Simvastatin Suppresses Glomerular Cell Proliferation and Macrophage Infiltration in Rats with Mesangial Proliferative Nephritis

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Abstract. Inhibition of 3-hydro-3-methylglutaryl coenzyme A reductase inhibits the production of mevalonate and has been shown to suppress proliferation in many cell types. Therefore, 3-hydro-3-methylglutaryl coenzyme A reductase inhibitors may have a beneficial effect in glomerular disease, because glomerular cell proliferation is a central feature in the active glomerular injury. This study examines the effect of simvastatin on glomerular pathology in a rat mesangial proliferative glomerulonephritis (GN) induced by anti-thymocyte antibody (anti-Thy1.1 GN). There was no difference in the degree of the antibody and complement-mediated initial injuries between simvastatin-treated and control GN rats. The most pronounced feature of simvastatin-treated GN was the suppression of the early glomerular cell proliferation. The proliferative activity was maximal at day 4 after disease induction (26.5 ± 7.0 of proliferating cell nuclear antigen-positive cells/glomerulus); however, approximately 70% of proliferation was suppressed by simvastatin treatment. At day 4 after disease induction, simvastatin administration also decreased α-smooth muscle actin expression in the glomerulus, which is a marker for mesangial cell activation. Inhibition of monocyte/macrophage recruitment into glomeruli by simvastatin was also a prominent feature. There was a 30% decrease in the number of glomerular ED-1+ cells by simvastatin treatment at day 2 after disease induction. Furthermore, simvastatin remarkably suppressed subsequent mesangial matrix expansion and type IV collagen accumulation in glomeruli. We also found that the platelet-derived growth factor expression was reduced in simvastatin-treated nephritic rats, which might simply reflect the reduction in mesangial cell proliferation and mesangial cellularity. There was no significant difference in plasma cholesterol or triglyceride levels between simvastatin- and vehicle-treated nephritic rats at day 2 and day 4, which corresponded to the times when simvastatin treatment resulted in a reduction in mesangial cell proliferation. In conclusion, this is the first report to find that mesangial cell proliferation and matrix expansion have been blocked by simvastatin in vivo. The protective effect of simvastatin in the matrix expansion in anti-Thy1.1 GN was partly by inhibition of mesangial cell proliferation and monocyte/macrophage recruitment into glomeruli, which were independent of a change in circulating lipids.

Many progressive glomerular diseases, including IgA nephropathy, membranoproliferative glomerulonephritis, lupus nephritis, and focal glomerulosclerosis are characterized by mesangial cell proliferation and accumulation of mesangial extracellular matrix. These processes may precede the development of glomerulosclerosis. Recent experimental studies clearly demonstrated the important role of proliferation and activation of mesangial cells in subsequent matrix expansion in the glomerulus (1,2). Therefore, the regulation of mesangial cell proliferation may provide useful insights into future therapeutic strategies for human progressive glomerular diseases (3). Products of mevalonate pathway play an important role in DNA replication and cell proliferation. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibits the production of mevalonate and has been shown to suppress proliferation in many cell types in vitro (4–11). This pharmacologic action of HMG CoA reductase inhibitors raised the possibility that they may have a beneficial effect in glomerular disease. Indeed, lovastatin inhibits proliferation of rat mesangial cells (12) and simvastatin inhibits platelet-derived growth factor (PDGF)-induced DNA synthesis in human mesangial cells in vitro. (13). These studies suggest that HMG CoA reductase inhibitors may suppress mesangial cell proliferation and prevent subsequent development of glomerular sclerosis in progressive glomerular diseases in vivo, in addition to their potential beneficial effect on glomerular injury by lowering serum lipids, as shown previously (14,15).

This study was designed to examine the effect of simvastatin on glomerular pathology in a rat model of mesangial proliferative glomerulonephritis induced by anti-thymocyte monoclonal antibody (anti-Thy1.1 GN). This model was chosen because previous studies have demonstrated that it is...
Table 1. Score for αSMA staining, mesangiolysis, mesangial matrix expansion, type IV collagen staining, and PDGF B-chain protein expression

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* Vehicle: vehicle-treated anti-Thy1.1 nephritic rats; simvastatin: simvastatin-treated anti-Thy1.1 nephritic rats (n = 8, respectively).
SMA, α-smooth muscle actin; PDGF, platelet-derived growth factor.

** P < 0.01 versus vehicle-treated rats on the same experimental day.

* P < 0.05 versus vehicle-treated rats on the same experimental day.

