12,000*g* for 2 minutes and supernatant removed for measurement of HBG enzymatic activity.

Western blot analysis

Western blot analysis was performed to measure the secretion of HBG from recruited human CD14$^+$ cells in the glomeruli as described [6]. Briefly, homogenates of isolated glomeruli (2 μg) from LPS-treated and saline-treated mice were subjected to electrophoresis in a 12.5% sodium dodecyl sulfate-polyacrylamide gel and then transferred to nitrocellulose membrane. The membrane was blocked with 5% dried milk, 0.1% Tween 20, PBS, and incubated with anti-human HBG antibody (×1000). It was then incubated with rabbit anti-gout immunoglobulin horseradish peroxidase conjugate (Cosmo Bio, Tokyo, Japan). Antigen-antibody complexes were visualized by chemiluminescence reagents (Amersham Pharmacia Biotech).

Analysis of proteinuria and serum creatinine

The concentration of albumin in urine was measured by single radial immunodiffusion (SRID) [23] using a specific antibody for mouse albumin [24]. Briefly, samples were applied to a 1% agarose gel containing anti-mouse albumin and incubated for 48 hours. The diameters of the expressed rings on the gel were measured and their concentrations were assessed by comparison with a standard curve. Concentration of plasma creatinine was measured using the Vision analyzer kit (Abbott Laboratories, North Chicago, IL, USA), which is based on the Jeffe reaction.

Statistical analysis

Data are expressed as mean ± SE. Statistical analysis was performed using the two-sample *t* test to compare data in different groups. $P < 0.05$ was found to be significant.

RESULTS

Susceptibility of CD34$^+$ cord blood cells to retroviral transfection

To examine the efficacy of CD34$^+$ cord blood cells to retroviral transfection, CD34$^+$ cells were established from freshly obtained human cord blood cells and retrovirally transfected with the human HBG gene, using centrifugation methods with concentrated viral supernatant. These cells were cultured in MethoCult GF H4434V for 14 days and DNA from each colony was subjected to PCR on the HBG gene. As shown in Figure 1, three different lineages of hematopoietic cell possessed the transgene (88.4 ± 5.9% in BFU-E, 79.7 ± 11.4% in CFU-E, and 81.1 ± 14.1% in CFU-G, respectively), and there were no significant differences between lineages, suggesting that CD34$^+$ cells from human cord blood cells

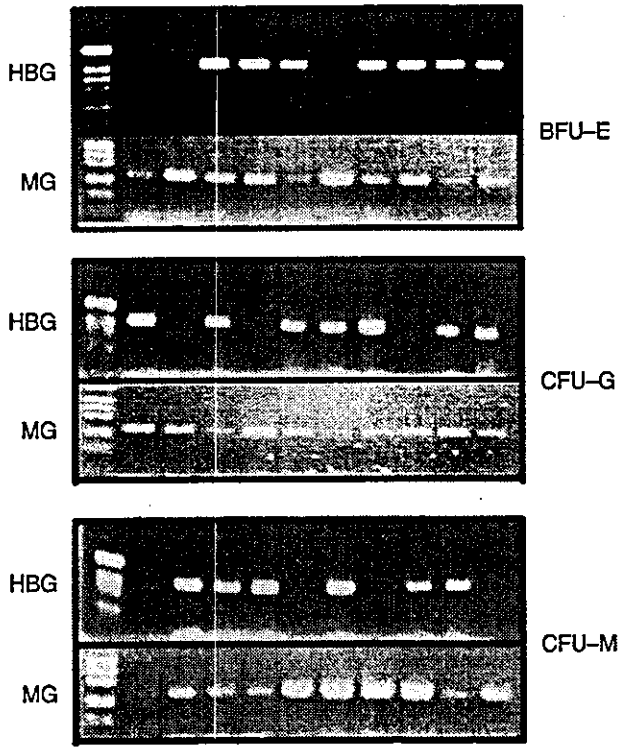

Fig. 1. Susceptibility of hematopoietic stem cells to retroviral transfection. Human cord blood–derived CD34$^+$ cells were transfected with human β glucuronidase (HBG) and cultured with erythropoietin, stem cell factor, granulocyte macrophage colony stimulating factor (GM-CSF), and interleukin-3 (IL-3) for 14 days. Well-isolated single colonies of BFU-E, CFU-M, and CFU-G were picked up and DNA was extracted and processed for PCR on human β-glucuronidase (HBG). The yield of DNA was monitored by human specific β_2 microgloblin (MG). After electrophoresis in a 2% agarose gel, the amplified products were visualized with ethidium bromide staining. Experiments were performed in quadruplicate and representative pictures are shown.

are susceptible to retroviral transfection in the precommited form.

Establishment of chimera mouse that has mouse body with human hematopoietic system

To establish the chimera mouse, the hematopoietic systems were exchanged for human cells, and HBG-transfected CD34$^+$ cells were transplanted to NOD/SCID mice. After 8 weeks, bone marrow cells from these mice were subjected to flow cytometric analysis on human HLA ABC antigen. As shown in Figure 2A, 24.1 ± 14.5% of bone marrow cells in these chimera mice expressed human HLA ABC antigen, showing successful chimerism in these mice. To confirm that these reconstituted bone marrow cells still maintained the transgene clonogenic assay was also performed on HBG. As shown in Figure 2B, the cells from these marrows still possessed the transgene (31.4 ± 10.8% in BFU-E, 38.9 ± 18.9% in CFU-E, and 33.8 ± 19.9% in CFU-G, respectively),

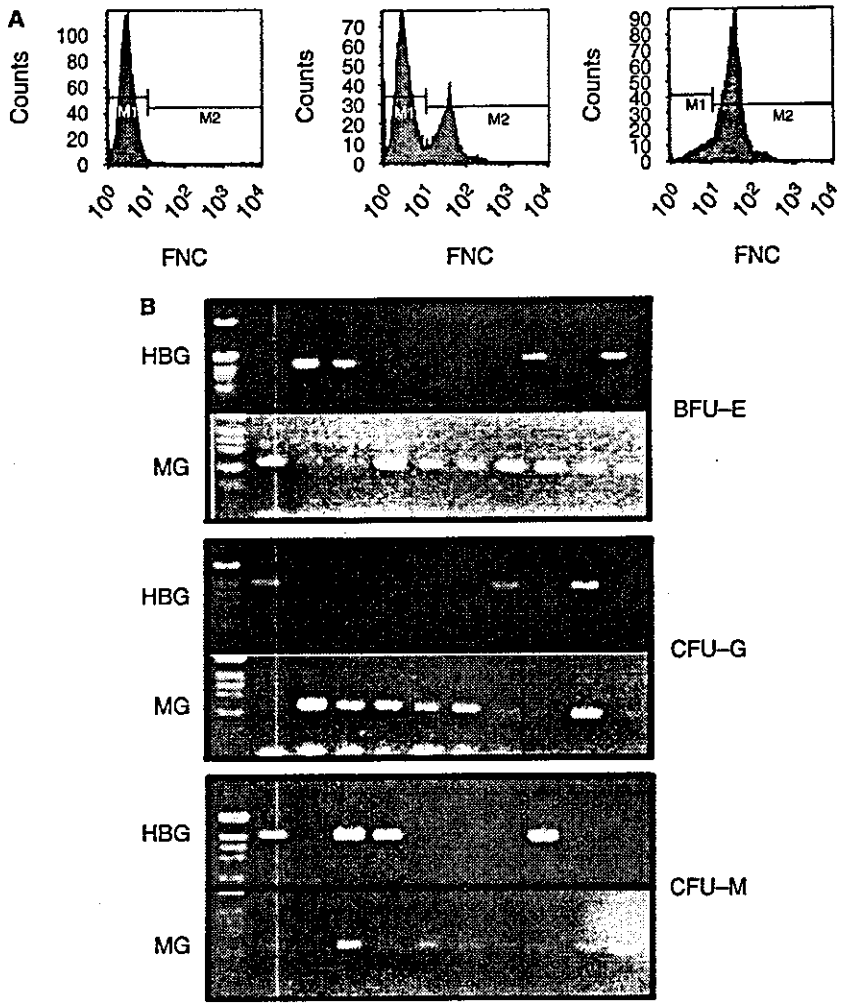

Fig. 2. Establishment of chimera mice. (*A*) Bone marrow of NOD/SCID mice was reconstituted with genetically-engineered human CD34$^+$ cells using a transplantation-based method. Eight weeks later, bone marrow was harvested from these mice and incubated with fluoroscein isothiocyanate (FITC)-conjugated human HLA ABC antibody. Ten thousand cells were subjected to flow cytometric analysis (center panel). As a control, bone marrow cells from NOD/SCID mice without bone marrow reconstitution (left panel), and human mononuclear cells from healthy volunteers (right panel), were also subjected to the same procedure. (*B*) Harvested bone marrow cells were also subjected to clonogenic assay on the human β glucuronidase (HBG) gene to confirm that the reconstituted bone marrow still possessed the transgene. Four mice per group were analyzed individually and representative data are shown.

showing a sustained engraftment of human hematopoietic cells expressing the HBG gene.

