Inhibition of Hydroxymethylglutaryl-Coenzyme A Reductase Reduces Th1 Development and Promotes Th2 Development

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Abstract—Several prospective clinical studies have indicated that hydroxymethylglutaryl-coenzyme A reductase inhibitors, statins, prevent cardiovascular events in part through their antiinflammatory properties. Because inflammation is positively and negatively regulated by T helper (Th) 1 cells and Th2 cells, respectively, we examined the effects of statins on the Th polarization in vitro and in vivo. Here we demonstrated that the statins tested, ie, cerivastatin, simvastatin, lovastatin, and atorvastatin, promoted Th2 polarization through both inhibition of Th1 development and augmentation of Th2 development of CD4+ T cells primed in vitro with anti-CD3 antibody and splenic antigen-presenting cells. Cerivastatin exerted most potent effect on modulation of Th1/Th2 development, and the effect was completely abrogated by an addition of mevalonate. Consistent with in vitro experiments, cerivastatin treatment decreased IFN-γ production of lymph node cells from mice immunized with ovalbumin emulsified in complete Freund’s adjuvant, indicating that Th1 development is also suppressed in an in vivo proinflammatory environment. In this murine model, cerivastatin significantly reduced mesangial matrix expansion of glomeruli in the kidney and attenuated proteinuria. The decrease of glomerular sclerosis by cerivastatin treatment was positively related to the suppression of interferon (IFN)-γ-producing Th1 response in draining lymph node cells. Hence, these findings strongly suggest that statins’ inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase regulates Th1/Th2 polarization in vivo and such a mechanism possibly plays a pathophysiological role in immune-related glomerular injury. (Circ Res. 2003;93:948-956.)

Key Words: statins ■ 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors ■ Th1/Th2 ■ inflammation ■ glomerular sclerosis

Naive CD4+ T cells that encounter antigen on antigen-presenting cells (APCs) develop into at least two distinct T helper (Th) cells, ie, Th1 cells secreting interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor-β and Th2 cells secreting IL-4, IL-5, and IL-10. The type of Th cells developed determines outcome of immune responses. Th1 cells mediate proinflammatory cellular immunity, whereas Th2 cells mediate humoral immunity and downregulate inflammatory responses. The development of naive CD4+ T cells into either Th1 or Th2 cells is influenced by several factors, including type of APC, costimulatory molecules, strength of signal delivered to T cell receptor (TCR), and cytokine milieu at early stage of naive CD4+ T cell activation.1,2 The process of Th development is tightly regulated to evoke appropriate immune responses. With the system unregulated, Th1 and Th2 cells mediate tissue-damaging inflammatory or allergic diseases, respectively.3 Statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, are potent inhibitors of cholesterol biosynthesis and have greatly improved the management of hypercholesteremia. Several large clinical trials have demonstrated that statins significantly decreased cardiovascular mortality and morbidity over the years.4,5 However, recent clinical and experimental studies have suggested that some benefits of statins in cardiovascular diseases may be attributed to mechanisms beyond the lipid-lowering effects, including antiinflammatory properties.5,6 Indeed, in some studies, statins have been shown to reduce serum concentration of C-reactive protein, a marker of inflammation, providing evidence for antiinflammatory effects of statins.5,7 Concerning the underlying mechanisms, statins inhibit IFN-γ-inducible major histocompatibility complex (MHC) class II expression on macrophages8 and block lymphocyte function–associated antigen-1 (LFA-1)–dependent stimulation of T cells,9 both of which might result in suppression of activation of proinflammatory Th1 cells. In addition, recent studies have demonstrated that treatment with atorvastatin prevents and...
reverses experimental autoimmune encephalomyelitis via suppression and augmentation of Th1 and Th2 immune responses, respectively.\textsuperscript{10,11} Simvastatin has been also reported to exhibit therapeutic potential in murine model of rheumatoid arthritis through inhibition of Th1 response.\textsuperscript{12} These studies have suggested that beneficial effects of statins on autoimmune diseases were attributable to suppression of Th1 response.

