Rosuvastatin is additive to high-dose candesartan in slowing progression of experimental mesangioproliferative glomerulonephrosis.

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Submitted 30 March 2007; accepted in final form 14 December 2007


Rosuvastatin is additive to high-dose candesartan in slowing progression of experimental mesangioproliferative glomerulonephrosis (GS). Progressive mesangioproliferative glomerulonephritis, mostly IgA nephropathy, is a major cause of end-stage kidney disease worldwide. In a chronic-progressive model of mesangioproliferative GS, we tested the renoprotective efficacy of rosuvastatin alone and in combination with a high-dose of the AT1 blocker candesartan. Treatment was started 1 wk after disease induction (anti-thy1 antibody injection into uninephrectomized rats) and continued until week 20. Tubulointerstitial expression of the key fibrosis mediator transforming growth factor (TGF)-β served as the main marker of disease progression. Compared with the untreated GS rats (475 ± 52 pg/ml), tubulointerstitial TGF-β1 protein expression was significantly reduced by both single therapies (rosuvastatin −47%, candesartan −51%, P < 0.01). Tubulointerstitial matrix accumulation (matrix score in GS: 64 ± 7%) was relatively reduced by −45 and −52%, respectively (P < 0.01). The combination of rosuvastatin and candesartan had significantly greater effects on tubulointerstitial TGF-β1 expression (−82% vs. GS) and matrix accumulation (−83% vs. GS) (P < 0.001 vs. GS, P < 0.05 vs. single therapy) than either drug alone. Similar additive beneficial effects were observed for renal fibronectin and tissue inhibitor of metalloproteinase-1 expression, cell proliferation, macrophage infiltration, proteinuria, and kidney function. In conclusion, rosuvastatin limits the progressive course of anti-thy1-induced GS toward chronic tubulointerstitial fibrosis and renal insufficiency to a degree comparable to the one achieved by a high dose of the AT1 antagonist candesartan. Combined treatment yields significantly greater actions on renal TGF-β overexpression and matrix accumulation, cell proliferation, and macrophage infiltration. The results suggest that rosuvastatin and the AT1 blocker independently interfere with separate key pathways involved in the progression of chronic mesangioproliferative GS.

renal fibrosis; transforming growth factor-β1; IgA nephropathy

MESANGIOPROLIFERATIVE glomerulonephritis, generally presenting as IgA nephropathy, is the most frequent primary glomerular disorder worldwide (7, 9). End-stage renal disease and the need for renal replacement therapy occur when disease initially present in glomeruli progresses to affect the whole kidney. At the histological level, progression of mesangioproliferative glomerulonephritis is characterized by glomerulosclerosis (GS) and fibrotic destruction of the corresponding nephron tubule (tubulointerstitial atrophy and fibrosis) that closely parallels the ongoing loss in renal function (7, 9, 11, 25). This sequence generally involves increasing proteinuria, as well as tubulointerstitial accumulation of extracellular matrix proteins, proliferation of renal cells, and infiltration with macrophages (4, 10, 21). So far, pharmacological inhibition of angiotensin II by angiotensin-converting enzyme (ACE) inhibitors or AT1-receptor antagonists is the only clinically established therapeutic approach in slowing the course of chronic-progressive renal fibrosis in a manner independent of the underlying disorder, including mesangioproliferative glomerulonephritis (7, 11, 27). The present investigation focuses on the question of whether renoprotection by angiotensin II inhibition can be further enhanced by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, i.e., statins, using an experimental model of progressive mesangioproliferative nephropathy.

In rats, injection of anti-thy1 antibody leads to mesangioproliferative glomerulonephritis by binding of the antibody to thy1-expressing mesangial cells, resulting in complement-dependent cell lysis (16). An acute, reversible, and −4–wk course of the disease occurs when a relatively low dose of anti-thy1 antibody is injected into animals with two kidneys (15, 17). By contrast, when a relatively high antibody dose is administered to animals with prior uninephrectomy, the initial acute mesangioproliferative glomerulonephritis is followed by slow progression of the disease toward GS, tubulointerstitial fibrosis, and renal insufficiency over several months (15, 26). The latter model has been termed anti-thy1-induced chronic GS or anti-thy1-induced renal fibrosis (11, 25). The course of experimental progressive mesangioproliferative GS closely mimics advancing mesangioproliferative nephropathy in humans and thus can serve as an experimental model for studying human mesangioproliferative disease. Overexpression of the key profibrotic cytokine transforming growth factor (TGF)-β has been found to play a central role in the course of anti-thy1-induced acute and chronic renal matrix expansion (4, 17). In acute anti-thy1 glomerulonephritis, overproduction of TGF-β is transient, while it is persistent and increases in chronic anti-thy1 GS.