Delivery into inflamed site by in vivo differentiated human cells

Since Verstegan et al [25] previously reported that human monocytes may spontaneously grow out in the SCID mice after transplantation with unfractionated human cord blood cells, we speculated that transplanted human CD34$^+$ cells may differentiate into mononuclear cells and effectively deliver transgene into inflamed glomeruli. Chimera mice were treated with LPS or saline once daily for 7 days and subjected to immmunohistochemical analysis, HBG enzymatic assay, and Western blot analysis of HBG expression. At day 7, kidneys were obtained from these mice and tissue sections were stained with human CD14 and HBG. As shown in Figure 3A, CD14$^+$ cells were detected in the glomeruli upon LPS treatment, and 19.3 ± 4.4% of those cells secreted HBG. The total HBG enzymatic activity in isolated glomeruli was not significantly elevated by LPS treatment (716.0 ± 68.8 nmol/hr/mg in saline-treated mice vs. 1085.9 ± 137.5 nmol/hr/mg in LPS-treated mice; P = 0.0821) (Fig. 3B). However, transplanted human HBG activity, which was estimated by subtraction of endogenous murine HBG activity from total HBG, was significantly elevated by LPS treatment (148.1 ± 52.0 nmol/hr/mg in saline-treated mice vs. 574.8 ± 102.7 nmol/hr/mg in LPS-treated mice; P = 0.0041). Furthermore, Western blot analysis revealed that isolated glomeruli from LPS-treated chimera mice contained higher amount of HBG protein compared with control chimera mice (Fig. 3C). Together, these data suggested that umbilical cord blood–derived CD34$^+$cells might differentiate into monocyte lineage cells while maintaining the expression of the foreign gene.

Although native macrophages may influence the behavior of donor macrophages, only a negligible number

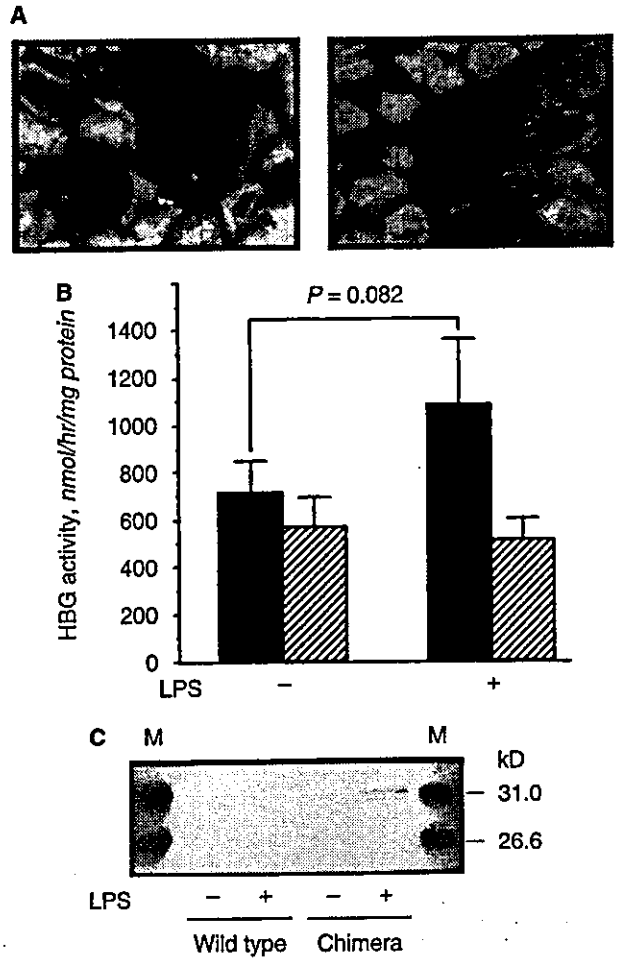

Fig. 3. Gene delivery using monocytes derived from human cord blood. Eight weeks after transplantation, chimera mice were treated with LPS to induce ICAM-1 expression in the glomeruli, or saline as a control. (*A*) Immunohistochemical analysis. Kidney sections from chimera mice with LPS (left) or saline (right) treatment were subjected to two-color immunofluorescent staining on human CD14 and human β glucuronidase (HBG). Green fluorescence represents CD14 positive cells and red fluorescence represents secreted human HBG. (*B*) HBG enzymatic activity of isolated glomeruli. Glomeruli from LPS-treated or saline-treated mice were isolated by sieving method and the homogenates were subjected to HBG bioassay. Symbols are: (■) total HBG activity; (▨) HBG activity remaining after immunoprecipitation with anti-human HBG antibody. (*C*) Secretion of human HBG from recruited cells. Two microgram protein of isolated glomeruli from LPS-treated and saline-treated mice were subjected to Western blot analysis of human HBG expression. As a control, glomeruli from wild type mice treated with LPS or saline were also examined under the same protocol. M is molecular weight marker.

of native macrophages could be detected in the glomeruli of the NOD/SCID mice treated with LPS (data not shown), probably because the number of native macrophages in NOD/SCID mice are small [26] and are functionally immature [27], especially when the response to LPS is impaired [28]. Therefore, donor macrophages may occupy the area of intercellular adhesion molecule-1 (ICAM-1) expression in the glomeruli without competition with native macrophages, by which the significance of transgene activity might be enhanced. On the other hand, recruitment of transplanted cells into glomeruli might affect renal function and, therefore, renal function after LPS treatment was examined in chimera mice. Both urine albumin and serum creatinine levels were under detectable level (the lowest detectable concentrations of albumin and creatinine are 20 mg/mL and 0.3 mg/dL, respectively), suggesting that accumulation of exogenous human CD14 cells, per se, did not affect renal function in chimera mice.

DISCUSSION

Transplantation-based gene therapy has been proposed as a novel therapeutic strategy for chronic inflammatory diseases such as glomerulonephritis [5]. This approach is based on the fact that inflammatory cells such as macrophages and neutrophils are capable of being recruited to and activated at the inflamed glomeruli and, therefore, genetic manipulation of bone marrow–derived cells or bone marrow, per se, using transplantation-based technology may modify inflammation. We previously reported that bone marrow reconstitution using genetically-modified hematopoietic progenitor cells to supply anti-inflammatory mononuclear cells to inflamed glomeruli confer resistance against glomerular inflammation [7]. As the next step toward the clinical use, we investigated the suitability of human umbilical cord blood as a source of hematopoietic stem cells, which may reduce the risks to patients, and showed that $CD34^+$ cells from human cord blood may reconstitute bone marrow; these cells may differentiate into monocyte lineage cells, which may deliver foreign genes into inflamed glomeruli.

In addition to fewer risks to the mother and infant, umbilical cord blood cells have several advantages as a source of hematopoietic stem cells for clinical allogeneic transplantation. For example, umbilical cord blood cells can be cryopreserved and easily shipped and thawed for use on demand, eliminating delays and uncertainties that presently complicate marrow collection from unrelated donors [29]. The amplification of allogeneic responses by neonatal T lymphocytes has been shown to be less than that of adult T cells, which may underlie umbilical cord blood reduced graft-versus-host reactivity [30, 31]. Although these advantages strengthen the rational of its clinical use, there are several obstacles to be overcome. First, the efficacy of retroviral transfection is quite low for human stem cells compared to mouse stem cells [32]. We tried several different methods and finally concluded that the most efficient transfection was done by a centrifugation method [14] using recombinant human fibronectin fragment CH-286–coated tubes [18] with concentrated viral supernatant [17]. This modification resulted

in the highest efficacy among our retrovirus-based transduction attempt. However, it may not be sufficient for therapeutic intervention, especially if the antagonist is used to cancel the effect of inflammatory cytokines. In terms of clinical application, further modification is needed. In this regard, attempts to establish a novel vector system, such as lentivirus vectors based on the human immunodeficiency virus, have been made [33]. The use of these vectors, however, raises concerns about their safety, which must be completely cleared before clinical use. Another approach to overcome the low gene transfection efficacy is selection or expansion of transfected stem cells ex vivo [34, 35] or in vivo [36, 37]. For instance, cotransfection with a gene that confers a reversible growth advantage in the presence of a synthetic drug may achieve a marked and sustained expansion of transfected hematopoetic cells [38]. These trials may improve the low transduction efficacy of human stem cells.

Only 5×10^6 cells could be collected from 50 mL umbilical cord blood cells in our method; it has been reported that the safest threshold needed for engraftment of autologous peripheral blood stem cells should be 7.8×10^6 $CD34^+$ cells/kg body weight [39], however, suggesting that cord blood samples harvested from one placenta may be insufficient and ex vivo expansion of umbilical cord blood progenitor cells may be necessary to engraft larger patients. For this purpose, Broxmeyer et al [40] reported that umbilical cord blood colony forming unit-granulocyte macrophage (CFU-GM) progenitor cells may be increased in vitro by 8- to 11-fold in the presence of stem cell factor (SCF) and granulocyte macrophage colony stimulating factor (GM-CSF). $CD34^+$ cells isolated from cord blood have also been reported to be able to expand in the presence of IL-11 and granulocyte colony stimulating factor (G-CSF) to a higher degree compared with those from human adult bone marrow (80-fold) [41], suggesting a significant level of self-renewal capacity for umbilical cord blood stem cells. Further study is necessary to show ex vivo expansion of umbilical cord blood progenitor cells to accelerate hematopoietic reconstitution.

Unfractionated marrow cells were used to reconstitute bone marrow and, therefore, cells other than monocyte-lineage cells possess the foreign gene, which may cause an unpredictable effect in a nontargeted organ. Hence, an on/off switching system, in which mononuclear lineage cells only are activated exclusively at the inflamed site, will be required. We recently succeeded in inflamed site-specific activation of terminally differentiated-monocyte lineage cells using an IL-1β promoter combined with a Cre/loxP system [42], so that transgene could be induced in the cells and the environment where IL-1β was produced [43]. Currently it is being examined whether bone marrow progenitor cells may maintain this regulation during the differentiation in our laboratory.