Therefore, to explore the effect of statins on Th1/Th2 development directly, we assayed type of Th cells generated from naive CD4\textsuperscript{+} T cells in vitro in the presence of several forms of statins. Furthermore, using mice inoculated with antigen in complete Freund’s adjuvant (CFA), we investigated the effects of the statins on the Th1/Th2 balance and assessed whether such changes are associated with regression of the kidney injury seen in the immunized mice.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and used at the age of 6 to 9 weeks. Mice were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (revised 1996, National Academy Press, Washington, DC). All experiments were approved by our institutional ethical committee for animal welfare.

Culture Media

RPMI1640 (Sigma), supplemented with 10% FBS (CSL Ltd), 5x10^{-3} mol/L 2-mercaptoethanol, and 100-µg/mL kanamycin, was used throughout. MEM (Sigma) with or without FBS was used in some procedures.

Reagents

Stock solutions of cerivastatin (Bayer) and pravastatin (Wako Pure Chemical Industries, Ltd) were prepared in dimethyl sulfoxide. The stocks were diluted to solutions of atorvastatin (Pfizer), simvastatin (Wako Pure Chemical Industries, Ltd) were prepared in distilled water. Stock solutions of cerivastatin (Bayer) and pravastatin (Wako Pure Chemical Industries, Ltd) were prepared in distilled water. Stock solutions of atorvastatin (Pfizer), simvastatin (Wako Pure Chemical Industries, Ltd), and lovastatin (Wako Pure Chemical Industries, Ltd) were made in dimethyl sulfoxide. The stocks were diluted to working solutions in culture medium for in vitro experiments or in saline for in vivo experiments. The nominal concentrations of dimethyl sulfoxide in the working solutions used in the in vitro experiments were 0.005% for atorvastatin and simvastatin and 0.01% for lovastatin. Mevalonate was purchased from Sigma.

Cell Preparations

CD4\textsuperscript{+} T cells were prepared from the spleens of C57BL/6 or BALB/c mice as described previously.\textsuperscript{13} Briefly, spleen cells depleted of red blood cells by treatment with Tris-buffered NH\textsubscript{4}Cl (pH 7.4) were passed through a nylon wool column, followed by depletion of CD8\textsuperscript{+}, I-E\textsuperscript{+}, Fc\textgamma RII/III\textsuperscript{+}, or CD24\textsuperscript{+} cells on a MACS column (Miltenyi Biotech). The recovered cells were confirmed to be >95% CD4\textsuperscript{+} and were used as naive CD4\textsuperscript{+} T cells. T cell-depleted spleen cells were prepared from the spleen as described\textsuperscript{13} and used as APC after 35-Gy irradiation.

In Vitro Priming of Naive CD4\textsuperscript{+} T Cells

Priming of naive CD4\textsuperscript{+} T cells (2x10\textsuperscript{5} cells/2 mL per culture) was carried out using 1 µg/mL anti-CD3 monoclonal antibody (mAb) (145-2C11) with irradiated T cell–depleted spleen cells (3x10\textsuperscript{6} cells/2 mL per culture) in wells of a 24-well plate (Iwaki Glass Co). In some experiments, naive CD4\textsuperscript{+} T cells were primed with plate-coated anti-CD3 mAb (2 µg/mL, 145-2C11) and anti-CD28 mAb (2 µg/mL, 37.51). Cultures received only medium or 0.01 to 30 µmol/L statins. In addition, some cultures were supplemented with 5 µg/mL anti-IL-4 mAb (11B11) and 1000 U/mL IL-12 (rmIL-12, Genetic Institute) (Th1 polarizing condition). On day 4, CD4\textsuperscript{+} T cells primed as described above were distributed into four wells in fresh medium containing 2.5 ng/mL IL-2 (hIL-2, Ajinomoto Co). On day 6, CD4\textsuperscript{+} T cells were recovered by a centrifugation over Ficoll and restimulated at 1x10\textsuperscript{6} cells/mL with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 µmol/L ionomycin for 5 hours in the presence of 5 µg/mL brefeldin A for the last 3 hours of culture.