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In studies of acute anti-thy1 glomerulonephritis, the concept has been put forward that the TGF-β overexpression is a valid target that enables comparing and optimizing currently used and new anti-fibrotic drugs (19). For the ACE inhibitor enalapril and the AT1 antagonist losartan, it was shown that angiotensin II inhibition reduces TGF-β expression and matrix accumulation more effectively at doses higher than those used for controlling blood pressure. We have recently applied this concept to progressive glomerular disease and the model of progressive anti-thy1-induced GS by asking what current clinically available intervention could be added to angiotensin II inhibition to yield greater renal protection. In a previous study (11), we found that one prime candidate for such an additive drug, i.e., mycophenolate mofetil, an anti-inflammatory transplant drug, was equally effective as a high-dose of the ACE inhibitor enalapril in chronic anti-thy1 GS. However, in the presence of high-dose angiotensin inhibition, mycophenolate mofetil did not provide additional protective actions on progressive renal fibrosis and renal insufficiency (11). This was a somewhat surprising finding, since chronic anti-thy1 GS shows a prominent tubulointerstitial round-cell infiltration, and therapeutic additivity had appeared to just be a logical consequence.

In the present study, we sequentially asked whether HMG-CoA reductase inhibitors yield additive renal protection beyond angiotensin II blockade in the chronic model of progressive mesangiproliferative nephropathy induced by anti-thy1 injection. HMG-CoA reductase is the rate-limiting step in endogenous cholesterol biosynthesis through which statins block the production of mevalonate (5). Like angiotensin inhibitors, statins have shown positive effects in a number of experimental models of renal disease, including acute anti-thy1 mesangio-proliferative glomerulonephritis (1, 13, 28). In our experiments, treatments with rosuvastatin or a high dose of the AT1 antagonist candesartan, or both interventions together, were started 1 wk after antibody injection, at a time when the glomerular lesions were already established. In week 20 after disease induction, the actions of both single and combined interventions on key pathways of renal disease progression, such as fibrogenesis, cell proliferation, and macrophage infiltration, were measured. Tubulointerstitial expression of the key fibrosis mediator TGF-β represented the main therapeutic target. Proteinuria, blood pressure, and renal function served as additional measures of disease severity and therapeutic efficacy.

METHODS

Materials. Unless otherwise indicated, materials, chemicals, or culture media were purchased from Sigma Chemical-Aldrich (Taufkirchen, Germany).

Animals. Male Wistar rats with a body weight of 150–180 g were obtained from Charles River (Sulzfeld, Germany). The animals were housed at a constant temperature with a 12:12-h dark-light cycle. Body weight was determined weekly. Animals were fed a normal protein diet (22.5% protein; Altromin, Lage, Germany) for at least 3 days before the start of the experiment to allow equilibration (25). Food and water intakes were monitored daily. Food and water intake was calculated by weighing the amount provided and the amount remaining after 24 h [24 h intake = amount provided (0 h) − amount remaining (24 h)]. Animal care and treatment conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by local authorities.

Induction of anti-thy1-induced chronic-progressive GS. Chronic-progressive GS was produced under anesthesia by surgically removing one kidney and intravenously injecting the monoclonal antibody MA1 1-22-3 [5 mg/kg body wt in phosphate-buffered saline (PBS), pH = 7.4] 3 days later (25). The antibody was produced from hybridoma cell lines, as previously described (11). MA1 1-22-3 antibody binds to a thy1-like antigen on the surface of mesangial cells of the kidney and causes a fast complement-dependent and nitric oxide-dependent mesangial cell lysis within the next 24 h. The progression in GS is linked to the uninephrectomy that is performed before anti-thy1 antibody injection, since the glomerular disease resolves over ~4 wk in animals with two kidneys (11, 15). Progression in chronic anti-thy1 GS is not primarily immune mediated. It is rather an experimental paradigm for a “one-hit, self-progressing kidney disease,” as it is seen in patients with deteriorating renal function and a transient glomerulonephritic episode in the past (18). Control animals with and without uninephrectomy were injected with equal volumes of PBS only.

Administration of rosuvastatin and candesartan. Rosuvastatin was given with the food at a daily dose of 10 mg/kg body wt. The dose was chosen on the basis of previous reports showing that this dose reduced hepatic cholesterol synthesis in rats by ~80% (14). The rosuvastatin-containing food was produced in our laboratory by using a standard powdered rat chow diet (22.5% protein, A1311, Altromin). The drug was mixed into the dry food powder in appropriate amounts, water was added to form pellets, and the air-dried pellets were subsequently given to the rosuvastatin-treated animals.

Candesartan was given with the drinking water at a dose of 10 mg/kg body wt. The drug inhibits the action of angiotensin II on the angiotensin type 1 receptor. The blood pressure-reducing dose of candesartan in hypertensive rats is ~1 mg/kg body wt (8, 15). In previous experiments, our laboratory has shown that at least double the blood pressure-reducing dose is needed to maximally limit TGF-β overexpression in an acute model of anti-thy1 glomerulonephritis (19). Thus, in the present study, rats were given 10 times the blood pressure-lowering dose.