Characterized by macrophage infiltration, mesangial cell proliferation, and subsequent mesangial matrix expansion (1,16–18). Although anti-Thy1.1 GN is not a model of progressive injury and is associated with a mild decline in renal function, it is thought to be the best model for studying the effect of simvastatin on mesangial cell proliferation and matrix expansion.

Materials and Methods

Experimental Protocol and Disease Model

The study was designed to determine whether simvastatin (an HMG CoA reductase inhibitor) reduces glomerular injury in experimental mesangial proliferative nephritis (anti-Thy1.1 model). Simvastatin (Banyu Pharmaceutical, Tokyo, Japan) was diluted to 0.2% in vehicle (0.5% carboxymethyl cellulose/0.1 M phosphate-buffered saline, Catayama Kagaku, Osaka, Japan). Ninety-six Sprague-Dawley rats were prepared (Nippon Kagaku Doubutu, Tokyo, Japan) and were divided into two groups: simvastatin-treated (n = 48) and vehicle-treated (n = 48). Administration of simvastatin (2 mg/kg twice a day by gastric tube) or vehicle (for control) was started from 2 d before disease induction and was continued to the day of nephrectomy. The amount of simvastatin administered per rat per body weight per day was decided according to a previous report (19). Six groups of simvastin- or vehicle-treated rats were studied: rats without disease induction (day 0, n = 8 of simvastatin-treated and n = 8 of vehicle-treated rats, respectively) and rats with anti-Thy1.1 GN at day 2 after disease induction (n = 8, respectively), at day 4 (n = 8, respectively), at day 7 (n = 8, respectively), at day 12 (n = 8, respectively), and at day 20 (n = 8, respectively). The disease was induced by a single intravenous injection of anti-rat Thy1.1 monoclonal antibody (2.5 ml/kg of 10% solution) (Cedarlane Laboratories Limited, Hornby, Ontario, Canada).

In each group, the rats were sacrificed, the kidneys were removed, and total RNA was extracted for Northern blot analysis, using specific probes for PDGF B-chain (20) and 28S ribosomal RNA (21). For each group, Northern blots were performed on glomerular RNA of two sets of four kidneys each. Kidneys from all rats in each group were also subjected to histologic examination. The number of proliferating cells, infiltrating monocytes/macrophages, and activated mesangial cells were assayed by enumerating the number of cells per glomerular cross section that stained for proliferating cell nuclear antigen (PCNA), ED-1, or α-smooth muscle actin (αSMA), respectively, as described previously (22,23). In addition, the degree of mesangiolysis was evaluated by periodic acid-Schiff (PAS)-stained sections (22). For the evaluation of mesangial matrix expansion, sections stained for type IV collagen as well as for PAS were used (1,22), because semiquantitative scores for the staining of type IV collagen mainly reflect changes in the extent rather than the intensity of mesangial matrix (1). Furthermore, the glomerular expression of PDGF B-chain was studied by immunohistochemistry (16). All slides were coded and then presented to two doctors for evaluation of both pathologic changes on PAS-stained sections and the immunohistochemical staining as shown below.

Renal Morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution (24) and embedded in paraffin. Four-micrometer sections were stained with PAS reagent and counterstained with hematoxylin. In the PAS-stained sections, the total number of nuclei per glomerular cross section was determined, and mesangiolysis was graded semiquantitatively on a scale of 0 to 4+ as
Immunohistochemical Staining for Proliferating Cells, Monocytes/Macrophages, αSMA, PDGF B-Chain, and Type IV Collagen

Kidney tissues from four rats in each group were fixed in methyl Carnoy's solution, and 4-μm sections were stained by the indirect immunoperoxidase method. Primary antibodies included:

- 19A2 (Coulter Immunology, Hialeah, FL), a murine monoclonal antibody against human PCNA, which is an auxiliary protein to DNA polymerase delta that is expressed in late G1, peaking during S phase, and extending to the M phase of the cell cycle;
- ED-1 (Chemicon International, Temecula, CA), a murine monoclonal IgG to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells;
- An IgG fraction of murine monoclonal antibody to αSMA (DAKO, Glostrup, Denmark), which has been shown to stain activated mesangial cells in vivo (25);
- An IgG fraction of polyclonal rabbit antihuman PDGF-BB (Genzyme Diagnostics, Cambridge, MA) (26); and
- An IgG fraction of polyclonal rabbit anti-mouse collagen IV (Collaborative Research, Bedford, MA).

