harvard Medical School animal facilities; experiments conformed to approved animal care protocols.

Mixed Lymphocyte Reaction

One-way mixed lymphocyte reaction (MLR) was performed as described previously, with bm12 splenocytes used as stimulators and B6 splenocytes as responders, with various concentrations of statins (cerivastatin, fluvastatin, and pravastatin) throughout culture. Cerivastatin was obtained from Bayer; pravastatin and fluvastatin were from Sankyo.

Dosing of Cerivastatin

Plasma concentrations of cerivastatin in humans are 7.7 nmol/L in low-dose (0.3 mg/d) and 29 nmol/L in high-dose (0.8 mg/d) regimens, respectively. Murine and human lymphocytes in vitro show comparable dose-response inhibition of MLR by statins (data not shown). We therefore targeted 7 to 30 nmol/L plasma concentrations of cerivastatin for this murine in vivo study, although this required higher weight per weight dosing in mice compared with humans. We divided B6 mice into 3 groups: normal diet group; a low-dose group, 25 ppm cerivastatin (25 mg cerivastatin/kg chow); and a high-dose group, 125 ppm cerivastatin (125 mg cerivastatin/kg chow). We used retro-orbital blood samples and measured plasma statin concentrations by high-performance liquid chromatography and plasma cholesterol levels by colorimetric assay (Sigma Diagnostics).

Vascularized Heterotopic Cardiac Transplantation

Cerivastatin or control chow administration to recipient B6 mice began 2 weeks before transplantation and continued until graft harvest. B/c (total allogeneic mismatch, to assess effects on acute rejection) or bm12 (MHC class II mismatch, to assess effects on GAD) donor hearts were transplanted heterotopically into B6 recipients without immunosuppression. We assessed graft function by palpation twice daily. In total allogeneic-mismatched combinations, grafts were harvested when the strength of beating declined to the point that failure would occur (by prior experience) within the next 12 hours (7 to 10 days after transplantation). In MHC II-mismatched combinations, we harvested grafts at 4, 8, or 12 weeks. The therapeutic efficacy of cerivastatin was assessed by scoring the duration of graft survival in total allogeneic mismatches and by scoring PR and the severity and extent of GAD in MHC II mismatches. We scored PR (0 to 4, based on extent of inflammatory infiltrate and associated myocyte injury) and GAD (0 to 4, based on extent of luminal stenoses averaged over ≥10 arteries) by blinded observers as described previously.

Graft Harvest

Harvested allografts were either used for extracting graft-infiltrating cells for flow cytometry or were transversely sectioned into 3 parts. For cellular extraction, hearts were digested at 37°C in 2 mg/mL collagenase (Sigma) and 2% bovine serum albumin in buffered saline, followed by staining and Ficoll density gradient centrifugation (Organon Teknika). In sectioned hearts, the most basal part was used for routine hematoxylin-and-eosin morphological examination. A second midtransverse section was frozen for immunohistochemical staining, and the apical portion was used for total RNA extraction for RNase protection assay (RPA).

Immunohistochemistry

For MHC II staining, 5-μm cryosections were fixed in acetone before incubation with 0.5% H<sub>2</sub>O<sub>2</sub>. Slides were incubated with 10% normal goat serum and stained with purified monoclonal anti-I-A/I-E antibodies (M5/114.15.2) or negative control (rat IgG2b, 2.5 μg/mL; PharMingen). Sections were incubated with biotinylated goat anti-rat IgG antibodies (1 μg/mL; Southern Biotechnology Associates, Inc), followed by streptavidin-peroxidase (DAKO). Antibody binding was visualized with 3-aminobenzidine (Sigma).

Cellular Surface Staining and Flow Cytometric Analysis

Graft-infiltrating cells were analyzed by flow cytometry after surface staining with methods described previously. Antibodies included anti-CD4-cyC (RM4-5), anti-CD8-phycoerythrin (PE) (53-6.7), anti-CD11b-FITC (M1/70), or anti-MHC II-PE (M5/114.15.2) and PE-conjugated rat IgG2b (negative control; PharMingen).