Experimental groups and design. A 24-h urine was collected 1 wk after anti-thy1 antibody injection. Based on the 24-h proteinuria actually achieved, the diseased animals were randomly assigned to the different treatment groups, which were as follows: 1) uninephrectomized, anti-thy1-injected animals, no treatment (GS; n = 15); 2) uninephrectomized, anti-thy1-injected animals treated with rosuvastatin (GS-statin; n = 14); 3) uninephrectomized, anti-thy1-injected animals treated with candesartan (GS-AT1 blocker; n = 10); and 4) uninephrectomized, anti-thy1-injected animals treated with rosuvastatin and candesartan (GS both; n = 10). In addition, groups of nonnephrectomized, PBS-injected controls (2-K Control; n = 4) and uninephrectomized, PBS-injected controls (1-K Control; n = 5) were included.

In week 20, after 19 wk of rosuvastatin and/or candesartan administration, the effects of single and dual treatments on proteinuria, blood pressure, and renal function, as well as on glomerular and tubulointerstitial fibrosis, cell proliferation, and macrophage infiltration, were determined. Blood creatinine and urea concentrations, calculated creatinine clearance, and blood hematocrit served as markers of renal function. Glomerular and tubulointerstitial changes were analyzed separately. Glomeruli were isolated by a graded sieving technique. Since the renal cortex consists mainly of tubulointerstitial tissue (>95%), it was considered representative of the tubulointerstitial. Analysis of glomerular and tubulointerstitial fibrosis involved a computer-based histological calculation of the matrix actually accumulated, molecular analysis of TGF-β1 as key mediator of tissue fibrosis, measurement of fibronectin as a marker of matrix protein synthesis, and the protease inhibitor tissue inhibitor of metalloproteinase-1 (TIMP-1) as a marker of matrix protein degradation. Tubulointerstitial and glomerular macrophage infiltration and cell proliferation were analyzed by immunohistochemistry using an anti-macro-
phage/monocyte (ED1) or a proliferating cell nuclear antigen (PCNA) antibody, respectively.

Measurement of systolic blood pressure and proteinuria. Systolic blood pressure and proteinuria were assessed in weeks 1, 10, and 20 after anti-thy1 antibody injection. Blood pressure was measured in restrained conscious animals by a tail-cuff method, as previously described (25). For collection of a 24-h urine, animals were housed individually in metabolic cages. Urinary protein was measured using a pyrogallol red microtiter plate technique, as previously described (25). Proteinuria is expressed as milligrams of protein per 24 h.

Termination of experiment. The animals were anesthetized with 0.1 mg ketanest/0.01 mg xylazine per 100 g body wt (Ketamin 10%, WDT, Garbsen, Germany; Rompun 2%, Bayer Vital, Leverkusen; Germany), as previously described (25). Following a midline abdominal incision, 5 to 10 ml heparinized blood were drawn from the abdominal aorta, and kidneys were subsequently perfused with 30 ml ice-cold PBS. Materials and tissues were subsequently processed, as described in the following sections.

Blood and urine analysis. Plasma and urine creatinine and plasma urea were measured spectrophotometrically in enzyme-based assays (25). The glomerular filtration rate (GFR) was calculated on the basis of serum and urinary creatinine concentrations and the corresponding urine volume and is expressed as milliliters per minute per 100 g body wt. Hematocrit was determined in blood plasma tubes after a 10-min centrifugation. Plasma cholesterol and triglyceride levels were measured by standard enzymatic methods following a 10-h fasting period.

Light and immunohistochemistry microscopy. For histological examination, cortical tissue was fixed in 10% neutral-buffered formalin or snap frozen in liquid nitrogen. All microscopic examinations were performed in a blinded fashion. Three-micrometer sections of paraffin-embedded tissue were stained with periodic acid–Schiff reagent to analyze tubulointerstitial and glomerular fibrosis by a computer-based morphometric analysis (25). Renal sections were examined on a Leica DM LB2 light microscope (Leica Microsystems, Wetzlar, Germany) connected to a Axiocam HRc video camera and the Axiovision 4.00 image analysis system (both Karl Zeiss Vision, Munich, Germany) using a 10 × 10 orthographic grid superimposed on digital images. The relative degrees of tubulointerstitial fibrotic lesions, i.e., matrix deposition, cell infiltration, tubular atrophy, and dilation, were calculated in 15 randomly selected cortical areas per animal, observed at ×200 magnification, and are expressed as the percentages of the area affected in relation to the total area analyzed. Glomerular matrix expansion was evaluated by calculating the relative size of mesangial matrix-occupying area (in percent) of 15 glomeruli from each rat (25).

For analysis of macrophage infiltration, paraffin-embedded tissues were incubated with a primary mouse anti-ED1 antibody (Serotec, Oxford, UK), in conjunction with a standard alkaline phosphatase and monoclonal anti-alkaline phosphatase technique (DakoCytomation, Hamburg, Germany), as previously described (11, 25). Glomerular macrophage infiltration was counted in at least 15 glomerular sections from each rat (glomerular macrophage number) and tubulointerstitial macrophage infiltration in at least 15 randomly selected cortical areas per sample (tubulointerstitial macrophage number), observed at ×200 magnification. Renal cell proliferation was analyzed using a primary mouse anti-PCNA-antibody (DakoCytomation) and a secondary goat anti-mouse antibody coupled with the Envision staining system (DakoCytomation) (11, 25). Renal cell proliferation was evaluated by counting PCNA-positive cells in at least 15 glomerular sections (glomerular cell proliferation) and at least 15 randomly selected interstitial areas from each rat observed at ×200 magnification (tubulointerstitial cell proliferation).