Recently, stem cell therapy has advanced with the discovery of several stem cells. We previously reported, using transplantation technology, that mesangial progenitor cells might exist in bone marrow cells [44], most likely in the fraction of mesenchymal stem cells because donor cells did not differentiate into residential mesangial cells when bone marrow was transplanted after elimination of mesenchymal stem cells by culture in IL-3, IL-6, and SCF for 72 hours [7]. Therefore, we have proposed two different therapeutic strategies for glomerular diseases, depending upon whether to differentiate bone marrow stem cells into mesenchymal or hematopoietic stem cells. In the case of diseases which need reconstitution of residential glomerular cells, such as congenital enzyme deficiency diseases, mesenchymal stem cells should be transplanted and, in contrast, diseases which need foreign cytokines and growth factors, such as glomerulonephritis, hematopoietic stem cells may used for gene delivery. Although we are still in the learning phase, because umbilical cord blood has been considered as a viable source of hematopoietic and mesenchymal stem cells [45], our success in this report allows us to proceed to the next step in the long avenue of research aimed to provide stress-free, tailor-made stem cell therapy for glomerular disease.

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Angiotensinogen gene variation and renoprotective efficacy of renin-angiotensin system blockade in IgA nephropathy

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Angiotensinogen gene variation and renoprotective efficacy of renin-angiotensin system blockade in IgA nephropathy.

Background. Blockade of the renin-angiotensin system (RAS) is well documented to be renoprotective; however, not all patients with glomerulonephritis respond well to this therapy. The interindividual variation in response to the RAS blockade may be in part genetically determined, whereas the results have been controversial.

Methods. We investigated whether the therapeutic efficacy of angiotensin-converting enzyme (ACE) inhibitors and/or angiotensin receptor blocker on renal prognosis is modified by the angiotensinogen gene (*AGT*) polymorphism in immunoglobulin A nephropathy (IgAN). In total, 259 patients with histologically proven IgAN were analyzed for clinical manifestations, renal survival, and their associations with *AGT* A(−20)C and M235T.

Results. The renal prognosis of 110 patients, who received ACE inhibitors/angiotensin receptor blocker during their clinical course, was significantly better than those without ACE inhibitors/angiotensin receptor blockers despite higher blood pressures and heavier proteinuria. The Cox proportional hazards regression model showed an increased hazard ratio (HR) for urinary protein (more than 1.0 g/day) of 3.346 ($P = 0.0001$), hypertension of 1.949 ($P = 0.01$), deteriorated renal function of 3.040 ($P < 0.0001$), no ACE inhibitor/angiotensin receptor blocker administration of 2.725 ($P = 0.0004$), and the T235 and C(−20) haplotype of 1.608 ($P = 0.0322$). Only in patients carrying at least one M235 and A(−20) haplotype did the administration of ACE inhibitors/angiotensin receptor blockers have no significant effect on the prognosis of renal function (Kaplan-Meier, log rank test, $\chi^2 = 0.700$; $P = 0.4028$), whereas it was significant in patients who had other haplotypes of *AGT* ($\chi^2 = 11.805$; $P = 0.0006$).

Conclusion. This study provides evidence that the M235T and A(−20)C genotype of *AGT* can influence the therapeutic efficacy of a RAS blockade on the renal survival in IgAN.

Key words: angiotensinogen, IgA nephropathy, gene polymorphism, renal survival.

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Immunoglobulin A nephropathy (IgAN), characterized by mesangial proliferative glomerulonephritis with predominant deposits of IgA1 in the mesangium, is the most prevalent form of glomerulonephritis and a major cause of end-stage renal disease (ESRD) in the world [1]. It has been reported that 9% to 50% patients with IgAN progress to ESRD within 20 years of disease onset, although the disease has a variable clinical course [2–4]. The mechanisms of the interindividual variation in the rate of disease progression remain to be elucidated, but it has been proposed that several genetic backgrounds are associated with the disease progression in IgAN [5, 6].

It has been well documented that increased production or activity of angiotensin II plays a detrimental role in the glomerular response to injury. Recently, both angiotensin-converting enzyme (ACE) inhibitor and angiotensin II receptor blocker therapies have been shown to decrease the proteinuria by improving glomerular permselectivity in IgAN [7, 8], although about half of the patients did not respond to the ACE inhibitor/angiotensin receptor blocker treatment [9].

The interindividual variation in response to the renin-angiotensin system (RAS) blockade may partly be genetically determined. It has been suggested that genetic backgrounds, which have an association with the local tissue activity of RAS in diseased kidney, may also determine the responsiveness to ACE inhibitor/angiotensin receptor blocker. The candidate gene variant proposed to date is an insertion/deletion (I/D) polymorphism in the angiotensin-converting enzyme gene (*ACE*), which has been shown to influence the concentration of ACE both in circulation and local tissue [10, 11]. However, the results from previous studies on the association between the *ACE* I/D polymorphism and the responsiveness to ACE inhibitor therapy are conflicting [12–16].

Another important gene variant, which has been proved to affect the activity of the RAS, is polymorphisms in the angiotensinogen gene (*AGT*, MIM 106150), although so far little information is available on its possible associ-

ation with the responsiveness to ACE inhibitor/angiotensin receptor blocker therapy. However, accumulating evidence indicates that *AGT* M235T variant is important in the pathogenesis of cardiovascular diseases such as hypertension. It has been suggested that changes in the 5′ upstream core promotor region of *AGT*, which is essential for the transcription of angiotensinogen mRNA, causes a functional alteration, which may contribute to the pathogenesis of cardiovascular diseases [17]. An adenine-to-cytosine transition at nucleotide –20 of the core promotor region [A(−20)C] has been shown to increase basal promotor activity of *AGT* by increasing the affinity of adenoviral major late transcription factor (MLTF) to this region of the promotor [18].

In this study, we investigated the possible interaction of *AGT* A(−20)C and M235T polymorphisms with the renoprotective efficacy of the RAS blockade on renal survival in patients with IgAN.

METHODS

Study population

The protocol of the present study was approved by the ethics committee of the institution involved. Japanese patients were eligible for inclusion in the analysis when (*1*) they had been diagnosed as having IgAN by kidney biopsy at our institute between 1976 and 2000; (*2*) they had no evidence of systemic diseases such as hepatic glomerulosclerosis, Schönlein-Henoch purpura, and rheumatoid arthritis; (*3*) they had been followed up for at least 12 months in our institute; and (*4*) written informed consent for genetic study was obtained. Patients who received immunosuppression therapy other than corticosteroids were excluded from the analysis. In general, kidney biopsy was indicated for patients with persistent proteinuria and hematuria sustained for at least 6 months, those with nephrotic range proteinuria, and those with reduced renal function.

In total, 259 IgAN patients were recruited for this study. In all cases, the diagnosis of IgAN was based on a kidney biopsy that revealed the presence of dominant or codominant glomerular mesangial deposits of IgA as assessed by immunofluorescence examination.

Baseline clinical data and survival analysis

Clinical characteristics of the patients before the start of any treatment, including age, gender, office blood pressure, level of urinary protein excretion (g/day), serum creatinine (mg/dL), and 24-hour creatinine clearance (mL/min/1.73 m^2 body surface area), were retrospectively reviewed from their medical records. Hypertension was defined by the use of one or more antihypertensive medications and/or a blood pressure greater than or equal to 140 mm Hg systolic or 90 mm Hg diastolic. The patients were followed up for the mean duration of 92.2 ± 67.7 (mean ± SD) months. In the survival analysis, the primary end point (progressive renal disease) was defined as the date at which the serum creatinine level was double that of the time at diagnosis, or when the patient underwent their first hemodialysis. All patients were treated according to a standardized procedure of our institute. Corticosteroids were administered to patients with a urinary protein excretion of more than 1.0 g/day at the time of renal biopsy with the exception of cases with deteriorated renal function (creatinine clearance <30 mL/min) and those over 65 years old. Patients with contraindication to steroid treatment, such as infectious diseases, glaucoma, refractory peptic ulcer, and severe osteoporosis, were also excluded from glucocorticoid therapy as judged by the physicians. An additional exception was a case in which no informed consent for corticosteroid therapy was available. Out of the 259 patients, 71 (27.4%) received corticosteroids, whereas 90 patients (34.7%) had a proteinuria of more than 1.0 g/day at the time of diagnosis. Antihypertensive agents were used in combination with or without ACE inhibitors/angiotensin receptor blocker to maintain the blood pressure lower than 140 mm Hg systolic and 90 mm Hg diastolic. The administration of glucocorticoids, antihypertensive agents, and ACE inhibitors/angiotensin receptor blockers was also recorded for each patient. In total, 110 patients received ACE inhibitors and/or angiotensin receptor blockers as antihypertensive agents after the diagnosis and during their clinical course. About half of the ACE inhibitors prescribed was enalapril (2.5~10 mg/day) in 56 patients, while others included temocapril (1~4 mg/day) in 18 patients, quinapril (5~10 mg/day) in 15 patients, lisinopril (5~10 mg/day) in seven patients, captopril (25~37.5 mg/day) in four patients, and delapril (7.5~30 mg/day) in three patients patients. Angiotensin receptor blockers were prescribed in 33 patients (25~50 mg/day of losartan in 16 patients, 2~8 mg/day of candesartan in 17 patients). Only 26 patients were administered both ACE inhibitors and angiotensin receptor blockers. Of 26 patients, 21 patients received ACE inhibitors and angiotensin receptor blockers simultaneously and five did so sequentially (ACE inhibitors to angiotensin receptor blocker). Since the choice of antihypertensive agents was not controlled but was left to the decision of each physician, there was a tendency for ACE inhibitors/angiotensin receptor blockers to be preferably administered to patients with a high-grade proteinuria (Table 2).