Intracellular Cytokine Staining

The T cells, restimulated as described above, were stained with biotin-anti-CD4 mAb in the presence of anti-FcR mAb (2.4G2) followed by incubation with streptavidin-Cychrome. They were then fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% saponin. Subsequently, they were incubated with FITC-anti-IFN-γ mAb (PharMingen) and PE-anti-IL-4 mAb (PharMingen) in the presence of 0.1% saponin for 1 hour at 4°C. The cells were gated on CD4\textsuperscript{+} T cells to exclude any residual APC and subjected to two-color analysis on a FACScan (Becton Dickinson). FITC- or PE-conjugated isotype-matched control mAbs were used as negative controls to set quadrant markers.

In Vivo Priming of T Cells

C57BL/6 or BALB/c mice were immunized subcutaneously with 100 µg ovalbumin (OVA) (Sigma) emulsified in CFA (Difco Laboratories). They were also injected intraperitoneally with either saline or 2 to 10 mg/kg cerivastatin in a volume of 100 µL per mouse on days −1, 0, 1, 2, 3, and 4. On day 9, single-cell suspension of subinguinal lymph node cells (LNCs) was cultured at 2x10\textsuperscript{6} cells/L per culture in 24-well plates or at 1x10\textsuperscript{6} cells/250 µL per culture in 48-well plates in the presence of 500 µg/mL OVA. Culture supernatants were harvested after 72 hours and stored at −30°C until assayed for cytokines by ELISA.

Cytokine ELISA

IL-4 and IFN-γ in culture supernatants were assayed by ELISA, as described previously,\textsuperscript{14} using paired mAbs specific for the corresponding cytokine. The lower detection limits of these assays were as follows: IL-4, 6 pg/mL; IFN-γ, 100 pg/mL.

Functional and Morphological Analyses of the Kidney of C57BL/6 Mice

On the last 2 days of the study of in vivo priming of T cells, a 24-hour urine specimen was collected, and the urine collected on the last day was used for determination of urinary excretions of protein. The concentration of protein in urine was determined using protein assay (BioRad).\textsuperscript{15} At the time the mice were killed, the right kidney was used for determination of urinary excretions of protein. The concentration of protein in urine was determined using protein assay (BioRad).\textsuperscript{15} At the time the mice were killed, the right kidney was used for morphological evaluation.

To assess the extent of injured glomerular capillaries, we semiquantitatively determined the area of the glomerulus having capillary damage. The severity of these lesions was graded according to the percentage of the glomeruli involved, as follows: 0, no lesions; 1+, 1% to 25%; 2+, 26% to 50%; 3+, 51% to 75%; and 4+, 76% to 100%. An overall glomerulosclerosis index was calculated by multiplying the severity score (0 to 4+) by the percentage of glomeruli affected and totaling the five values.

To assess the accumulation of mesangial matrix in the glomerulus, we used RGB color analysis of glomerular mesangium stained by periodic acid Schiff reagent and determined the areas showing the same pattern of RGB wavelength as the mesangium using an Olympus microscope and WinRoof color analyzing software (Mitani Corp) according to the previous method.\textsuperscript{16} The measurement was expressed as the percentage of mesangial matrix to the whole area of a glomerulus tested. We scanned 100 glomeruli per each mouse.

Statistical Analysis

Results are expressed as mean±SEM. Student’s t test and one-way ANOVA were performed with STATISTICA software (StatSoft) and a Windows 98 computer system. Differences between groups were considered significant at P<0.05.
Results

Statins Inhibit Th1 Development and Promote Th2 Development In Vitro

To study the effect of statins on Th polarization, naive CD4+ T cells from C57BL/6 mice were primed with anti-CD3 mAb together with irradiated T cell–depleted spleen cells as APCs in the absence or presence of various concentrations of statins, ie, cerivastatin, simvastatin, lovastatin, atorvastatin, and pravastatin. Six days later, the phenotypes of Th cells generated were assayed by intracellular staining of IFN-γ and IL-4 on restimulation with PMA and ionomycin. As shown in Figure 1A, any of the statins except pravastatin dose-dependently inhibited Th1 development and promoted Th2 development, resulting in the promotion of Th2 polarization. Cerivastatin exhibited the most remarkable Th2 polarization effect considering the dose-response relationship.