Cortical and glomerular TGF-β1, fibronectin, and TIMP-1 protein expression. Glomeruli from individual rats were isolated by a graded sieving technique (160-, 125-, and 71-μm mesh metal sieves), as described previously (25). For cultures of renal cortical tissue, a piece of cortical tissue was weighed and minced extensively with a razor blade (25). Glomeruli or cortical tissues were suspended in DMEM supplemented with 0.1 U/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Glomeruli were cultured at a density of 2,000 glomeruli/ml for 48 h, and minced cortical tissue at a density of 10 mg/ml protein. After 48-h incubation at 37°C and 5% CO2, supernatants were harvested and stored at −70°C until analysis of TGF-β1, fibronectin, or plasminogen activator inhibitor type 1 content. TGF-β1 content of culture supernatant was measured after acid activation, using a commercially available ELISA kit (TGF-β1 DuoSet, R&D Systems, Wiesbaden, Germany), according to the manufacturer’s instructions. Fibronectin levels were determined with modified inhibitory ELISA, according to published methods. TIMP-1 concentrations were measured with a commercially available ELISA kit (TIMP-1 DuoSet, R&D Systems). Three samples from each animal were analyzed.

Cortical TGF-β1, fibronectin, and TIMP-1 mRNA expression. Cortical total RNA was extracted with TRIzol reagent (Gibco BRL, Berlin, Germany), according to the manufacturer’s instructions. The mRNA expression of TGF-β1, fibronectin, TIMP-1, and GAPDH were determined by a “two-step” RT-PCR, as previously described (25). A cDNA copy was created with reverse transcriptase from an RNA PCR Core kit (Roche Applied Biosystems). Real-time PCR was performed on a LightCycler (Roche Applied Biosystems) in a 384-well microplate format according to the manufacturer’s instructions. The expression in the samples was normalized against the expression of GAPDH, and mRNA expression was calculated as 2−ΔΔCt.

Proteinuria and urinary analysis. Urine volume and is expressed as milliliters per minute per 100 g body weight. The glomerular filtration rate (GFR) was calculated on the basis of serum and urinary creatinine concentrations and the corresponding urine volume and is expressed as milliliters per minute per 100 g body wt. Hematocrit was determined in blood plasma tubes after a 10-min centrifugation. Plasma cholesterol and triglyceride levels were measured by standard enzymatic methods following a 10-h fasting period.

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Fig. 1. Proteinuria (A) and systolic blood pressure (B) 1, 10, and 20 wk after induction of anti-thy1 chronic-progressive glomerulosclerosis (GS). Treatments with rosuvastatin (+statin), high-dose candesartan (+AT1 blocker), or both (+both) were started 7 days after injection of anti-thy1 antibody into uninephrectomized rats. Nonnephritic animals with (1-K Control) and without uninephrectomy (2-K Control) received an injection with similar volumes of PBS. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. GS; α*P < 0.01 vs. GS+statin; #P < 0.05 vs. GS+statin and GS+AT1 blocker.
performed using the LightCycler System and SYBR Green I as double-stranded DNA binding dye (Roche Diagnostics, Mannheim, Germany). The following primer pairs were used (annealing temperature in parentheses): TGF-β1: sense 5'-GGTTGGCAGCCGAGAGGCTGTA-3' and antisense 5'-GGCATGGTAGCCCTTGGGCT-3' (64°C); fibronectin: sense 5'-GGTCCAAAATCGGTCATGTTCACA-3' and antisense 5'-CTGAATTGGAACCGTGTAAGG-3'(64°C); TIMP-1: sense 5'-CCCAGAAAATCAGCAGACCA-3' and antisense 5'-CCTGCTGATTTCACAGC-3' (66°C); and GAPDH: sense 5'-CCACCTTCCAGGAGGAT-3' and antisense 5'-GATGACCTTGCCACCAGCCT-3' (59°C). For analysis, a relative quantification method was used, as previously described (25). Briefly, amplification is given as $N = N_0 \times E^{\Delta C P}$ (where $N$ is number of amplified molecules; $N_0$ is initial number of molecules; $E$ is amplification efficiency; and $\Delta C P$ is crossing point deviation expressing as $\Delta C P = C_{P_{\text{target}}} - C_{P_{\text{GAPDH}}}$. In this quantification method, measured $C_{P}$ is defined as the point at which the fluorescence rises above the background fluorescence. Finally, $N_0$ of the target gene was calculated and compared relative to expression of GAPDH mRNA as housekeeping gene.

Statistical analysis. The results are expressed as means ± SE. Statistical analysis between groups was performed by Kruskal-Wallis U-testing. A value of $P < 0.05$ was considered significant.