Determination of the *AGT* genotype

Genomic DNA from the peripheral blood cells of 259 Japanese patients with histologically confirmed IgAN was isolated by an automatic DNA isolation system (NA-1000, Kurabo, Osaka, Japan). The M235T variant of *AGT* at exon 2 was determined as described previously [19]. To determine the A to C transition at nucleotide

Table 1. The genotype distributions, allele frequencies, and estimated haplotype frequencies of *AGT* polymorphisms

		A(−20)C		M235T
Genotype	AA	160	MM	3
	AC	85	MT	87
	CC	14	TT	169
	Total	259		259
Allele	A	0.782	M	0.180
	C	0.218	T	0.820
Haplotype		T235 and C(−20)		0.2182
		T235 and A(−20)		0.6023
		M235 and A(−20)		0.1795

−20 in the 5′ upstream region of the core promoter in the *AGT* gene, the following primers were used: 5′ primer, 5′-AGAGGTCCCAGCGTGAGTGTC-3′ (nucleotides −166 to −144); 3′ primer, 5′-AGCCCACAGC TCAGTTACATC-3′ (nucleotides 81 to 101) [20]. Polymerase chain reaction (PCR) was performed in a final volume of 50 μL containing 100 ng DNA, 10 pmol of each primer, 250 mmol/L of each of the four desoxynucleoside triphosphates (dNTPs), 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol Tris-HCl at pH 8.4, and 2 U of *Taq* polymerase (Takara, Shiga, Japan). The PCR conditions were as follows: 30 cycles of 94°C for 30 seconds, 64°C for 1 minute, and 72°C for 1 minute. After PCR, 265 bp products, including the 5′ upstream core promoter region, were obtained. Next, 8.5 μL of unpurified product was digested with 2 U of *Eco*O109I (Takara) for at least 3 hours at 37°C. These samples were separated by 3% agarose gel electrophoresis, and visualized by ethidium bromide staining.

Statistical analysis

Statview 5.0 statistical software (Abacus Concepts, Inc., Berkeley CA, USA) was used for statistical analysis. Continuous variables were compared using the Mann-Whitney U-test. Chi-square analysis was used when comparing allele frequencies and categoric variables between the groups. Hardy-Weinberg equilibrium was tested by a chi-square test with 1 *df*. The Kaplan-Meier method and Cox proportional hazards regression model were used to analyze the time course from renal biopsy to end point. In the Cox regression model, we first tested each variable by univariate analysis, including age, gender, body mass index, urinary protein, hypertension, deteriorated renal function at the time of diagnosis, steroid therapy, and administration of ACE inhibitors/angiotensin receptor blockers, as well as the gene polymorphisms. Deteriorated renal function was defined as a serum creatinine level of more than 1.2 mg/dL or creatinine clearance of less than 70 mL/min/1.73 m^2 body surface area. The covariates, which were statistically significant in the univariate analysis, were then included in the multivariate analysis. The effects of these covariates were expressed by a hazard ratio (HR). A value of $P < 0.05$ was considered to indicate statistical significance. Haplotype analysis, based on a maximum likelihood method, was performed using ARLEQUIN software Version 2.0 (Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva, Switzerland). Pairwise linkage disequilibrium coefficients were calculated and expressed as the D′ = D/D max or D/D min, according to Thompson et al [21].

RESULTS

Genotype distribution, allele frequency, and estimated haplotype frequency of the *AGT* gene variant

Table 1 summarizes the results of genotyping for the *AGT* polymorphisms of A(−20)C and M235T in this study. The expected frequencies of the genotypes, assumed to be under the Hardy-Weinberg equilibrium, were no different from the observed genotype distribution. The genotype and allele frequencies of *AGT* A(−20)C and M235T were consistent with those of previous reports for the Japanese population [22, 23]. The estimated frequencies of the haplotypes are also shown in Table 1. The haplotype analysis revealed that the two alleles were in a tight linkage disequilibruim with the linkage disequilibrium coefficient (D′) of 1.000 ($P <$ 0.0001). Only three haplotypes: T235 and C(−20); T235 and A(−20); and M235 and A(−20), were observed, indicating that the polymorphism at −20 of *AGT* only existed in a subset of the 235T alleles (Table 1).

Characteristics of the patients and comparisons between patients who did or did not receive ACE inhibitors/angiotensin receptor blockers

Table 2 shows the clinical manifestations of the patients both at the baseline and during the observation. Out of 259 patients 110 (42.5%) received ACE inhibitors/angiotensin receptor blockers after the diagnosis and during their clinical course. Comparisons were made between patients who did or did not receive ACE inhibitor/angiotensin receptor blocker therapy. At baseline, there was no difference in gender, serum creatinine, or creatinine clearance. However, the patients who received the ACE inhibitor/angiotensin receptor blocker therapy were found to be older, have heavier urinary protein, and higher blood pressures than compared with those who did not. During the mean observation period of 92.2 ± 67.7 months, 30.5% (79) of patients progressed to progressive renal disease. Despite the obvious clinical risk factors at the baseline such as heavier proteinuria and higher blood pressure, the incidence of progressive renal disease was significantly lower in patients with the ACE inhibitor/angiotensin receptor blocker administration ($\chi^2 =$ 15.786, $P < 0.0001$). As a consequence of the higher level of proteinuria, a higher proportion of patients with ACE

Table 2. Clinical characteristics of patients with immunoglobulin A nephropathy (IgAN) and comparisons between patients who did or did not receive angiotensin-converting enzyme inhibitor/angiotensin receptor blockade (ACEI/ARB)

		ACEI/ARB administration			
	Total $N = 259$	Received $N = 110$	Not received $N = 149$	P value	χ^2
At the time of renal biopsy					
Age *year*	37.0 ± 13.4	39.1 ± 13.8	35.5 ± 13.0	0.0492	
Gender *male* %	46.3	49.1	44.3	0.4442	0.585
Urinary protein excretion *g/day*	1.31 ± 1.31	1.44 ± 1.21	1.21 ± 1.39	0.0231	
Serum creatinine *mg/dL*	1.00 ± 0.63	0.92 ± 0.32	1.06 ± 0.78	0.6138	
Creatinine clearance *mL/min*	88.8 ± 33.0	90.7 ± 30.1	87.3 ± 35.0	0.3437	
Blood pressure *mm Hg*					
Systolic	127.6 ± 18.2	130.6 ± 17.8	125.2 ± 18.3	0.0135	
Diastolic	77.3 ± 13.3	78.9 ± 13.5	76.0 ± 13.1	0.1021	
Incidence of hypertension %	35.6	44.0	29.2	0.0144	5.986
During observation					
Observed period *month*	92.2 ± 67.7	91.7 ± 74.9	92.6 ± 62.2	0.3238	
Incidence of progressive renal disease %	30.5	17.3	40.3	<0.0001	15.786
Corticosteroid %	27.5	39.0	18.8	0.0005	12.192
ACEI/ARB %	42.5	100	0		
Blood pressure *mm Hg*					
Systolic	128.4 ± 16.6	130.0 ± 15.6	127.2 ± 17.3	0.0749	
Diastolic	77.5 ± 11.6	78.3 ± 10.6	76.9 ± 12.4	0.2524	

Values are mean ± SD.

Table 3. Cox proportional hazard model to test the significance of clinical covariates and genotypes of *AGT* polymorphisms as predictors of renal survival

Variable	P value	HR	95% CI
Urinary protein excretion >1.0 g/day	0.0001	3.346	1.817, 6.162
Hypertension	0.0100	1.949	1.173, 3.238
Deteriorated renal function	<0.0001	3.040	1.795, 5.155
No ACEI/ARB administration	0.0004	2.725	1.561, 4.755
T235 and C(−20) haplotype of AGT	0.0322	1.608	1.074, 3.039

Abbreviations are: HR, hazard ratio; deteriorated renal function, a serum creatinine level of more than 1.2 mg/dL or creatinine clearance of less than 70 mL/min/1.73 m^2 body surface area at the time of diagnosis; ACEI/ARB, angiotensin-converting enzyme inhibitor and/or angiotensin receptor blocker; CI, confidence interval.

inhibitor/angiotensin receptor blocker therapy were treated with glucocorticoids. The mean blood pressure during the clinical course was no different between the two groups.