Total RNA Extraction and RNase Protection Assay

Total RNA was isolated from allografts with TriZol reagent (Invitrogen). RPA was performed (RiboQuant, PharMingen) with 10 μg of RNA from each heart according to the manufacturer’s recommendations.

Murine Endothelial Cell Isolation and Culture

Endothelial cells (ECs) were isolated from murine hearts as described previously and cultured on Fib coll density gradient centrifugation (Organon Teknika). Sections were incubated with biontinylated goat anti-rat IgG antibodies (1 μg/mL; Southern Biotechnology Associates, Inc), followed by streptavidin-peroxidase (DAKO). Antibody binding was visualized with 3-aminobenzidine (Sigma).

Chemokine ELISA

ELISA was performed as described previously. Antibody pairs were as follows: purified anti-RANTES (regulated on activation and normally T-cell expressed and secreted) and biontinylated anti-RANTES monoclonal antibodies (R&D Systems Inc) or purified anti-MCP-1 (monocyte chemotactant protein-1) and biontinylated anti-MCP-1 monoclonal antibodies (PharMingen).

Statistical Analysis

Comparisons between 2 groups for frequency of graft-infiltrating cells, normalization of RPA data, and ELISA used 1-way ANOVA.

Results

Cerivastatin and Total Cholesterol Levels in Mice Receiving Statin-Formulated Chow

Cerivastatin levels were measured from plasma collected at the time of graft harvest; 25 and 125 ppm cerivastatin yielded

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Plasma Statin and Cholesterol Levels

<table>
<thead>
<tr>
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<th>Plasma Cerivastatin Concentration (Mean ± SD), nmol/L</th>
<th>Total Cholesterol (Mean ± SD), mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no drug)</td>
<td>0.0 ± 0.1 (n=22)</td>
<td>75.5 ± 13.8 (n=15)</td>
</tr>
<tr>
<td>Low-dose diet, 25 ppm cerivastatin (25 mg/kg chow)</td>
<td>10.1 ± 8.4 (n=28)</td>
<td>82.4 ± 9.6 (n=15)</td>
</tr>
<tr>
<td>High-dose diet, 125 ppm cerivastatin (125 mg/kg chow)</td>
<td>21.9 ± 20.1 (n=35)</td>
<td>72.6 ± 21.6 (n=29)</td>
</tr>
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approximate plasma concentrations of 10.1 and 21.9 nmol/L, respectively. These levels fall within the therapeutic range for humans (7.7 to 29 nmol/L). There were no differences in plasma cholesterol levels among the groups (Table).

Statins Inhibit MLR, but Only at Supratherapeutic Doses

Statin inhibition of allogeneic responses in vitro was assessed by 1-way MLR with murine splenocytes derived from naïve bm12 and B6 mice; splenocytes were cultured with pravastatin (hydrophilic), fluvastatin (lipophilic), or cerivastatin (lipophilic). Consistent with previous reports, pravastatin had no effect on MLR even at the highest concentrations tested (Figure 1). In contrast, cerivastatin significantly inhibited MLR proliferation at concentrations greater than 1 μmol/L, and fluvastatin inhibited MLR proliferation at concentrations above 10 μmol/L (Figure 1). Notably, the concentrations of cerivastatin inhibitory for MLR proliferation exceed those clinically achievable in humans. The results indicate that clinically achievable concentrations of cerivastatin in vivo (<30 nmol/L) were neither cytotoxic nor did they directly inhibit lymphocyte proliferation. At concentrations of cerivastatin <100 nmol/L, IFN-γ production was not affected (data not shown).