For all staining, negative controls consisted of substitution of the primary antibody with an equivalent concentration of an irrelevant murine monoclonal antibody. Quantification of proliferating (PCNA\textsuperscript{+}) cells and macrophages (ED-1\textsuperscript{+} cells) was performed by examining, in a blinded manner, all glomeruli containing more than 20 discrete capillary segments each (range, 22 to 36; mean 28.7 ± 8.3) in each biopsy and enumerating the number of positive cells per glomerular cross section.

For the evaluation of the staining for αSMA, PDGF B-chain, or type IV collagen, each glomerulus was graded semiquantitatively, as described previously (1,16): 0, diffuse, very weak, or absent mesangial matrix staining and no localized increase of staining; 1+, diffuse, weak mesangial matrix staining with 1 to 25% of the glomerular tuft showing focally increased staining; 2+, 25 to 50% of the glomerular tuft demonstrating focal, strong staining; 3+, 50 to 75% of the glomerular tuft staining strongly in a focal manner; and 4+, >75% of the glomerular tuft staining strongly.

Isolation of Glomerular RNA

Glomeruli were isolated by differential sieving (27). To preserve the integrity and stability of the glomerular RNA, all isolation steps were done at 4°C using diethylpyrocarbonate (Sigma, St. Louis, MO)-treated phosphate-buffered saline, autoclaved or baked sieves, and glassware and using as little time as possible for the preparation. Less than 20 min elapsed from nephrectomy to dissolution of the isolated glomeruli in RNAzol\textregistered B solution (Cinna/Biotex Laborato-
ries, Houston, TX). Total RNA was extracted from isolated glomeruli from all groups of rats with RNAzol\textregistered B following the manufacturer's instructions as reported previously (2,23).

Northern Blot Analysis

Total glomerular RNA was denatured and electrophoresed through a 1% agarose gel (15 μg of RNA/lane), transferred to a nylon membrane (Hybond N\textsuperscript{+}, Amersham, Arlington Heights, IL), and baked at 80°C for 2 h. Examination of the membrane under ultraviolet light in the presence of ethidium bromide demonstrated good resolution and integrity of the 28S and 18S ribosomal bands (23).

Figure 1. Total glomerular cells and proliferating cell nuclear antigen (PCNA)-positive cells/glomerulus (cells/glm) in simvastatin-treated and vehicle-treated glomerulonephritis (GN) rats. Total glomerular cells in both groups decreased below normal at day 2 after disease induction, respectively, followed by a rapid increase at day 4. The increase in glomerular cellularity from day 4 to day 20 was significantly suppressed by simvastatin treatment. As reported previously, the degree of PCNA expression accurately predicts the subsequent cellular response, and the number of PCNA\textsuperscript{+} cells increased from day 2 in vehicle-treated GN rats. The number of PCNA\textsuperscript{+} cells was significantly lower in simvastatin-treated GN rats at days 2, 4, and 7, compared with vehicle-treated GN rats, respectively (n = 8 in each group). □, vehicle-treated GN rats; ■, simvastatin-treated GN rats. *P < 0.01 versus vehicle-treated GN rats in the same experimental day, respectively.

reported previously (22). Furthermore, mesangial matrix expansion was evaluated on the same sections, and each glomerulus was graded as follows: 0, no matrix expansion; 1+, matrix expansion below 25% of the glomerular tuft; 2+, matrix expansion between 25 and 50% of the glomerular tuft; 3+, matrix expansion between 50 and 75% of the glomerular tuft; 4+, matrix expansion over 75% of the glomerular tuft. A minimum of 20 glomeruli was examined per biopsy (range, 20 to 47).
Figure 2. Immunostaining for PCNA cells in vehicle-treated (A through F) and simvastatin-treated (G through L) GN rats. The increase in the PCNA\(^*\) cells/glom began at day 2, peaked at day 4, and then slowly decreased during the course of the disease in this model. There was no difference in the number of PCNA\(^*\) cells/glom between both groups at day 0 (A and G); however, simvastatin treatment was associated with a drastically reduced proliferative response at days 2, 4, and 7. Immunoperoxidase, \(\times600\). A and G: day 0; B and H: day 2; C and I: day 4; D and J: day 7; E and K: day 12; F and L: day 20 (after disease induction, respectively).

The DNA probes used for Northern blot analysis were as follows:

- PDGF B-chain. Murine PDGF B-chain genomic DNA (a 326-bp fragment) was cloned into the Smal site of the transcription vector pGEM1 at a site between the SP6 and T7 promoters (provided by Dr. Daniel F. Bowen-Pope, University of Washington, Seattle, WA) (20).