Identification of risk factors for the progression to progressive renal disease

Cox proportional hazards regression analysis was used to test the significance of the clinical covariates at the time of diagnosis and the genotypes of the *AGT* polymorphism. In the multivariate analysis, a urinary protein excretion of more than 1.0 g/day, hypertension, deteriorated renal function, no ACE inhibitor/angiotensin receptor blocker administration, and the T235 and C(−20) haplotype of *AGT* were identified as significant and independent risk factors (Table 3), whereas steroid therapy, age, and gender were not. The T235 and C(−20) haplotype of *AGT* was demonstrated to be a independent risk factor for progressive renal disease with an increased HR of 1.608 (95% CI, 1.074 to 3.039; $P = 0.0322$) even after adjusting for the other significant covariates, including a urinary protein of more than 1.0 g/day (HR, 3.346; 95% CI, 1.817 to 6.162; $P = 0.0001$), hypertension (HR, 1.949; 95% CI, 1.173 to 3.238; $P = 0.0100$), deteriorated renal function at the time of renal biopsy (HR, 3.040; 95% CI, 1.795 to 5.155; $P < 0.0001$), and no administration of ACE inhibitors/angiotensin receptor blockers (HR, 2.725; 95% CI, 1.561 to 4.755; $P = 0.0004$). In the univariate analysis, the M235 and A(−20) haplotype was not a significant prognostic factor (HR, 0.776; 95% CI, 0.477 to 1.261; $P = 0.3055$). The therapeutic efficacy of ACE inhibitors/angiotensin receptor blockers on the renal survival was confirmed by Kaplan-Meier analysis (Fig. 1A). The mean survival of renal function in patients, who received ACE inhibitors/angiotensin receptor blockers, was significantly longer than that of those without ACE inhibitors/angiotensin receptor blockers (198 ± 11.6 months vs. 139.0 ± 6.8 months, respectively; log rank test $\chi^2 = 10.728$, $P = 0.0011$). Figure 1B shows the renal survival within a subgroup of patients who received steroid therapy ($N = 71$) with or without ACE inhibitors/angiotensin receptor blockers, as well as those with ACE inhibitor/angiotensin receptor blocker therapy but no steroids ($N = 67$). The therapeutic efficacy was clearly seen within the group with steroid therapy (Kaplan-Meier log rank test, $\chi^2 = 10.032$; $P = 0.0015$), indicating that the renoprotective effect of ACE inhibitors/angiotensin receptor blockers was independent of steroid treatment. In addition, the renal survival of patients with ACE inhibitors/angiotensin receptor blockers but no ste-

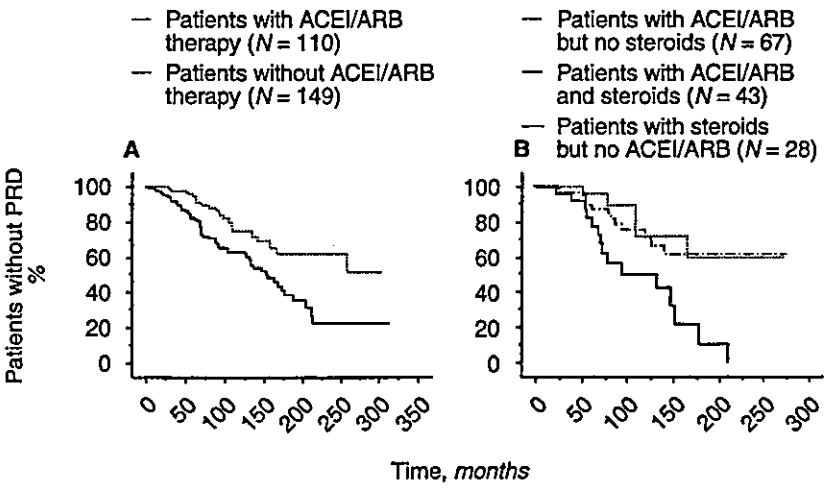

Fig. 1. Renal survival rate in the total 259 immunoglobulin A nephropathy (IgAN) patients, and in 71 cases, which were treated with corticosteroids, as well as those with ACE inhibitors/angiotensin receptor blockers (ACEI/ARB) but no steroids. (*A*) In the total 259 patients, the renal survival of patients, who received ACE inhibitors/angiotensin receptor blockers (N = 110) was significantly better than that in those who did not (N = 149; Kaplan-Meier log rank test; χ^2 = 10.728, P = 0.0011). (*B*) In the patients, who received corticosteroids, the renal survival of patients, who received ACE inhibitors/angiotensin receptor blockers (N = 43) was significantly better than that in those who did not (N = 28; χ^2 = 10.032, P = 0.0015). The renal survival of patients with ACE inhibitors/angiotensin receptor blockers but no steroids (N = 67) was no different from that of those with both ACE inhibitors/angiotensin receptor blockers and steroids (χ^2 = 0.235; P = 0.6277), but was significantly better than that of those with steroids but no ACE inhibitors/angiotensin receptor blockers (χ^2 = 11.519; P = 0.0007). PRD is progressive renal disease.

Table 4. Cox proportional hazard model to test the significance of clinical covariates on patients with or without the T235 and C(−20) haplotype of *AGT*

	Patients with T235 and C(−20) (N = 99)			Patients without T235 and C(−20) (N = 160)		
Variable	P value	HR	95% CI	P value	HR	95% CI
Urinary protein excretion >1.0 g/day	0.0010	4.263	1.792, 10.139	0.0048	1.981	1.082, 8.029
Hypertension	0.0268	1.974	1.122, 6.141	0.0085	2.538	1.268, 5.079
Deteriorated renal function	0.0010	1.351	1.098, 2.364	<0.0001	8.197	3.759, 17.857
No ACEI/ARB administration	0.0069	1.952	1.208, 5.368	0.0012	3.620	1.663, 7.880

Abbreviations are: HR, hazard ratio; deteriorated renal function, a serum creatinine level of more than 1.2 mg/dL or creatinine clearance of less than 70 mL/min/1.73 m² body surface area at the time of diagnosis; ACEI/ARB, angiotensin-converting enzyme inhibitor and/or angiotensin receptor blocker; CI, confidence interval.

roids was no different from that of those with both ACE inhibitors/angiotensin receptor blockers and steroids (χ^2 = 0.235; P = 0.6277), but was significantly better than that of those with steroid but no ACE inhibitors/angiotensin receptor blockers (χ^2 = 11.519; P = 0.0007).

Renoprotective efficacy of ACE inhibitor/angiotensin receptor blocker therapy and the AGT haplotype

Next to examine whether the *AGT* polymorphism affects the renoprotective effect of ACE inhibitors/angiotensin receptor blockers, the Cox proportional hazards analysis was performed on patients with or without the T235 and C(−20) haplotype (Table 4), and on those with or without M235 and A(−20) haplotype (Table 5). The therapeutic efficacy was significant both in patients with and without the T235 and C(−20). On the other hand, the HR of no ACE inhibitor/angiotensin receptor blocker administration was 1.092 (95% CI, 0.661 to 3.418; P = 0.4701) in the patients carrying the M235 and A(−20) haplotype of *AGT*, whereas it was significant in those with other haplotypes (HR, 2.715; 95% CI, 1.417 to 5.202; P = 0.0026). Figure 2 shows a comparison of the renal survival rates in patients with or without ACE inhibitor/angiotensin receptor blocker administration, who did (Fig. 2A) and did not carry (Fig. 2B) the M235 and A(−20) haplotype of *AGT*, respectively. Again, within a subgroup of patients with at least one haplotype of M235 and A(−20) (N = 90), the renal survival rate was no different between groups with or without ACE inhibitor/angiotensin receptor blocker treatment (Kaplan-Meier log rank test, χ^2 = 0.700, P = 0.4028). In contrast, a remarkable therapeutic efficacy on the renal survival rate was observed in the patients lacking the M235 and A(−20) haplotype (N = 169; Kaplan-Meier log rank test, χ^2 = 11.805, P = 0.0006). Table 6 shows the comparisons of clinical characteristics between groups of patients with and without the M235 and A(−20) haplotype. No significant difference was observed in any point between the two groups. The blood pressures both at baseline and during the observation as well as the incidence of hypertension were numerically higher in the patients without M235 and A(−20), although the differences were

Table 5. Cox proportional hazard model to test the significance of clinical covariates on patients with or without the M235 and A(−20) haplotype of *AGT*

	Patients with M235 and A(−20) (N = 90)			Patients without M235 and A(−20) (N = 169)		
Variable	P value	HR	95% CI	P value	HR	95% CI
Urinary protein excretion >1.0 g/day	0.0418	3.255	1.045, 10.143	0.0032	2.763	1.407, 5.426
Hypertension	0.0113	2.214	1.329, 5.914	0.0288	1.930	1.070, 3.480
Deteriorated renal function	0.0054	4.032	1.511, 10.753	0.0013	2.747	1.484, 5.076
No ACEI/ARB administration	0.4701	1.092	0.661, 3.418	0.0026	2.715	1.417, 5.202

Abbreviations are: HR, hazard ratio; deteriorated renal function, a serum creatinine level of more than 1.2 mg/dL or creatinine clearance of less than 70 mL/min/1.73 m² body surface area at the time of diagnosis; ACEI/ARB, angiotensin-converting enzyme inhibitor and/or angiotensin receptor blocker; CI, confidence interval.

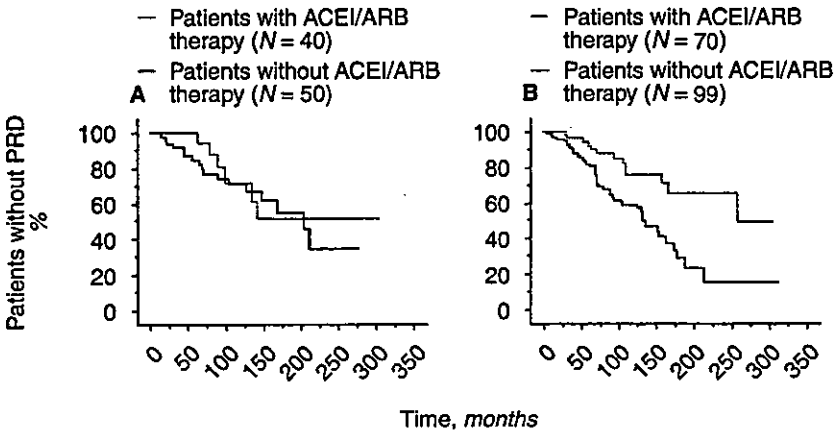

Fig. 2. Renal survival rate in patients with (N = 90), and without the M235 and A(−20) haplotype (N = 169). (*A*) In patients with the haplotype M235 and A(−20), the renal survival of patients, who received ACE inhibitors/angiotensin receptor blockers (ACEI/ARB) (N = 40) was not significantly different from that in those who did not (N = 50; Kaplan-Meier log rank test; χ^2 = 0.700, P = 0.4028). (*B*) In contrast, in the patients without the M235 and A(−20) haplotype, the renal survival of patients, who received ACE inhibitors/angiotensin receptor blockers (N = 70) was significantly better than that in those who did not (N = 99; χ^2 = 11.805, P = 0.0006). PRD is progressive renal disease.