Cerivastatin Does Not Affect PR or Graft Survival in Fully Allogeneic Cardiac Transplants

In total allogeneic-mismatched murine heart transplantation (B/c donor hearts and B6 recipients), graft survival averaged 9.2 ± 1.0 days (mean ± SD, n=6) for control chow, 9.5 ± 1.0 days (n=6) for low-dose statin, and 9.3 ± 0.8 days (n=6) for high-dose statin. Rejection scores, which reflected the extent of inflammation, did not differ among groups (Figure 2).
of mononuclear cell infiltrate and associated myocyte injury, were comparable for all allografts at beat cessation (data not shown). The results indicate that cerivastatin has no effect on inflammatory cell recruitment or graft survival in total allogeneic-mismatched allografts.

Cerivastatin Attenuates GAD in MHC Class II–Mismatched Recipient Allografts We performed MHC class II–mismatched murine heart transplantation using bm12 hearts and B6 recipients. Grafts were harvested 12 weeks after transplantation, a time point at which GAD lesions typically are well developed. In animals receiving control chow, graft arteries developed characteristically thickened intima, whereas animals that received either concentration of cerivastatin had significantly reduced GAD (Figure 2), and animals that received high-dose cerivastatin had significantly reduced PR. The GAD scores were 2.8±1.3 (n=11, mean ± SD) for control chow, 1.4±1.2 (n=9) for low-dose statins, and 0.6±1.2 (n=11) for high-dose statins, whereas the PR scores were 2.6±0.6 (n=11, mean ± SD) for control chow, 2.1±1.0 (n=9) for low-dose statins, and 1.9±0.7 (n=11, P<0.05 versus control) for high-dose statins.

T-Lymphocyte and Macrophage Accumulation in Grafts Is Significantly Decreased in Cerivastatin-Treated Recipients Because PR scores reflect graft-infiltrating cell number, it appeared that statin treatment had caused a significant reduction in inflammatory cell infiltration at the 12-week time point, when there are well-developed GAD lesions. To quantify the decrease in graft inflammatory cells by statin treatment, infiltrating cells were recovered and counted from cardiac allografts harvested 4 and 8 weeks after transplantation. High-dose statin-treated allografts had significantly fewer infiltrating cells than controls at all time points (Figure 3); low-dose statin-treated allografts had significantly less inflammation at 8 weeks after transplantation.

We also performed flow cytometry using anti-CD4, anti-CD8, and anti-CD11b antibodies to identify which cell types were diminished in allografts from cerivastatin-treated recipients. With high-dose cerivastatin, the numbers of CD8-positive cells decreased significantly compared with controls harvested at all time points. The numbers of CD4-positive cells tended to decrease in 4-week allografts and decreased significantly in 8-week allografts in high-dose cerivastatin-treated groups compared with controls. The numbers of CD11b-positive cells decreased significantly in 4-week allografts in the high-dose cerivastatin group and in 8-week allografts in both low- and high-dose cerivastatin-treated groups compared with controls.

Cerivastatin Does Not Modulate MHC II Expression MHC expression and subsequent strength of the allogeneic response may affect cell recruitment. Statins can also directly limit MHC II expression in vitro or can potentially alter the local cytokine milieu. We therefore examined whether clinically achievable levels of cerivastatin modulate MHC II expression by performing immunohistochemical staining for MHC II in cerivastatin-treated or control recipient cardiac allografts harvested 4 and 8 weeks after transplantation. Although the cellular MHC II staining intensity was not
changed, the number of MHC II–positive cells in grafts in statin-treated hosts was reduced, especially around the vessels (Figure 4A). Because macrophages make up the majority of the inflammatory cells at later time points, we extracted the graft-infiltrating lymphocytes from control- and cerivastatin-treated recipient allografts 8 weeks after transplants and performed 2-color flow cytometry for MHC II and CD11b (macrophages). Macrophage MHC II expression did not differ significantly between any groups (Figure 4C); CD11b-positive cells in control, low-dose, and high-dose cerivastatin-treated recipient allografts showed similar percentages of MHC II–positive cells (81.3±1.7, 79.0±1.6, and 78.7±1.9% [mean±SD, n=4], respectively); the mean fluorescence channels for MHC II expression were also comparable (3020±260, 2800±140, and 2960±40, respectively). The results suggest that cerivastatin treatment does not affect the level of MHC II expression by macrophages; the reduction in the number of MHC II–positive cells was proportional to the overall reduction in the number of graft-infiltrating cells.