To determine whether the enhancement of Th2 polarization by statins is limited to Th1-prone C57BL/6 mice (Figure 1B) or could be reproduced in Th2-prone BALB/c mice, naive CD4+ T cells from BALB/c mice were primed in the absence or presence of 0.01 to 0.1 μmol/L cerivastatin. Naive CD4+ T cells from BALB/c mice developed predominantly Th2 cells, and the Th2 development was additionally enhanced by 0.01 μmol/L or more of cerivastatin (Figure 2). On the other hand, because Th1 cells were scarcely detected even in the absence of cerivastatin, we could not detect significant suppression of Th1 development by cerivastatin.

In addition, as shown in Figure 3, the enhanced Th2 polarization by cerivastatin was reversed by the addition of mevalonate, the immediate downstream metabolite of HMG-CoA. The addition of increasing doses of mevalonate evidently attenuated both the inhibition of Th1 and augmentation of Th2 development by cerivastatin, and 0.3 mmol/L or more of mevalonate completely reversed the effects of cerivastatin. Taken together, these results indicate that statins promote Th2 polarization irrespective of genetic background of mice strain and the effect is related to their class effects of inhibitors of HMG-CoA reductase.

Cerivastatin Has Direct Effects on Naive CD4+ T Cells, Resulting in Suppression of Th1 Development

To determine whether statins directly affect Th development independently of APC, naive CD4+ T cells were primed with plate-bound anti-CD3 mAb plus anti-CD28 mAb in the absence of APC together with or without 0.1 μmol/L ceriv-
Cerivastatin. Although the proportion of Th1 cells developed in culture driven by plate-bound anti-CD3 mAb plus anti-CD28 mAb was significantly higher than that developed in culture with anti-CD3 mAb and T cell–depleted spleen cells in the absence (open bars) or presence (filled bars) of 0.03 μmol/L cerivastatin together with increasing concentrations of mevalonate. Results are expressed as percentages of Th1 (A) and Th2 cells (B). Data shown are representative of 3 experiments with similar results.

Cerivastatin Does Not Induce Apoptosis in Th1
As for the mechanism of the Th2 polarization induced by statins, we were unable to neglect the contribution of selective apoptosis in Th1 cells. To examine this possibility, the proportion of apoptotic cells within CD4⁺ T cells cultured for 4 days with anti-CD3 mAb and APC in the absence or presence of 0.1 μmol/L cerivastatin was assessed by Annexin V staining. The percentage of Annexin V⁺ cells in the CD4⁺ T cell population remained unchanged by the addition of cerivastatin (9.7±0.5% and 9.9±1.1% in the presence and absence of cerivastatin, respectively). Furthermore, when naive CD4⁺ T cells were primed under the condition where Th1 polarization was promoted (anti–IL-4 mAb plus IL-12), Th1 development was not suppressed by cerivastatin (Figure 4C). Taken together, these results indicate that the suppression of Th1 development by cerivastatin is not attributable to a selective induction of apoptosis in Th1.

Cerivastatin Suppresses Th1 Development In Vivo
To test whether cerivastatin has similar effects in vivo, cytokine production profiles of draining LNCs from mice immunized with OVA and treated with cerivastatin were assessed. As shown in Figure 6, cerivastatin treatment suppressed IFN-γ production of LNCs from both C57BL/6 and BALB/c mice after restimulation with OVA ex vivo. LNCs from C57BL/6 mice immunized with OVA did not produce detectable IL-4, which is consistent with results reported,20 and cerivastatin treatment did not augment IL-4 production (data not shown). On the other hand, LNCs from BALB/c mice immunized with OVA and restimulated with OVA ex vivo produced detectable IL-4. However, the IL-4 production of LNCs was not augmented by cerivastatin treatment (data not shown). The suppression of IFN-γ production by the
treatment with cerivastatin was not attributable to the alteration of cell composition within LNCs, because the numbers of total LNCs and the proportion of CD4+/H11001, Mac-1+/H11001, or B220+/H11001 cells in cerivastatin-treated mice were essentially equal to those in untreated mice (data not shown).

Cerivastatin Ameliorates Functional and Morphological Alterations in the Kidney
To assess pathophysiological role of the alterations of Th polarization induced by statins for organ injury in immune-related diseases, we investigated the effects of cerivastatin on functional and morphological alterations in the kidney of C57BL/6 mice immunized with OVA emulsified with CFA. In this model, urinary excretion of protein was significantly higher in C57BL/6 mice immunized with OVA/CFA (control) group than the untreated mice without immunization (0.72±0.17 versus 0.35±0.11 mg/dL, P<0.001). This increase was significantly decreased by 20% in mice treated with 10 mg/kg cerivastatin (0.59±0.16 mg/dL, P<0.05) (Figure 7).