Table 6. Comparisons of clinical characteristics between patients with and without the M235 and A(−20) haplotype of the *AGT* polymorphism

	With M235 and A(−20) (N = 90)	Without M235 and A(−20) (N = 169)	P value	χ^2
At the time of renal biopsy				
Age *year*	37.6 ± 13.7	36.7 ± 13.3	0.5688	
Gender *male* %	40.0	49.7	0.1359	2.224
Urinary protein excretion *g/day*	1.24 ± 1.38	1.35 ± 1.27	0.5220	
Serum creatinine *mg/dL*	0.96 ± 0.42	1.02 ± 0.72	0.7332	
Creatinine clearance *mL/min*	90.0 ± 36.1	88.1 ± 31.3	0.8980	
Blood pressure *mm Hg*				
Systolic	125.5 ± 16.3	128.7 ± 19.2	0.1712	
Diastolic	76.4 ± 13.3	77.8 ± 13.4	0.3202	
Incidence of hypertension %	27.6	39.8	0.0522	3.691
During the observation				
Observed period *month*	92.1 ± 71.2	92.2 ± 66.0	0.7413	
Incidence of progressive renal disease %	25.6	33.1	0.2070	1.592
Glucocorticoid %	24.7	29.1	0.4634	0.538
ACEI/ARB %	44.4	41.4	0.6392	0.220
Dosage of enarapril *mg* (N = 56)	4.6 ± 2.1 (N = 18)	4.5 ± 1.9 (N = 38)	0.9013	
Duration of enarapril *months* (N = 56)	82.6 ± 58.1	84.9 ± 66.3	0.8579	
Blood pressure *mm Hg*				
Systolic	125.2 ± 14.8	126.8 ± 17.2	0.2410	
Diastolic	75.8 ± 11.1	78.5 ± 11.8	0.1761	

ACEI/ARB is angiotensin-converting enzyme inhibitor and/or angiotensin receptor blocker. Values are mean ± SD.

not statistically significant. In patients who received enalapril (N = 56), the average dosage of drug and the administration duration were no different between patients with or without the M235 and A(−20) haplotype.

DISCUSSION

A characteristic of the present study was that the renoprotective efficacy of ACE inhibitors/angiotensin receptor blockers on the prognosis of renal function, but not

on proteinuria over a short-term period, in IgAN was evaluated by a sufficiently long-term observation, and its association with *AGT* gene polymorphism was analyzed by multivariate and time-to-event analysis. The therapeutic efficacy was independent of other clinical variables such as proteinuria, hypertension, deteriorated renal function at the baseline, and steroid therapy. The important and interesting finding in the present study is that a subgroup of patients, who carry at least one M235 and A(−20) haplotype of *AGT*, did not respond well to the ACE inhibitor/angiotensin receptor blocker therapy, whereas the treatment was remarkably effective in patients without this haplotype. The antiproteinuric effect of ACE inhibitors/angiotensin receptor blockers may be associated with the effect on renal survival. However, accurate assessment of the quantitative antiproteinuric effect over a long-term period was difficult in this retrospective style study, because consistent measurements of urinary protein excretion were not always available for each subject. In fact, even within patients who had stable renal functions over a long period, we sometimes observed fluctuations in the urinary protein excretion of up to 50% or more. Therefore, in this study the renoprotective effect was evaluated by long-term renal survival, which would have more important clinical implications.

It is important to predict the renoprotective effects of antihypertensive agents in individual patients with renal disease. It has been reported that polymorphism in the *ACE* gene is associated with the therapeutic efficacy of ACE inhibitors on proteinuria over a relatively short-term observation period in IgAN and diabetic nephropathy, whereas the results from previous studies are conflicting [12–16]. In addition to the possible reasons for the conflicting conclusions, which include differences in the ethnicity, study design and relatively small sample sizes, there is the possibility that the contribution of the *ACE* I/D polymorphism is of insufficient magnitude to lead to any obvious or definite conclusion from the different study designs in various ethnic populations.

Genetic variations in *AGT* have been shown to be associated with variations in plasma angiotensinogen levels. The M235T polymorphism, which is in a complete linkage disequilibrium with the A(−6)G polymorphism of the *AGT*, is associated with an increased risk of essential hypertension in multiple populations [24, 25]. In addition, association of *AGT* A(−20)C with essential hypertension in the Japanese population has been reported [20, 26, 27]. Recently, we provided evidence that both the M235T and A(−20)C polymorphisms in *AGT* were associated with the progression of renal dysfunction in 137 patients with IgAN, whose renal function was preserved at the time of renal biopsy [28]. Our present result, that the T235 and C(−20) haplotype is an independent risk factor for progressive renal disease in all patients with IgAN, which included those with deteriorated renal function at baseline, is consistent with both our previous study and a previous report in the Caucasian population [29]. The prognostic significance of the T235 and C(−20) haplotype observed in this study was relatively weak in comparison with that of our previous result. This may partly be a consequence of an association between T235 and C(−20) and progression of renal dysfunction because deteriorated renal function at the baseline, which was excluded from the previous analysis, was included as a covariate in the Cox proportional hazard model in the present study. Although there was no significant difference in blood pressures between groups with each *AGT* haplotype in our patients with IgAN, we cannot provide any evidence to support or deny the possible association between the *AGT* polymorphisms and hypertension from the results of this study because a substantial proportion of the study population had renal dysfunction, which is likely to be the cause of hypertension and may affect the plasma AGT level.

Although we could not provide data on the circulating and/or local tissue activity of angiotensin II in each genotype, both T235 and C(−20) alleles of *AGT* have been found to be functionally associated with higher transcriptional activity of the gene [22, 30]. Therefore, it is likely that the more rapid progression of renal dysfunction in patients carrying the haplotype T235 and C(−20) is due to the higher activity of angiotensin II than those without this haplotype. Conversely, it is not surprising that patients carrying the M235 and A(−20) haplotype, who are assumed to have lower transcriptional activity of *AGT*, would be resistant to the efficacy of the RAS blockade because of their lower angiotensin II activity in tissues.

There is a possibility that the group with M235 and A(−20) received lower doses of ACE inhibitors/angiotensin receptor blockers than the other group, although in some of patients the average dose and duration of enalapril administration were no different because their blood pressures tended to be, but not significantly, lower at the baseline. This may explain in part the apparent lack of efficacy of ACE inhibitors/angiotensin receptor blockers in the group with M235 and A(−20).

To elucidate the precise molecular mechanism of the finding in this study, the local tissue activity of angiotensin II in the kidney and its response to the ACE inhibitor/angiotensin receptor blocker administration in patients with each set of *AGT* haplotype needs to be investigated by a randomized controlled prospective study. Studies in other ethnic populations are also needed because there are substantial differences in the haplotype frequencies of *AGT* between Caucasian and Japanese populations [23]. It is known that single nucleotide polymorphisms with high allele frequencies are more statistically informative than those with low allele frequencies in association studies [31].

This study was not a randomized controlled study and, in fact, the ACE inhibitors/angiotensin receptor blockers were preferably prescribed to patients with a heavier proteinuria and higher blood pressure at the time of diagnosis, both of which were independent clinical risk factors for the progression of renal dysfunction. However, the remarkable renoprotective effect of the ACE inhibitor/angiotensin receptor blocker therapy was observed in this study. Despite more severe clinical manifestations at baseline, the renal outcome was significantly better in the patients who received ACE inhibitor/angiotensin receptor blocker treatment than that in those who did not. To confirm the results of the present study, a randomized controlled prospective study with a large-scale population of patients is necessary. However, making conclusions about the long-term renal survival of IgAN in a prospective study is difficult, because the actual prognosis of renal function in each case can only be determined after a sufficiently long observation period. In fact, a substantial proportion of our patients had stable renal function and only one third of them progressed to the end point during the mean observation period of 92.2 months. Therefore, we believe that the Cox hazard regression analysis, and time-to-event analysis, using past precise medical records, is an adequate and feasible method to investigate the long-term renal prognosis. Moreover, with this approach, no assumption of linearity was necessary and information could be drawn from patients with a partial follow-up. Therefore, even if the bias as a result of this being a retrospective study is taken into account, this study provides a new theory suggesting that the interindividual variation in responsiveness to the RAS blockade is associated with *AGT* polymorphism.

ACE inhibitors are well established as renoprotective drugs both in diabetic and nondiabetic renal disease [8, 32]. Angiotensin receptor blockers are the other inhibitors of the RAS, although it has distinct pharmacologic properties from ACE inhibitors. At present we have no knowledge or data on comparisons of the long-term renoprotective effect of either ACE inhibitors or angiotensin receptor blockers alone, with a combination of both drugs [8], but similar renal vasodilation by both classes of drugs has been reported in studies on direct comparisons of renal hemodynamic effects in patients with hypertension or renal diseases [33, 34]. The combination of ACE inhibitors and angiotensin receptor blockers has been reported to be at least additive in decreasing protein excretion in patients with IgAN [35, 36], whereas the results of larger trials are still controversial [37, 38]. In the present study, the numbers of subjects who were treated with each drug were not large enough to analyze separately. Further investigation using a fixed medication protocol is also needed on this matter.