RESULTS

Body weight and food intake. Body weight did not differ between the groups when anti-thy1 chronic GS was induced (data not shown). Proteinuria was similar between the diseased groups before treatment was started (Fig. 1A). By week 20, at the end of the experiment, mean body weight had reached 536 ± 12 g in the normal (2-K Control) and 568 ± 12 g in the uninephrectomized control group (1-K Control). In comparison, final body weight was significantly lower in all diseased animal groups (GS: 487 ± 16 g, GS-statin: 516 ± 15 g, GS-AT1 blocker: 512 ± 8 g, GS-both: 483 ± 14 g; $P < 0.05$ vs. control), indicating chronic renal disease and insufficiency. The differences between the diseased groups were not significant. Food and water intakes did not vary significantly between the groups throughout the experiment.

Disease severity in anti-thy1-induced chronic GS. Compared with the normal controls, anti-thy1-induced chronic GS 20 wk after disease induction was characterized by significant elevations in proteinuria (567 ± 56 mg/day, Fig. 1A), systolic blood pressure (142 ± 3 mmHg, Fig. 1B), and in tubulointerstitial TGF-β protein (475 ± 52 pg/ml, Fig. 2B) and mRNA expres-
sion (12.5-fold increase, Fig. 3A). Tubulointerstitial TGF-β overexpression was paralleled by significant increases in histological tubulointerstitial matrix accumulation (matrix score 64 ± 7%, Fig. 2A), protein expression of fibronectin (7,255 ± 512 ng/ml), and TIMP-1 (6,066 ± 1,503 ng/ml, Figs. 2, B–D) (all P < 0.05 vs. 1-K Control and 2-K Control). Fibronectin mRNA expression was elevated 7.4-fold and TIMP-1 20.0-fold (all P < 0.001 vs. 1-K Control and 2-K Control). At the glomerular level, GS animals showed increased histological matrix accumulation (matrix score 65 ± 5%) and protein expression of TGF-β1 (168 ± 23 pg/ml) and fibronectin (6,415 ± 1,172 ng/ml) (Figs. 4, A–C; all P < 0.05 vs. 1-K Control and 2-K Control). Glomerular TIMP-1 expression was not significantly different in all groups (Fig. 4D).

In parallel to the degree of kidney fibrosis, renal function was markedly impaired in the untreated chronic anti-thy1 GS group. This was indicated by increased blood creatinine (1.4 ± 0.2 mg/dl) and urea concentrations (144 ± 14 mg/dl), decreased GFR (0.24 ± 0.03 ml·min⁻¹·100 g body wt⁻¹), and lower blood hematocrit levels (0.37 ± 0.01%, Fig. 5, A–D, respectively; all P < 0.05 vs. 1-K Control and 2-K Control). In addition, untreated chronic anti-thy1 renal fibrosis was characterized by significantly increased infiltration of ED1-positive cells into the tubulointerstitium (ED1: 50 ± 4 cells/section, Fig. 6A) and the glomeruli (ED1: 3.9 ± 0.5 cells/cross section, Fig. 6C). Renal cell proliferation was augmented as well. PCNA-positive cells reached a count of 35 ± 4 cells per tubulointerstitial section and 3.8 ± 0.3 cells per glomerular section (Fig. 6, B and D, respectively; all P < 0.05 vs. 1-K Control and 2-K Control). Finally, blood lipids were also significantly altered in anti-thy1 GS, showing cholesterol fasting levels of 194 ± 10 mg/dl and triglyceride fasting levels of 187 ± 21 mg/dl (Figs. 7, A and B, respectively; all P < 0.05 vs. 1-K Control and 2-K Control).

Effects of separate treatments with rosuvastatin and candesartan. Compared with the untreated GS animals, treatment with rosuvastatin alone reduced tubulointerstitial TGF-β protein expression by −47% and mRNA expression by −54%
With high-dose candesartan alone, tubulointerstitial TGF-β1 protein and mRNA expression were reduced by 51% and 52%, respectively. The discrepancy between separate treatments was not significantly different, indicating comparable anti-fibrotic efficacy (P not significant for all parameters, GS statin vs. GS AT1 blocker). Concomitantly, comparable benefits from each separate treatment were documented on the basis of proteinuria, blood pressure, tubulointerstitial matrix accumulation and protein expression of fibronectin and TIMP-1, and tubulointerstitial mRNA expression of fibronectin and TIMP-1. Noticeably beneficial effects were also monitored for glomerular histological matrix expansion, renal macrophage infiltration and cell proliferation, as well as proteinuria and kidney function (Figs. 1–6, Table 1). The reductions achieved were significantly greater than those accomplished with either rosuvastatin or high-dose candesartan alone. Consistent with tubulointerstitial TGF-β expression, the additive renoprotective actions of the dual therapy were also documented by the multiple measures that recorded tubulointerstitial and glomerular matrix accumulation, renal macrophage infiltration and cell proliferation, as well as proteinuria and kidney function (Figs. 1–6, Table 1). The relative changes with each intervention are summarized in Table 1. Blood cholesterol and triglyceride levels were significantly reduced by rosuvastatin, whereas high-dose candesartan had no significant effect (Fig. 7).