Chemokine mRNA Expression Is Decreased in Cerivastatin-Treated Recipient Allografts

Given the reduction in graft-infiltrating cells, we hypothesized that cerivastatin treatment modulates chemokine expression. We therefore performed RPA to measure chemokine mRNA expression from allografts harvested 8 and 12 weeks after transplantation. RANTES and MCP-1 mRNA expression were significantly decreased in cerivastatin-treated recipient allografts compared with controls (Figure 5); lymphotactin, eotaxin, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and IFN-γ-inducible protein 10 (IP-10) mRNA levels were not affected by statin treatment (data not shown). Statin treatment also did not affect intra- graft IFN-γ, interleukin-6, and TNF-α mRNA expression in grafts up to 2 weeks after transplantation (data not shown).

Cerivastatin Inhibits RANTES and MCP-1 Expression by Murine ECs

We hypothesized that cerivastatin attenuated in vivo inflammatory cell accumulation in allografts by inhibiting EC
chemokine expression. We therefore stimulated murine ECs by IFN-\(\gamma\) and TNF-\(\alpha\) in the absence or presence of different concentrations of cerivastatin or pravastatin for 24 hours and measured RANTES and MCP-1 protein expression in the culture supernatants. Both RANTES and MCP-1 protein expression decreased in the presence of cerivastatin (IC\(_{50}\) \(\approx 30\) nmol/L; Figure 6A) but not in the presence of pravastatin (Figure 6B). To examine whether intermediates of the cholesterol biosynthetic pathway would reverse the inhibitory effects of cerivastatin, we added L-mevalonate in the cultures in the presence of the highest dose (100 nmol/L) of cerivastatin. L-Mevalonate completely reversed the inhibition by cerivastatin.

**Discussion**

This study provides compelling in vivo evidence for inhibition of GAD by cerivastatin independently of any effect on systemic cholesterol levels. The absence of a cerivastatin-induced cholesterol effect in the present study is consistent with previous work showing that statins do not significantly lower cholesterol levels in mice.\(^5\) In the absence of any hypocholesterolemic effect, treatment of B6 recipient mice with clinically achievable doses of cerivastatin resulted in a significant dose-dependent reduction of graft inflammatory cells and a significant attenuation of GAD in MHC II-mismatched cardiac allografts compared with controls.

Large clinical trials have demonstrated that statins are effective in the prevention of acute coronary events and in the reduction of cardiovascular-related morbidity and mortality in patients with atherosclerosis.\(^6\) A significant reduction in cardiac rejection, lower incidence of vasculopathies, less intimal thickening, and increased survival were also observed in cardiac transplantation patients receiving pravastatin or simvastatin.\(^6,15\) In vitro experiments also show that statins may have anti-inflammatory properties and modulate immune responses, including effects relating to inflammatory cell recruitment, differentiation, proliferation, and cytokine expression (eg, IFN-\(\gamma\) and TNF-\(\alpha\)).\(^6\) However, it is not known whether these effects occur in vivo in the setting of clinical levels of statins.\(^6\) Notably, in the total allogeneic-mismatched transplant model in the present study (without immunosuppression), cerivastatin treatment did not affect intragraft proinflammatory cytokine expression (data not shown). We attribute this to redundant pathways of cytokine elaboration not amenable to inhibition by clinically achievable doses of cerivastatin alone.