- 28S ribosomal RNA. A bovine 280-bp cDNA probe was used to detect 28S ribosomal RNA (provided by Drs. Luisa Iruela-Arispe and Helene Sage, University of Washington) (21).

Probes of PDGF B-chain and 28S ribosomal RNA were labeled with [\(\alpha\text{-}^{32}\text{P}\)]-deoxyctydine 5-triphosphate (3000 Ci/mmol, New England Nuclear Research Products, Tokyo, Japan) by random primer extension. Membranes were prehybridized in a solution containing 50% (vol/vol) formamide, 5\(\times\) saline-sodium phosphate-ethylenediaminetetra-acetic acid (SSPE), 5\(\times\) Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS), and 100 \(\mu\)g of salmon sperm DNA per milliliter. Hybridization was performed with the same solution, to which 2 \(\times\) \(10^6\) cpm/ml of \(^{32}\)P-labeled cDNA was added for 24 h at 42\(^\circ\)C. Blots were then washed three times in 2\(\times\) SSPE, 0.1% SDS for 5 min each at room temperature and then in 0.1% SSPE, 0.1% SDS at 50\(^\circ\)C. After drying, membranes were exposed to nonpreflashed Kodak X-OMAT film (Kodak, Tokyo, Japan) with enhancing screens at -70\(^\circ\)C. The obtained autoradiograms were read by linear densitom-
macrophages/glom

Figure 3. The number of glomerular macrophages (ED-1* cells) in vehicle-treated and simvastatin-treated GN rats. There was a 30% decrease in glomerular macrophage infiltration at day 2 after disease induction by simvastatin treatment (12.0 ± 1.7 in simvastatin-treated, n = 8, and 17.5 ± 2.6 in vehicle-treated GN rats, n = 8). ■, vehicle-treated GN rats; □, simvastatin-treated GN rats. *P < 0.01 versus vehicle-treated GN rats in the same experimental day, respectively.

Administration of Simvastatin to Normal Rats
We also performed another set of experiments to document the effects of simvastatin on normal control rats and excluded any non-specific toxic effect of the drug. We prepared 17 Sprague Dawley rats. At first, nephrectomy was done on five rats before simvastatin treatment. Administration of simvastatin (2 mg/kg twice a day by gastric tube) was started on 12 rats and was continued to the day of renal biopsy. Renal biopsy was performed on days 2, 4, 7, and 12 (n = 6, respectively; renal biopsy was done at days 2 and 7 on six rats and at days 4 and 12 on another set of six rats). Renal tissues were fixed in methyl Carnoy’s solution followed by PAS staining. Immunohistochemistry for PCNA was done on all tissue sections.

Measurement of Serum Lipids and Serum Creatinine (n = 8 in each group)
Fasting tail vein blood samples were obtained from rats lightly anesthetized with ether just before nephrectomy. Serum cholesterol and triglycerides were measured using an autoanalyzer (ASTRA, Beckman Instruments, Brea, CA), and serum creatinine was measured by enzyme assay, using a creatinine assay kit (Yatron, Tokyo, Japan).

Measurement of Urine Protein Excretion (n = 8 in each group)
Twenty-four-hour urine collections were obtained from rats that were individually housed in metabolic cages. Rats were fasted during the collection period, but were allowed free access to water. Urine protein excretion was measured by a sulfoalicylic acid method (27), using a whole serum standard (Lab Trol, Dade Diagnostics, Aquado, Puerto Rico).

Statistical Analyses
All values are expressed as mean ± SD unless stated otherwise. Statistical significance (defined as P < 0.05) was evaluated using the t test or one-way ANOVA with modified t test performed using the Bonferroni correction (28).

Results
Simvastatin Treatment in Normal Rats
Simvastatin treatment (2 mg/kg twice a day) was well tolerated in normal rats (the number of PCNA+ cells/glomerulus [cells/glom] was 1.4 ± 0.4 at day 2, 1.4 ± 0.1 at day 4, 1.3 ± 0.6 at day 7, and 1.4 ± 0.3 at day 12) and was associated with normal renal morphology by PAS staining. The number of PCNA+ cells/glom in normal rats (before simvastatin treatment, n = 5) was 1.3 ± 0.5.
Simvastatin Treatment in Anti-Thy1.1 Nephritis

Effect of Simvastatin on Mesangiolysis. The injection of anti-Thy1.1 antibody in rats is associated with an acute complement-dependent mesangiolysis with a loss of mesangial cells that peaks at 24 to 48 h (16–18). Simvastatin-treated rats had equivalent mesangiolysis as vehicle-treated rats at all times studied (Table 1).