In untreated mice, the glomeruli of the kidney exhibited an apparently normal appearance (Figure 8A). The interstitium was not expanded. In contrast, in untreated, immunized mice, the glomerulus exhibited a significant expansion of mesangial matrix with an increase in number of mesangial cells (areas indicated by arrows) (Figure 8B) along with normal tubules (Figure 8C). In the immunized mice treated with cerivastatin, proliferation of mesangial cells decreased and the areas of mesangium became small (Figure 8D).

The improvement of glomerular injury was assessed using two different methods. As shown in Figure 8E, overall glomerulosclerosis index significantly decreased in cerivastatin-treated mice compared with the untreated, immunized group. Moreover, the mesangial area determined by RGB wavelength analyses was significantly lower in the cerivastatin-treated group than the immunized mice without treatment (Figure 8F).

In this model, plasma total cholesterol levels of mice treated with 10 mg/kg cerivastatin (89.4±5.0 mg/dL) were never different from those of the control group (86.7±4.9 mg/dL, P=0.23).

Discussion
In the present study, we demonstrate that four statins, cerivastatin, simvastatin, lovastatin, and atorvastatin, promote Th2 polarization via suppression of Th1 development and augmentation of Th2 development from naive CD4+ T cells primed with anti-CD3 mAb and splenic APC in vitro. Considering the dose-response relationship, cerivastatin exhibited the most potent effect on Th2 polarization. This effect
was seen in two genetic backgrounds, C57BL/6 and BALB/c mice that are prone to develop Th1 and Th2, respectively, and was completely abrogated by the addition of mevalonate, the immediate downstream metabolite of HMG-CoA. These findings indicate that the effects of these statins are attributable to a class-effect of inhibitors of HMG-CoA reductase and indebted to mevalonate depletion. Statins have been shown to suppress production of IL-12,\textsuperscript{10,11} that plays a pivotal role in Th1 development.\textsuperscript{2} Therefore, APC can be the target for the immunomodulatory activities of statins. However, our analysis in this study revealed that statins also had a direct effect on naive CD4\textsuperscript{+} T cells on TCR stimulation, thereby resulting in an inhibition of Th1 development. Inhibition of Th1 development was also seen even when naive CD4\textsuperscript{+} T cells were primed with plate-coated anti-CD3 and anti-CD28 mAbs in the absence of APC. This inhibition of Th1 development was not attributable to the selective induction of apoptosis in Th1 cells, which is in accord with previous studies.\textsuperscript{10–12} Recently, it has been shown that STAT4 phosphorylation in T cells from experimental autoimmune encephalomyelitis mice was reduced by atorvastatin.\textsuperscript{10} Therefore, it is possible that statins directly affect STAT4 signaling pathway, resulting in the inhibition of Th1 development.\textsuperscript{21} It also has been shown that atorvastatin inhibits activation of nuclear factor-κB,\textsuperscript{22} which preferentially promotes Th1 development.\textsuperscript{23} Therefore, it is possible that statins inhibit Th1 development via attenuation of nuclear factor-κB signaling in naive CD4\textsuperscript{+} T cells. Alternatively, statins might weaken the strength of signals delivered by TCR via modulation of cholesterol microdomains, which has been shown to suppress Th1 development.\textsuperscript{2,24,25} On the other hand, the enhancement of Th2 development by statins was indicated to be mediated by modulation of APC functions. Augmentation of Th2 development was seen only when naive CD4\textsuperscript{+} T cells were primed in the presence of APC. Statins have been demonstrated to bind to the LFA-1 L-site that is critical to intercellular adhesion molecule-1 binding,\textsuperscript{9} and blocking of LFA-1/intercellular adhesion...
molecule-1 interaction has been shown to promote Th2 development. Therefore, it is also possible that blocking of LFA-1-mediated T cell costimulation by statins might promote Th2 development. On the other hand, statins have been reported to downregulate the expressions of MHC class II, CD40, CD80, and CD86 on APC induced by IFN-γ. In this study, however, the expressions of these molecules on APCs interacting with naive CD4+ T cells in the presence of anti-CD3 mAb were not suppressed by statins (Figure 5). Similarly, Leung et al.12 have also demonstrated that simvastatin treatment did not affect MHC class II expression. Accordingly, the expressions of these molecules seemed not to play an important part in the enhanced Th2 development by statins. Modulation of cytokine production by APC could be another candidate of the mechanism for inducing Th2 development. Additional research will be required to elucidate the mechanisms involved in the effects of statins on Th1/Th2 development.