Effects of combined rosuvastatin and high-dose candesartan treatment on anti-thy1-induced chronic GS. Compared with the untreated GS animals, dual rosuvastatin and candesartan therapy lowered tubulointerstitial TGF-β1 mRNA expression by 86% and its protein expression by 82% (Fig. 2B and 3A, Table 1). The reductions achieved were significantly greater than those accomplished with either rosuvastatin or high-dose candesartan alone. Consistent with tubulointerstitial TGF-β expression, the additive renoprotective actions of the dual therapy were also documented by the multiple measures that recorded tubulointerstitial and glomerular matrix accumulation, renal macrophage infiltration and cell proliferation, as well as proteinuria and kidney function (Figs. 1–6, Table 1). Dual intervention lowered fasting blood cholesterol levels below those observed in the undiseased controls (Fig. 7). Blood triglyceride fasting concentrations with dual therapy were similar to those seen in the control animals.

Taken together, the results show that dual statin and high-dose AT1 intervention produces marked additive actions on the progression of anti-thy1-induced disease toward GS, tubuloin-
terstitial fibrosis, and renal insufficiency. Additive actions were documented for key pathways of renal disease progression, such as TGF-β overexpression and fibrogenesis, cell proliferation, and macrophage infiltration. Separate treatment with rosuvastatin slows progression to a degree comparable to the one derived from high-dose pharmacological angiotensin II inhibition.

DISCUSSION

Lipid abnormalities are frequently found in patients with impaired kidney function (1, 13). Although the pathophysiology of lipid alterations in chronic kidney disease is still insufficiently understood, it is known that dyslipidemia develops in the early stages of renal insufficiency and worsens with the decline in GFR. In chronic kidney disease, the pattern of lipid changes is characterized by high triglycerides, low HDL, and elevated small dense LDL concentrations. These likely contribute to the excessive risk of cardiovascular morbidity and mortality found in patients with impaired renal function (1). On the other hand, abnormal lipid levels have also been implicated in the progression of chronic renal diseases. Epidemiological cohort studies in patients with kidney disease have identified dyslipidemia as an important and independent marker for renal disease progression (1, 13, 23). In experimental settings, an exclusive 4% increase in dietary cholesterol content resulted in marked interstitial fibrosis, TGF-β overexpression, and round-cell infiltration within 3 mo in uninephrectomized rats (6).

Since renal fibrosis and atherosclerosis share a number of cellular and molecular features, and given the documented pleiotropic benefits of statins in vascular disease (5), we addressed the question of whether HMG-CoA reductase inhibition is additive to high-dose angiotensin II blockade in a model of progressive human mesangioproliferative nephropathy. The major findings of the present study are as follows: 1) rosuvastatin limits progression in chronic anti-thy1-induced renal fibrosis with an efficacy comparable to a high dose of the AT1 blocker candesartan in a direct side-by-side comparison; 2) combined rosuvastatin and candesartan administration yields a substantial and robust additive reduction in the progression of chronic anti-thy1-induced disease toward GS, tubulointerstitial damage, and renal insufficiency; and 3) addi-
tivity of statin and angiotensin inhibition was documented for a number of key pathways of renal disease progression, i.e., renal matrix protein accumulation, cell proliferation, and leukocyte infiltration. Both the therapeutic and mechanistic implications of these clinically relevant findings are discussed in the following paragraphs.

Using tubulointerstitial expression of the key fibrosis marker and mediator TGF-β as the main therapeutic target, a marked reduction of 50% in its mRNA and protein expression was found when rosuvastatin (+statin), high-dose candesartan (+AT1 blocker), or both (+both) were started 7 days after injection of anti-thy1 antibody into uninephrectomized rats. Nonnephritic animals without (2-K Control) and with uninephrectomy (1-K Control) received a PBS injection. GFR, glomerular filtration rate. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. GS; #P < 0.05 vs. GS+statin and GS+AT1 blocker.

The beneficial effects of rosuvastatin monotherapy in experimental chronic anti-thy1-induced renal fibrosis are in line with the positive action of simvastatin previously reported in the acute model of anti-thy1-induced glomerulonephritis (28).

Cotreatment with rosuvastatin and high-dose candesartan resulted in a significant additional reduction in tubulointerstitial TGF-β mRNA and protein expression by a total of ~80%. Again, extra benefits were observed as well as indexes of renal matrix protein overproduction, cell proliferation, and macrophage infiltration as central pathways in the progression of kidney disease (4, 10, 13, 21). The AT1 blocker candesartan was given in doses clearly higher than required to control blood pressure in order to block the renin-angiotensin pathway as completely as possible (19). Thus, beyond therapeutic additivity, the robust extra renoprotection by dual therapy indicates that, on the mechanistic level, both interventions interfere independently with multiple key pathways in the progression of chronic mesangioproliferative GS.