The acute mesangiolysis is associated with a reduction in glomerular cell number that was also similar between groups (total glomerular cellularity of 69.1 ± 4.3 at day 0 and 59.0 ± 5.7 cells/glom at day 2 in vehicle-treated rats versus 70.1 ± 3.8 at day 0 and 57.1 ± 2.9 cells/glom at day 2 in simvastatin-treated rats). These studies suggest that simvastatin did not block the acute mesangial injury induced by anti-Thy1.1 antibody.

Effect of Simvastatin on Mesangial Cell Proliferation. Previous studies in the anti-Thy1 model have shown that mesangial cell proliferation begins at day 2, peaks between day 3 and day 5, and is resolved by day 14 (16,17,23,24). This pattern was observed in vehicle-treated rats, with a marked increase in the number of PCNA+ cells at day 2 (14.9 ± 2.0 positive cells/glom, n = 8), day 4 (26.5 ± 6.5 positive cells/glom, n = 8), and day 7 (8.0 ± 2.1 positive cells/glom, n = 8) compared with normal rats (1.3 ± 0.3, n = 8). Simvastatin treatment was associated with a drastically reduced proliferative response at all time periods, including day 2 (3.9 ± 1.0, n = 8, P < 0.01), day 4 (8.0 ± 2.7, n = 8, P < 0.01), and day 7 (2.0 ± 1.4, n = 8, P < 0.01).

The increase in glomerular cell proliferation in vehicle-treated rats was associated with a marked increase in glomerular cellularity. After the nadir of glomerular cellularity noted at day 2 (59.0 ± 5.7 cells/glom), a marked rebound in cellularity was observed in vehicle-treated rats, with a near doubling of the cell count by day 7 (85.2 ± 4.0 cells/glom at day 4 and 100.7 ± 6.6 cells/glom at day 7). In contrast, simvastatin treatment was not associated with the expected rebound in cellularity (57.1 ± 2.9 cells/glom at day 2, 75.5 ± 3.4 cells/glom at day 4, P < 0.01 versus vehicle-treated rats; 74.4 ± 3.9 cells/glom at day 7, P < 0.01 versus vehicle-treated rats) as shown in vehicle-treated rats, consistent with the effect of simvastatin to reduce the mesangial cell proliferation (Figures 1 and 2).

Effect of Simvastatin on the Initial Macrophage Infiltration in Glomeruli. Induction of anti-Thy1.1 GN resulted in an early glomerular influx of monocytes/macrophages (maximal at day 2 after disease induction), which then slowly decreased during the course of the disease, as reported previously (17,29). There was a 30% decrease in glomerular macrophage infiltration at day 2 after disease induction by simvastatin treatment (17.5 ± 2.6 in vehicle-treated and 12.0 ± 1.7 in simvastatin-treated nephritic rats, P < 0.01) (Figures 3 and 4).

Effect of Simvastatin Treatment on de Novo αSMA Expression in Mesangial Cells. Mesangial cell proliferation in glomerulonephritis in the rat is associated with a distinct phenotypic change in which mesangial cells assume smooth muscle cell characteristics (22). Therefore, de novo αSMA expression is a marker for mesangial cell activation (22). An increased αSMA expression was present by day 2 after disease induction in both groups, followed by a higher increase at days 4, 7, and 12. Simvastatin treatment markedly reduced αSMA expression at day 4 after disease induction compared with control GN rats (3.5 ± 0.3 versus 2.0 ± 0.3, P < 0.01) (Table 1, Figure 5).

Effect of Simvastatin Treatment on Mesangial Matrix Expansion and Type IV Collagen Accumulation in Glomeruli. Anti-Thy1.1 GN is characterized by initial macrophage infiltration and mesangial cell proliferation, and subsequent mesangial matrix expansion by accumulation of extracellular matrix protein. Significant higher matrix expansion by PAS-stained sections (from day 4 to day 20) and higher staining scores for type IV collagen (from day 2 to day 20) than those of day 0 were shown in vehicle-treated rats, respectively, and were maximal at day 7, which followed maximal glomerular cell proliferation at day 4. Simvastatin treatment dramatically

Figure 5. Immunostaining for α-smooth muscle actin (αSMA) in vehicle-treated (A) and simvastatin-treated (B) GN rats at day 4 after disease induction. Expression of smooth muscle cell phenotype by mesangial cells was prominent in vehicle-treated GN rats (staining score 3.5 ± 0.3/glomerular cross section); however, simvastatin treatment markedly reduced αSMA expression (2.0 ± 0.3, P < 0.01 versus vehicle-treated GN rats). Immunoperoxidase, ×600.
suppressed mesangial matrix expansion and type IV collagen accumulation from day 4 to day 20 (Table 1, Figures 6 and 7).