CONCLUSION

This study provides the first evidence that *AGT* polymorphisms can influence, not only the prognosis of renal function, but also the renoprotective efficacy of a RAS blockade on the long-term renal survival in IgAN patients. Although further studies in other ethnic populations and investigations on the association of the *AGT* haplotypes with local tissue activity of angiotensin II are necessary, the results of this study, if confirmed, suggest that more active ACE inhibitor/angiotensin receptor blocker usage may be applied for patients with no M235 and A(−20) haplotype of *AGT* even in an early phase of the disease. Further work needs to be undertaken to assess whether patients with the M235 and A(−20) haplotype can responding some way to the RAS blockade or to other classes of antihypertensive agents.

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Hepatocyte growth factor regulates proteoglycan synthesis in interstitial fibroblasts

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Hepatocyte growth factor regulates proteoglycan synthesis in interstitial fibroblasts.

Background. Hepatocyte growth factor (HGF) is a clinically important growth factor with therapeutic potential for the treatment of interstitial fibrosis and chronic renal failure. Proteoglycans are components of the renal interstitium, which have multiple actions, including growth regulation. In this study, we examined the effects of HGF on proteoglycan synthesis in interstitial fibroblasts, and the mechanisms of these effects.

Methods and Results. Expression and agonist-induced activation of the HGF receptor c-Met was detected in rat renal interstitial fibroblasts (NRK-49F) by reverse transcription-polymerase chain reaction (RT-PCR) analysis and immune complex/immunoblot assay. Moreover, stimulation of the cells with HGF resulted in a marked increase (five- to tenfold) in phosphorylation of extracellular signal-related protein kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (MAPK), but not of c-Jun NH_2 terminal kinase (JNK). Treatment with HGF resulted in a time- and dose-dependent increase ($P < 0.01$) in both cell-associated and secreted proteoglycan synthesis to two- to fourfold of control levels. This effect was attenuated by the MAPK/ERK kinase (MEK) inhibitor PD98059 and the p38 MAPK inhibitor SB203580. Ion-exchange chromatography suggested that chondroitin sulfate/dermatan sulfate proteoglycans were up-regulated after HGF treatment. Northern blot, RT-PCR, Western blot, and promoter activity assays revealed that HGF caused a significant increase in decorin mRNA and protein, as well as in biglycan mRNA, protein, and promoter activity, suggesting transcriptional control of gene expression. Since the effects of biglycan on fibroblast proliferation are still unclear, the effects of biglycan were examined by thymidine assay, and biglycan was found to attenuate transforming growth factor-β (TGF-β)–induced changes in cell proliferation.

Conclusion. These results suggest that HGF causes an increase in the small leucine-rich proteoglycans biglycan and decorin by ERK1/2- and p38 MAPK-mediated pathways in fibroblasts. These findings may be relevant for understanding potential mechanisms by which HGF can exert TGF-β inhibitory actions in the kidney.

Key words: hepatocyte growth factor, proteoglycan, fibroblast, biglycan, decorin.

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Recent studies have suggested that hepatocyte growth factor (HGF) is an important therapeutic candidate for the treatment of renal disease. HGF is a relatively recently discovered growth factor, which was originally identified and cloned as a mitogen for mature hepatocytes [1]. However, later studies suggested that HGF also has multiple actions in the kidney, many of which may be of therapeutic benefit [2–4]. For example, injection of recombinant HGF into a mouse model of chronic renal disease was shown to inhibit the onset of tubulointerstitial fibrosis [5]. Similarly, administration of HGF by injection [6] or by systemic administration of naked plasmid [7] has been reported to ameliorate chronic renal fibrosis seen in the unilateral ureteral obstruction model of interstitial fibrosis, whereas neutralization of endogenous HGF with anti-HGF antibodies produces the opposite effect [6]. A beneficial effect of HGF has also been found in chronic allograft nephropathy [8]. From these results emerges the concept that HGF not only plays a role in the pathogenesis of renal disease, but also may be useful for the therapy of interstitial fibrosis.

An important objective in nephrology is the development of new treatments to arrest and/or reverse the progression of chronic renal failure. These results from several laboratories suggest that HGF may indeed hold promise for the treatment of renal disease, but at present many of the mechanisms by which HGF can influence the progression of renal disease are still undefined.

Proteoglycans are important components of the extracellular matrix of the renal interstitium and are known to fulfill a variety of important functions, including the control of collagen deposition, and the activation and inactivation of cytokines and growth factors. In particular, the proteoglycan decorin is thought to be a natural inhibitor of transforming growth factor-β (TGF-β) [9], and administration of this proteoglycan by injection [10] or gene transfer [11] has been shown to arrest the progression of renal disease.

Although proteoglycans play an important role in the processes of growth and extracellular matrix deposition,

the effects of HGF on proteoglycan synthesis are undefined. In this study, our aims were to (*1*) to examine if HGF receptors are present in fibroblasts, and to understand the signal transduction mechanisms activated by HGF; (*2*) to characterize the effects of HGF on proteoglycan subtype synthesis in interstitial fibroblasts, and the mechanisms of these effects; and (*3*) to examine potential consequences of the up-regulation of proteoglycans by HGF. Our results suggest that HGF can up-regulate growth modulatory proteoglycans, which may be relevant for our understanding of the actions of HGF in the renal interstitium.

METHODS

Culture of rat renal interstitial fibroblasts (NRK-49F) and analysis of c-Met mRNA

The rat renal interstitial fibroblast cell line NRK-49F was obtained from the American Type Culture Collection, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Total RNA was purified by the acid guanidine-phenol-chloroform method [12], then 1 μg was subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for c-Met mRNA using the primers and protocols described by Oh et al [13]. In some experiments, reverse transcriptase was omitted to ensure the lack of contamination with genomic DNA. To confirm that the results were not specific to the NRK-49F cell line, experiments were also performed using human neonatal dermal fibroblast primary cultures [neonatal human fibroblast (NHF) cells], which were obtained from Sanko Junyaku (Tokyo, Japan).

Analysis of agonist-induced c-Met phosphorylation

Analysis of agonist-induced c-Met phosphorylation was performed using an immune complex-immunoblot analysis technique [14]. In brief, NRK-49F was made quiescent by placing it in serum-free DMEM for 48 hours. The cells were treated with HGF (20 ng/mL) for 5 minutes, and then reactions were terminated by rinsing with ice-cold phosphate-buffered saline (PBS), followed by lysis in immunoprecipitation buffer [50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L ethyleneglycol tetraacetate (EGTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mg/mL leupeptin, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), and 2 mmol/L sodium orthovanadate]. The lysates were first precleared with Pansorbin (Calbiochem, San Diego, CA, USA), then lysates containing equal amounts of protein (~0.5 mg) were incubated with polyclonal anti-Met antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at 4°C for 2 hours. The resulting immune complexes were collected by precipitation with Pansorbin, washed three times with immunoprecipiation buffer, resuspended in 1 × Laemmli electrophoresis buffer, and heated at 95°C for 5 minutes. Proteins were resolved by electrophoresis on 4% to 12% sodium dodecyl sulfate-polyacrylamide gel elctrophoresis (SDS-PAGE) gels, and transferred to nitrocellulose membranes. Tyrosine phosphorylation of the receptors was visualized by Western blotting of the membranes with monoclonal anti-phosphotyrosine antibody (PY99, 1:500) (Santa Cruz Biotechnologies) and horseradish peroxidase-coupled secondary antibody, using the enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK). In parallel experiments, blots were treated with anti-Met antibody (1:200) to confirm equal loading of samples.

Assessment of mitogen-activated protein kinase phosphorylation

Phosphorylation of the mitogen-activated protein kinase (MAPK) extracellular signal-related protein kinase (ERK)1/2, p38 MAPK, and c-Jun NH_2 terminal kinase (JNK) were assessed by Western blot. Quiescent cells were stimulated with HGF (20 ng/mL) for the indicated times, then the reactions were stopped by the addition of ice-cold 1 × Laemmli electrophoresis buffer. Proteins were resolved by electrophoresis on 12% SDS-PAGE gels, then phosphorylated MAPKs were detected using antiphospho-ERK1/2, antiphospho-p38 MAPK, and antiphospho-JNK antibodies (Cell Signaling, Beverly, MA, USA). The relative intensities of the bands were assessed using computer densitometry software (Scion Image, Frederick, MD, USA).

Proteoglycan synthesis assays

Synthesis of cell-associated and medium-secreted proteoglycan was determined as described by us previously [15, 16]. Cells in 24-well plates were made quiescent in serum-free media for 48 hours. Following serum deprivation, cultures were incubated in DMEM containing ^{3}H-glucosamine (2 μCi/mL) or sulfate-free medium containing ^{35}S-sulfate (5 μCi/mL) in the presence of HGF (20 ng/mL unless otherwise stated) for 48 hours. The medium was harvested and 300 μL of the supernatant was incubated with 25 μL of 25 mmol/L $MgSO_4$, and 120 μL of 2.5% cetylpyridinium chloride (CPC) in the presence of 5 μg of carrier chondroitin sulfate for 1 hour at 37°C. Precipitated proteoglycans were collected on nitrocellulose filters by vacuum filtration, washed with 1.0% CPC in 20 mmol/L NaCl and radiocounted in a liquid scintillation counter. In some experiments, samples were treated overnight at 37°C with chondroitinaseABC (10 mU) in 33 mmol/L Tris HCl, 33 mmol/L sodium acetate, 80 μg/mL bovine serum albumin (BSA) (pH 8.0), or chondroitinase AC (10 mU) in 33 mmol/L Tris HCl, 80 μg/mL BSA (pH 6.0), or heparitinase III (10 mU) in 100 mmol/L sodium acetate, 10 mmol/L calcium acetate (pH 7.0) prior to CPC precipitation. For determination of cell-associated proteoglycan synthesis,

the cell layers were rinsed with PBS and lysed in 1 mol/L NaOH. A total of 300 μL of each sample were neutralized with 2 N acetic acid, and digested with Pronase E (1 mg/mL) at 55°C for 18 hours. After addition of chondroitin sulfate (100 μg/mL) as a carrier, cell-associated proteoglycans were precipitated for 3 hours at 37°C with 1% CPC in 20 mmol/L NaCl. The precipitate was collected on nitrocellulose filters and treated as described above.