Additivity of dual statin and angiotensin-inhibiting therapy has previously been reported in rat models of hypertensive 5/6 nephrectomy, diabetic nephropathy, and nephrotic syndrome (12, 20, 29). These therapeutically important findings are now expanded into a progressive model of human mesangioprolif...
erative nephropathy as a leading cause of end-stage kidney disease worldwide (7, 9). This notion yields two major perspectives. On the one hand, these models of renal disease differ substantially in their kind of primary tissue injury. In 5/6 nephrectomy, it is hypertension; in diabetes, hyperglycemia; in nephritic syndrome, massive proteinuria; and in chronic anti-thy1 disease, immune-induced glomerulonephritis, respectively. On the other hand, the relative uniform additivity of combined statin and angiotensin-inhibiting therapy in all of these models further underscores the concept of common final pathway operating

Fig. 6. Tubulointerstitial and glomerular macrophage infiltration (A and C) and cell proliferation (B and D) 20 wk after induction of anti-thy1 chronic-progressive GS. Shown are ED1-positive cells (A and C) representing macrophages, and proliferating cell nuclear antigen (PCNA)-positive cells indicating proliferation (B and D). Treatments with rosvastatin (+statin), high-dose candesartan (+AT1 blocker), or both (+both) were started 7 days after injection of anti-thy1 antibody into uninephrectomized rats. Non-nephritic animals without (2-K Control) and with uninephrectomy (1-K Control) served as controls. Analysis was performed using specific murine primary antibodies in conjunction with a standard alkaline phosphatase and monoclonal anti-alkaline phosphatase technique. Tubulointerstitial results are expressed as positive cells per cortical section observed at ×200 magnification, and glomerular results as positive cells per glomerular cross section. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. GS; #P < 0.05 vs. GS + AT1 blocker; ##P < 0.05 vs. GS + statin and GS + AT1 blocker.

Fig. 7. Blood fasting cholesterol (A) and triglyceride (B) concentrations 20 wk after induction of anti-thy1 chronic-progressive GS. Treatments with rosvastatin (+statin), high-dose candesartan (+AT1 blocker), or both (+both) were started 7 days after injection of anti-thy1 antibody into uninephrectomized rats. Non-nephritic animals without (2-K Control) and with uninephrectomy (1-K Control) served as controls. Blood was drawn after a 12-h fasting period. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. GS; #P < 0.05 and ##P < 0.01 vs. GS + statin and GS + AT1 blocker.
in chronic renal disease. This common final pathway does not apply only to mechanism, but to therapeutic intervention.

Beyond the study in S/56 nephrectomy and diabetic nephropathy, the present investigation in chronic anti-thy1 GS points at a predominantly intrarenal mode of action of dual therapy on the progression toward renal failure. In contrast to diabetic and hypertensive nephropathy, the advance of the disease in chronic anti-thy1 GS primarily follows an intrarenally determined pathological program that is initiated after a certain point of no return has been passed. This course is seen in patients with primary glomerular disease who progress toward end-stage renal disease after a single episode of glomerulonephritis. In diabetic and hypertensive nephropathy, extrarenal stimuli, i.e., high blood pressure or glucose levels, are continuously damaging the kidneys and thereby drive the disease progress as well.

In the present study, blood pressure was lower in the rosuvastatin-treated animals, and blood lipids in the candesartan treatment group. The relative changes were computed for each parameter as follows: (mean of treatment group/mean of untreated GS group) × 100. Treatments with rosuvastatin (+statin), high-dose candesartan (+AT1 blocker), or both (+both) were started 7 days after injection of anti-thy1 antibody into uninephrectomized rats. TGF-β, transforming growth factor-β; TIMP-1, tissue inhibitor of metalloproteinase-1; ED1, macrophage/monocyte 1; PCNA, proliferating cell nuclear antigen; GFR, glomerular filtration rate. AT1 blocker; dP < 0.01 vs. GS; *P < 0.05 vs. GS; #P < 0.01 vs. GS; $P < 0.01 vs. GS + statin; †P < 0.05 vs. GS + AT1 blocker; ‡P < 0.05 and ‡P < 0.01 vs. GS + statin and GS + AT1 blocker.

### Table 1. Relative changes in proteinuria, blood pressure, tubulointerstitial and glomerular matrix accumulation, cell infiltration, renal excretory function, and blood lipids 20 wk after induction of anti-thy1 chronic-progressive glomerulosclerosis