**Effect of Simvastatin Treatment on Immunostaining for PDGF B-Chain and Expression of PDGF B-Chain mRNA.** Whereas normal rat glomeruli had minimal PDGF B-chain protein (by immunostaining) or mRNA (by Northern blot analysis), in anti-Thy1.1 GN a marked increase in PDGF B-chain mRNA and protein was observed in mesangial areas in the proliferative phase, as reported previously (2,16). Simvastatin treatment significantly reduced the PDGF B-chain protein expression compared with nephritic control rats from day 4 to day 20 (Table 1, Figure 8). The decrease in PDGF B-chain mRNA expression in simvastatin versus control rats was observed from day 2. PDGF B-chain mRNA expression in simvastatin-treated rats, which was normalized for equivalent amounts of 28S ribosomal RNA per lane, respectively, was 32% (day 2), 24% (day 4), 50% (day 7), and 43% (day 12) of vehicle-treated rats (Figure 9). These results demonstrated that the decrease in PDGF gene expression by simvastatin treatment preceded the decrease in PDGF protein.

*Figure 6.* Periodic acid-Schiff-stained sections in vehicle-treated (A through C) and simvastatin-treated (D through F) GN rats. As shown in Table 1, matrix expansion in glomeruli was suppressed from day 4 to day 20 by simvastatin treatment. Day 7 to day 20 are shown here. Marked matrix expansion with hypercellularity was remarkable at day 7 in vehicle-treated GN rats, which then slowly decreased during the course of the disease. Suppression of mesangial matrix expansion by simvastatin treatment was shown at days 7, 12, and 20, respectively (A and D; day 7; B and E; day 12; C and F; day 20). Periodic acid-Schiff staining, ×600.
Figure 7. Immunostaining for type IV collagen in vehicle-treated (A through C) and simvastatin-treated (D through F) GN rats. Type IV collagen accumulation was observed in the mesangial area. As shown in Table 1, increased staining score type IV collagen was shown from day 2 to day 20 in this model and was maximal at day 7, which followed maximal glomerular cell proliferation at day 4, and simvastatin treatment dramatically suppressed type IV collagen accumulation from day 4 to day 20. Immunostaining at days 7, 12, and 20 are shown here. Staining intensity for type IV collagen and its expansion were remarkably suppressed at day 7 (D) in simvastatin-treated GN rats compared with vehicle-treated GN rats (A). The reduction of staining score for type IV collagen was also shown at day 12 (E) and day 20 (F) by simvastatin treatment compared with vehicle-treated group (B and C, respectively). (A and D: day 7; B and E: day 12; C and F: day 20). Immunoperoxidase, ×600.

Effect of Simvastatin Treatment on Serum Lipids in Mesangial Proliferative Nephritis. In control nephritic rats, a rise in cholesterol was noted at day 7 of disease. This was blocked by simvastatin treatment. The level in simvastatin-treated rats also tended to be lower than vehicle-treated rats with nephritis at other time points, but statistical significance was only observed at days 7 and 20 (Figure 10). No effect of simvastatin was noted in triglyceride levels during the course of the nephritis (Figure 10).

Urinary Protein Excretion and Serum Creatinine Concentration. Urinary protein excretion and serum creatinine concentration are less relevant in the anti-Thy1.1 model because renal dysfunction and increase in the urinary protein excretion are transient and not so prominent. In control ne-
Figure 8. Immunostaining for PDGF B-chain in vehicle-treated (A and B) and simvastatin-treated (C and D) GN rats. As shown in Table 1, simvastatin treatment significantly reduced the PDGF B-chain protein expression compared with nephritic control rats from day 4 to day 20. Immunostaining at days 4 and 7 are shown here. Diffuse expression of PDGF B-chain protein in the mesangial area was prominent in vehicle-treated GN rats at day 4 (A); however, the expression was decreased in simvastatin-treated rats (C). The reduction in the staining for PDGF B-chain was also shown in the simvastatin-treated rats (D) at day 7 compared with vehicle-treated rats (B). Immunoperoxidase, ×600.