Although high-dose statin treatment may reduce MHC II expression in vitro,\(^8\) the in vivo levels achieved in the present study did not diminish inflammatory cell MHC II expression as assessed by direct immunohistochemical staining or by flow cytometric evaluation of extractable cells. Quantification of the intensity of immunohistochemical staining is problematic, and the flow cytometric analysis may not be completely representative because of systematic losses of cells expressing low-level MHC II. Nevertheless, the observation that therapeutic levels of statins apparently do not
and MCP-1 mRNA expression but did not affect MIP-1/H9251
were measured by ELISA. Mev indicates L-mevalonate (100
(A) and RANTES (B) protein levels from culture supernatants
stimulated with IFN-γ (500 U/mL) and TNF-α (10 ng/mL). MCP-1
attenuated the development of atherosclerosis and the pathogenesis of
GAD.26 Targeted disruption of MCP-1 or the receptor for
MCP-1 (CCR2) attenuated the development of atherosclerosis
in apolipoprotein E knockout mice.26 Several investigators
demonstrated that high-dose statins inhibit MCP-1 expression
by both ECs and macrophages.27

By inhibiting L-mevalonate synthesis, statins prevent the
synthesis of important downstream isoprenoid intermediates of
the cholesterol biosynthetic pathway and block the modifi-
cation of various regulatory proteins.16,28 Because the inhibi-
tion of RANTES and MCP-1 production in vitro was
reversed by administration of L-mevalonate, the statin effect
may be due to a biosynthetic pathway that involves a
mevalonate intermediate or could involve a mevalonate-
sensitive modification of proteins such as Ras, Rho, PI,
kinese, or nitric oxide.16,29 Preliminary experiments using
inhibitors for Rho kinase, PI, kinase, or nitric oxide synthase
suggested that PI, kinase in particular may mediate increased
RANTES and MCP-1 expression by murine ECs stimulated
by IFN-γ and TNF-α (data not shown).

The present study provides new insights into the mecha-
nisms underlying the beneficial effects of statin treatment in
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cholesterol levels in mice nevertheless reduce chemokine
release from ECs and inhibit inflammatory cell accumulation
in cardiac allografts, resulting in diminished GAD.

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Figure 6. Cerivastatin inhibits MCP-1 and RANTES expression
by murine ECs. Cultured murine ECs were incubated with indi-
cated concentrations of drugs for 24 hours and concurrently
by IFN-γ (100 μg/mL). *P<0.0001, **P<0.001 vs control (cerivastatin 0
nmol/L).

A MCP-1 protein

B RANTES protein

Development of GAD.23 Several basic studies suggest a benefi-
cial effect on organ transplant rejection by RANTES
blockade.24,25 MCP-1 is an important chemokine in the
progression of atherosclerosis and the pathogenesis of
GAD.26 Despite structural similarities, chemokines within a particular
cell type may have widely varying targets and/or effector func-
tions.18,19 The present study demonstrated that clinically
achievable levels of cerivastatin inhibited intragraft RANTES
and MCP-1 mRNA expression but did not affect MIP-1α,
MIP-β or MIP-2, lymphotactin, eotaxin, or IP-10 mRNA
production. Moreover, therapeutic levels of cerivastatin re-
duced RANTES and MCP-1 elaboration by murine ECs in
culture.

Macrophages and particularly ECs in vessels of rejecting
human allografts express RANTES.22 RANTES expression
correlated with mononuclear cell infiltration and preceded the
dependent MLR stimulation (Figure 1). The apparent overall
decrease in MHC class II antigen expression seen in grafts
from statin-treated recipients in the present study is attribut-
able to the decreased frequency of inflammatory cells.

Emigration of leukocytes from the peripheral circulation
into an allograft is an essential component of allograft
rejection. ECs play a critical role in inflammatory cell
recruitment via adhesion molecules and/or chemokine ex-
pression.17 Chemokines are a large family of soluble mole-
cules expressed by ECs, smooth muscle cells, or lymphocytes
that attract, and in many cases activate, a variety of inflam-
matory and noninflammatory cells.18,19 Chronic allograft
endothelial injury leads to macrophage migration, as well as
the eventual accumulation and proliferation of intimal smooth
muscle–like cells.19–21

To date, more than 50 chemokines have been identified.
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