Among the statins tested, only pravastatin did not behave as a modulator of Th1/Th2 balance. The reason for the negative findings of pravastatin is not clear; however, it may be attributable to its lipophobic properties that hinder the translocation of pravastatin across the cell membranes.

In accordance with results obtained in in vitro experiments, OVA-specific IFN-γ production of draining LNCs from C57BL/6 and BALB/c mice immunized with OVA was suppressed by cerivastatin treatment. The mice were inoculated with CFA, an oil that contains inactivated mycobacteria, and this was a standard procedure for inducing antigen-specific Th1 development. Thus, cerivastatin alters Th1/Th2 balance mainly through inhibition of Th1 response, even in potent Th1-provoking circumstances. These results accord with recent studies showing that atorvastatin or simvastatin is capable of inhibiting Th1 responses in murine autoimmune models. In the present study, in vitro Th2 development was clearly enhanced by cerivastatin; however, Th2 development was not significantly altered in ex vivo study. This failure to promote Th2 development is consistent with previous data on simvastatin. Considering these data, statins conceivably inhibit Th1 development without enhancing compensatory Th2 development in vivo.
More interestingly, we clearly demonstrated that the treatment with cerivastatin was associated with a significant improvement of morphological injury in glomeruli and a decrease in proteinuria in OVA-immunized mice. These effects were not associated with cholesterol reduction. The decrease of glomerular sclerosis by cerivastatin treatment was positively related to the suppression of IFN-γ-producing Th1 response in draining LNCs (r = 0.54, P < 0.05). These results strongly suggest that the Th1/Th2 alterations and the following changes of cytokines expression might be responsible for the attenuation of inflammatory lesions in the glomeruli. In fact, recent studies have found that Th1 cells may be more important in mediating glomerular sclerosis than humoral factors related to immune mechanism.\textsuperscript{29,30} Furthermore, Radeke et al\textsuperscript{31} have shown that OVA-specific Th1 cells alone are sufficient to induce glomerulonephritis together with antigen by using SCID mice lacking humoral immunity. Taken together, it seems highly possible that cerivastatin treatment protects renal glomeruli against inflammatory process, probably through inhibition of Th1 response. In fact, it is well-known that statins have demonstrated beneficial effects in different models of progressive renal dysfunction, and part of the benefit is actually independent of the lipid-lowering effect.\textsuperscript{31} The non–lipid-mediated mechanisms consist of inhibition of chemokines, such as monocyte chemotactant protein-1,\textsuperscript{32} and reduction of proliferation of mesangial cells,\textsuperscript{33,34} renal epithelial tubular cells,\textsuperscript{35} and vascular smooth muscle cells.\textsuperscript{36,37}

Finally, our present results, if combined with data reported by other investigators,\textsuperscript{10–12} provide evidence that the beneficial effects of statins on the inflammatory diseases are mediated, at least in part, by the inhibition of Th1 development. The suppression of IFN-γ production of LNCs by cerivastatin treatment was associated with reduced mesangial matrix expansion of glomeruli of the kidney and attenuation of proteinuria, suggesting that HMG-CoA reductase inhibitors regulate in vivo the Th1/Th2 polarization and that such an immune-related mechanism possibly plays a role in improvement of the immune-induced glomerular injury with treatment of statins.

Acknowledgments

The study was supported in part by a Grant-in-Aid for Young Scientist from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (13770185). The authors express great thanks to Bayer, Wuppertal, Germany, and Pfizer, Sandwich, UK, for their kind gifts of cerivastatin and atorvastatin, respectively.

References


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_Circ Res._ 2003;93:948-956; originally published online October 16, 2003;
doi: 10.1161/01.RES.0000101298.76864.14

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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