Figure 9. Northern blot analysis of total glomerular RNA (15 μg/lane) obtained in normal rats (nl), in simvastatin-treated GN rats at day 2 (d2), day 4 (d4), day 7 (d7), and day 12 (d12), and in vehicle-treated GN rats at day 2, day 4, day 7, and day 12 for the gene expressions of PDGF B-chain and 28S ribosomal RNA (28S rRNA). The decrease in PDGF B-chain mRNA expression in simvastatin versus control rats was observed from day 2. PDGF B-chain mRNA expression in simvastatin-treated rats, which was normalized for equivalent amounts of 28S ribosomal RNA per lane, respectively, was 32% (day 2), 24% (day 4), 50% (day 7), and 43% (day 12) of vehicle-treated rats. From these, the decrease in PDGF gene expression by simvastatin treatment preceded the decrease in PDGF protein.
phritic rats, urinary protein excretion was markedly increased at day 2, and then decreased gradually from day 4 to day 20 (Table 2). The remarkable increase in urinary protein excretion at day 2 may reflect mesangiolysis. There was no difference in the intensity of mesangiolysis between vehicle-treated and simvastatin-treated rats, and there was no difference in the amount of proteinuria at day 2 between the two groups. The urinary protein excretion at day 4 in simvastatin-treated rats was significantly lower than that in vehicle-treated rats, which may be due to suppression of active proliferation in the glomerulus by simvastatin (Table 2). The increase in serum creatinine concentration was prominent at day 2 in both groups, which may be due to mesangiolysis. Then it decreased to normal range gradually. In simvastatin-treated rats, serum creatinine concentration at day 7 was significantly low. Suppression of glomerular disease by simvastatin may contribute to the inhibition of renal dysfunction in this model.

Discussion

Mevalonate metabolism produces isoprenoid intermediates that are important for DNA replication and cell proliferation (8). Therefore, the inhibition of HMG CoA reductase by HMG CoA inhibitor suppresses cell proliferation in many cell types in vitro by blocking mevalonate metabolism production (4–11). The in vitro antiproliferative effect of HMG CoA reductase inhibitor was demonstrated in cultured human and rat glomerular mesangial cells (12,13). The in vivo ameliorative effect of HMG CoA reductase inhibitor on experimental glomerular injuries through lowering serum lipids was reported (15); however, part of the beneficial effect of HMG CoA reductase inhibitor on experimental glomerular diseases may be via a direct effect on mesangial cell proliferation.

In the present study, we demonstrated that treatment of anti-Thy1.1 GN rats with simvastatin inhibits the initial macrophage infiltration, early glomerular cell proliferation, and subsequent mesangial matrix expansion in this model. Anti-Thy1.1 GN is characterized by initial mesangiolysis, platelet and monocyte/macrophage infiltration, subsequent mesangial cell proliferation, and final mesangial matrix expansion (1,16–18). Experiments on the administration of simvastatin in normal rats showed that there was no toxic effect of the drug and that renal morphology remained normal. There was no difference in the degree of mesangiolysis or hypocellularity at day 2 after disease induction between simvastatin-treated and control nephritic rats. The observation that the severity of the initial glomerular injury in both groups was equivalent suggests that the treatment with simvastatin did not alter the disease by modulating the initial antibody and complement-mediated injury.

The most pronounced feature of simvastatin-treated anti-Thy1.1 GN was suppression of early glomerular cell proliferation, as evaluated by both total glomerular cellularity and the number of PCNA+ cells per glomerulus. The proliferative activity in this model was maximal at day 4 after disease induction (26.5 ± 7.0 of PCNA+ cells/glom; however, approximately 70% of proliferation was suppressed in simvastatin-treated GN rats. Simvastatin administration in nephritic rats also decreased αSMA expression in the glomerulus at day 4 after disease induction. These observations suggest that the suppression of glomerular cell proliferation in simvastatin-treated nephritic rats was mainly due to the suppression of mesangial cell proliferation, because the major source of the proliferating cells in anti-Thy1.1 GN is the mesangial cell (17), and αSMA expression is a marker for mesangial cell activation (22).