<table>
<thead>
<tr>
<th>Variable/Group</th>
<th>GS + Statin, %</th>
<th>GS + AT1, Blocker, %</th>
<th>GS + Both, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria</td>
<td>-30*</td>
<td>-55*</td>
<td>-87*</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>-7*</td>
<td>-14*</td>
<td>-18*</td>
</tr>
<tr>
<td>Tubulointerstitial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrix score</td>
<td>-46*</td>
<td>-40*</td>
<td>-79*</td>
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<tr>
<td>TGF-β protein</td>
<td>-47*</td>
<td>-51*</td>
<td>-82*</td>
</tr>
<tr>
<td>Fibronectin protein</td>
<td></td>
<td>-35*</td>
<td>-44*</td>
</tr>
<tr>
<td>TIMP-1 protein</td>
<td>-39*</td>
<td>-51*</td>
<td>-77*</td>
</tr>
<tr>
<td>TGF-β mRNA</td>
<td>-54*</td>
<td>-52*</td>
<td>-86*</td>
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<td>Fibronectin mRNA</td>
<td>-29</td>
<td>-37</td>
<td>-77*</td>
</tr>
<tr>
<td>TIMP-1 mRNA</td>
<td>-26</td>
<td>-45</td>
<td>-75*</td>
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<tr>
<td>ED1-positive cells</td>
<td>-31*</td>
<td>-39</td>
<td>-68*</td>
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<tr>
<td>PCNA-positive cells</td>
<td>-62%</td>
<td>-60</td>
<td>-65%</td>
</tr>
<tr>
<td>Glomerular</td>
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<tr>
<td>Matrix score</td>
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<td>-30*</td>
<td>-46*</td>
</tr>
<tr>
<td>TGF-β protein</td>
<td>-35*</td>
<td>-46*</td>
<td>-65%</td>
</tr>
<tr>
<td>Fibronectin protein</td>
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<td>-22</td>
<td>-36w</td>
</tr>
<tr>
<td>ED1-positive cells</td>
<td>-43*</td>
<td>-30</td>
<td>-66w</td>
</tr>
<tr>
<td>PCNA-positive cells</td>
<td>-25*</td>
<td>-29</td>
<td>-30w</td>
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<td>Renal function</td>
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<tr>
<td>Plasma creatinine</td>
<td>-34*</td>
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<tr>
<td>Plasma urea</td>
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<td>+135w</td>
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<tr>
<td>GFR</td>
<td>+20w</td>
<td>+16w</td>
<td>+23w</td>
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<tr>
<td>Blood lipids</td>
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<tr>
<td>Cholesterol</td>
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<td>-78w</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>-32*</td>
<td>-23</td>
<td>-58w</td>
</tr>
</tbody>
</table>

Results are expressed in relation to the untreated glomerulosclerosis (GS) animal group. The relative changes were computed for each parameter as follows: (mean of treatment group/mean of untreated GS group) × 100. Treatments with rosuvastatin (+statin), high-dose candesartan (+AT1 blocker), or both (+both) were started 7 days after injection of anti-thy1 antibody into uninephrectomized rats. TGF-β, transforming growth factor-β; TIMP-1, tissue inhibitor of metalloproteinase-1; ED1, macrophage/monocyte 1; PCNA, proliferating cell nuclear antigen; GFR, glomerular filtration rate. AT1 blocker; dP < 0.01 vs. GS; *P < 0.05 vs. GS; #P < 0.01 vs. GS; $P < 0.01 vs. GS + statin; †P < 0.01 vs. GS + AT1 blocker; ‡P < 0.05 and ‡P < 0.01 vs. GS + statin and GS + AT1 blocker.

Beyond animal experiments, the present study suggests that statins may confer renoprotection in patients with progressive forms of mesangioproliferative glomerulonephritis, even when they are already receiving renin-angiotensin system inhibition as standard therapy. In addition, the results indicated that statins should be more actively considered in mesangioproliferative nephropathy patients who do not tolerate angiotensin II inhibition. Interestingly, a significant renoprotective effect of statin therapy has recently been shown in a meta-analysis on the progression of various human chronic kidney diseases (1, 23). Furthermore, addition of atorvastatin to a preexisting regime with angiotensin II inhibition was reported to further reduce proteinuria and the rate of functional decline in a relatively large group of patients with idiopathic chronic glomerulonephritis (3). More clinical data on this issue will likely be generated by the ongoing Study of Heart and Renal Protection (SHARP) trial. SHARP is attempting to enroll 6,000 subjects with impaired renal function and assess the effects of lipid lowering with simvastatin, or a combination of simvastatin and the cholesterol inhibitor ezetimibe, on both major vascular events and the progression toward end-stage renal disease (2). In addition, the ongoing PLANET study (Prospective evaLuation of proteinurIA and reNal function in diabETic and nondiabetic patients with progressive renal disease) will determine whether rosuvastatin or atorvastatin, added to renin-angiotensin inhibition, reduces proteinuria in patients with established proteinuria. In a development of direct interest for this study, a recent post hoc analysis of five blinded, placebo-controlled trials comprising 525 patients with a mean estimated GFR of ~70 mL·min⁻¹·1.73 m² showed that a short-term treatment with rosuvastatin improved estimated GFR by 0.8 mL·min⁻¹·1.73 m², while it decreased by 1.5 mL·min⁻¹·1.73 m² in the placebo group (24).
In conclusion, in a model of progressive mesangio proliferative GS, rosuvastatin, in addition to high-dose candesartan, slows significantly its advancing course toward chronic renal fibrosis and insufficiency. The therapeutic and mechanistic additivity of HMG-CoA reductase inhibition and AT1-blockade involves independent and separate actions on renal fibrogenesis, cell infiltration, and inflammation as key pathways in mediating the progression of chronic renal disease.

GRANTS

This study was supported in part by grants from AstraZeneca, United Kingdom; Deutsche Forschungsgemeinschaft (PE 558/2-3), Bonn, Germany; and Sonnenfeldstiftung, Berlin, Germany. Y. Wang-Rosenke and D. Khadzhynov are recipients of doctoral degree grants from the Deutscher Akademischer Austauschdienst, Bonn, Germany (A/01/03941 and A/06/09454).

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