Inhibition of monocyte/macrophage recruitment into glomeruli by simvastatin was also a prominent feature in the present study. Macrophages are thought to be important effector cells in glomerular injury and matrix expansion. Therefore, regulation of macrophage infiltration might lessen subsequent glomerular damage. Recently, Park et al. reported that lovastatin treatment...
Table 2. Urinary protein excretion and serum creatinine concentration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
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</thead>
<tbody>
<tr>
<td>Urinary protein excretion (mg/d)</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0</td>
</tr>
<tr>
<td>simvastatin</td>
<td>2.4</td>
</tr>
<tr>
<td>Serum creatinine (mg/d)</td>
<td></td>
</tr>
<tr>
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<td>0.74</td>
</tr>
<tr>
<td>simvastatin</td>
<td>0.76</td>
</tr>
</tbody>
</table>

a Vehicle: vehicle-treated anti-Thy1.1 nephritic rats; simvastatin: simvastatin-treated anti-Thy1.1 nephritic rats (n = 8, respectively).
b P < 0.05 versus vehicle-treated rats on the same experimental day.

simvastatin reduced the glomerular macrophage infiltration in nephritic rats induced with puromycin aminonucleoside (30). The mechanism by which HMG CoA reductase inhibitors block monocyte/macrophage infiltration in glomeruli is not completely understood, but it may relate to the ability of these agents to inhibit important monocyte chemokines and adhesion molecules (31–33). Recently, Keane’s group reported that the mevalonate pathway can mediate chemokine (MCP-1) expression in mesangial cells (34), so it is tempting to speculate that simvastatin could be acting by blocking MCP-1 expression.

Abnormal accumulation of extracellular matrix in the glomerulus is a major biological feature of progressive glomerular injury (35), and is preceded by mesangial cell proliferation in anti-Thy1.1 GN (16,17). However, if only one injection of anti-Thy1.1 antibody is given, most glomeruli recover their normal architecture by 6 wk (1). Thus, mesangial matrix expansion in anti-Thy1.1 GN is reversible, and the regulation of mesangial cell proliferation may therefore modulate mesangial matrix expansion. In this regard, the suppression of matrix expansion and the accumulation of type IV collagen in glomeruli in simvastatin-treated rats with nephritis might be due to the inhibition of mesangial cell proliferation. However, it is also possible that simvastatin could act by direct effects on mesangial matrix production.

We found that simvastatin reduced mesangial cell proliferation in our model. Previous studies have suggested that mesangial cell proliferation in this model is largely mediated by PDGF (16,17) and, to a lesser extent, by basic fibroblast growth factor (36) and endothelin (23). Because HMG CoA reductase inhibitors can inhibit PDGF-mediated mesangial cell proliferation in vivo, it is likely that these agents could inhibit proliferation in vivo via a similar mechanism.

We also found that the PDGF expression was reduced in simvastatin-treated nephritic rats. Previously, we have reported that PDGF is primarily expressed by mesangial cells in this model (17), so the reduction in expression may simply reflect the reduction in mesangial cell proliferation and mesangial cellularity. This is further supported by the observation that lovastatin does not block PDGF expression by mesangial cells exposed to serum (37) despite its ability to inhibit the proliferative response (12).

Many glomerular diseases are frequently associated with lipid abnormalities, and experimental evidence suggests that lipids may be important modulators in the progression of glomerulosclerosis (33). In the present study, there was no significant difference in plasma cholesterol or triglyceride levels between simvastatin- and vehicle-treated GN rats at days 2 and 4, which corresponds to the times when simvastatin treatment resulted in a reduction in mesangial cell proliferation. This suggests that the inhibitory effects of simvastatin on mesangial cell proliferation and macrophage infiltration was independent of a change in circulating lipids in anti-Thy1.1 GN.

A significant difference between vehicle-treated GN and simvastatin-treated GN rats in urinary protein excretion was observed only at day 4, and serum creatinine concentration was significantly low only at day 7 in simvastatin-treated rats. These results are not surprising, as anti-Thy1.1 GN is associated with only a mild decline in renal function and a small amount of urinary protein excretion in proliferative phase and in subsequent matrix expansion phase, although renal dysfunction and urinary protein excretion are moderate from mesangiolysis to early proliferative phase. This model is also reversible. The importance lies in the ability of simvastatin to remarkably suppress the proliferation and matrix expansion.

In conclusion, this is the first time that mesangial cell proliferation and matrix expansion have been blocked by simvastatin in vivo. The protective effects of simvastatin in the matrix expansion in anti-Thy1.1 GN was partly by inhibition of mesangial cell proliferation and monocyte/macrophage recruitment into glomeruli. These results indicate that HMG CoA reductase inhibitor may be considered as a therapeutic strategy in progressive glomerular diseases.

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