Ion exchange chromatography

To separate proteoglycans in the media on the basis of differences in charge density, ion exchange chromatography was performed as described previously using DEAE-Sephacel (Amersham) [15, 16]. After application of media containing ^{35}S-sulfate–labeled proteoglycans from control and HGF-treated cells, unbound radioactivity was removed from the column by washing with 30 mL of wash buffer [8 mol/L urea, 50 mmol/L Tris (pH 7.5), 2 mmol/L EDTA, 0.1 mol/L NaCl, 0.5% Triton X-100]. Bound radioactivity was eluted with a NaCl gradient (0.1 to 0.7 mmol/L in the same buffer) and the radioactivity in the collected fractions were quantified by scintillation counting.

Northern blot analysis

Total RNA was purified by the acid guanidine-phenol-chloroform method [12] and quantified by measurement of absorbance of 260 nm in a spectrophotometer. Total RNA (20 μg) was denatured with formamide and formaldehyde at 65°C for 10 minutes and fractionated by electrophoresis through a 1.0% formaldehyde-agarose gel. RNA was stained with ethidium bromide to verify integrity and equal loading, transferred to a nylon filter (Pall BioSupport, East Hills, NY, USA), then cross-linked using an ultraviolet irradiator (Stratagene, La Jolla, CA, USA). Prehybridization was conducted at 42°C for 2 hours in a buffer containing 6 × standard sodium citrate (SSC) [0.9 mol/L sodium chloride, 0.09 mol/L sodium citrate (pH 7.0)], 5 × Denhardt's solution [0.1% (wt/vol) polyvinylpyrrolidone, 0.1% (wt/vol) ficoll type 400, and 0.1% (wt/vol) BSA], 50% formamide, 0.1% SDS, and sheared, denatured salmon sperm DNA (100 μg/mL). The cDNA probe for biglycan [17] was generously provided by Dr. Dreher (Weis Center for Research, Danville, PA, USA). The probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Clontech (Palo Alto, CA, USA). Probes were radiolabeled with α-^{32}P deoxycytidimine triphosphate (dCTP) by the random primer synthesis method (RadPrime DNA Labeling System, Gibco-BRL, Grand Island, NY, USA). After hybridization, the filter was washed in 0.2 × SSC, 0.1% SDS at 42°C. Bands were visualized, and incorporated radioactivity was quantified by scanning with a laser image analyzer (model BAS 2000, Fuji Film, Tokyo, Japan).

RT-PCR

RT-PCR was performed as described by us previously [16]. One microgram total RNA was reverse transcribed in a reaction mixture containing 10 mmol/L Tris HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L desoxynucleoside triphosphate (dNTP), 1 U RNase inhibitor, 2.5 μmol/L (50 pmol) random hexamers and 2.5 U Moloney murine leukemia virus reverse transcriptase in a volume of 20 μL. The reverse-transcribed product was amplified with proteoglycan core protein sense and antisense primers in a reaction mixture containing 10 mmol/L Tris HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 15 pmol of each primer, 5 μCi ^{32}P dCTP, and 2.5 U Taq polymerase using a Perkin-Elmer-Cetus Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) for 24 cycles. The sequences of the primers for biglycan, decorin, versican, perlecan, and GAPDH were as reported previously [16, 18]. Preliminary experiments confirmed that the amplifications were performed in the linear phase of the amplification cycle. In some experiments, reaction products were subcloned into the plasmid pCDNA3.1His/Topo (Invitrogen, Groningen, The Netherlands) and sequenced using an automated sequencer. Reaction products were resolved by electrophoresis through 8% polyacrylamide gels. Gels were dried using a gel dryer prior to imaging using a laser image analyzer.

Western blot analysis of proteoglycan core proteins

Western blot analysis of proteoglycan core proteins in the media of NHF cells treated with HGF (20 ng/mL) was performed after chondroitinase ABC digestion as described previously [16] using antihuman biglycan (LF-51) and antihuman decorin (LF-136) antibodies [19, 20], which were generously provided by Dr. Fisher (National Institute of Dental and Craniofacial Research, Bethesda, MD, USA).

Transient transfection and luciferase assays

Plasmids containing various lengths of the 5′-flanking region of the human biglycan gene cloned upstream of the luciferase gene in vector pGL2-Basic (Promega, Madison, WI, USA) [21] were generously provided by Dr. Ungefroren (University of Hamburg, Germany). The constructs used were Bgn (−1212, +42), Bgn (−686, +42), Bgn (−153, +42), Bgn (−78, +42), and Bgn (−46, +42). The numbers in parentheses refer to the positions of the 5′- and 3′-nucleotides relative to the major transcription start site (5′ end of exon 1) of the biglycan gene. To assess the promoter activity of luciferase constructs with and without HGF treatment, NRK-49F cells in 24-well plates were transfected with various biglycan promoter luciferase plasmids (0.5 μg) using lipofectamine plus (Gibco-BRL) as recommended by the manufac-

turers. Some of the cells were treated with HGF (20 ng/mL) after completion of the transfection procedure. A Renilla luciferase construct (pRL-TK) was used to normalize for changes in transfection efficiency, and the luciferase activities were assessed 24 hours after transfection by the dual luciferase assay (Promega) exactly as recommended by the manufacturer.

Thymidine incorporation studies and enzyme-linked immunosorbent assay for active TGF-β1

Subconfluent NRK-49F cells in 24-well plates were made quiescent by treating with serum-free medium for 48 hours. TGF- β1 and biglycan were preincubated together at 37°C for 24 hours, then applied to the quiescent cells. After 24 hours, ^{3}H- thymidine (1 μCi/mL) was added, and the cells were harvested after a further 24 hours for the assessment of thymidine incorporation by trichloroacetic acid (TCA) precipitation [22]. The activity of TGF-β1 in the culture medium in the presence or absence of biglycan was assessed by measurement of active TGF-β1 using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), exactly as recommended by the manufacturer. Total TGF-β1 in the same samples was estimated after acid activation of latent TGF-β1 following the manufacturer's protocols.

Statistics

Results are expressed as the mean ± SEM. Statistical comparisons were made by analysis of variance (ANOVA) followed by Scheffe's F test. *P* values less than 0.05 were considered statistically significant.

Materials

Cell culture materials, radioisotopes, and electrophoresis materials were obtained from Gibco-BRL, Amersham, and BioRad, respectively. Recombinant human TGF-β1 was obtained from R&D Systems. Other reagents, including biglycan purified from bovine articular cartilage, were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

RESULTS

Analysis of c-Met expression and activation in interstitial fibroblasts

To examine if rat interstitial fibroblasts (NRK-49F) express the c-Met receptor, RT-PCR was performed using the primers and protocols described by Oh et al [13]. As shown in Figure 1A, a 725 bp band corresponding to reverse transcribed and amplified c-Met mRNA was readily detectable, whereas the band was not seen in the absence of reverse transcriptase treatment, suggesting expression of c-Met mRNA. Similar results were seen with primary cultures of human fibroblasts (NHF cells).

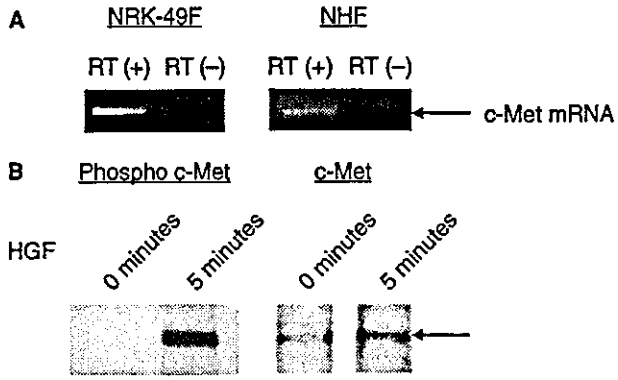

Fig. 1. Expression and activation of hepatocyte growth factor (HGF) receptor c-Met in NRK-49F and neonatal human fibroblast (NHF) cells. (*A*) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of c-Met mRNA. Total RNA from NRK-49F or neonatal human fibroblast (NHF) cells were treated with or without RT, then PCR was performed using primers specific for c-Met mRNA. (*B*) Activation of c-Met by HGF. NRK-49F cells were treated for the indicated times with HGF, then phosphorylation of c-Met was examined by immunoprecipitation with anti-Met antibody followed by immunoblot with antiphosphotyrosine antibody (left panel), or anti-Met antibody (right panel) as described in the Methods section.

To confirm that the receptors were functionally active, cells were stimulated with HGF, and the tyrosine phosphorylation of the receptors after agonist treatment was examined by immunoprecipitation with c-Met antibody followed by immunoblot with antiphosphotyrosine antibody. As shown in Figure 1B, treatment with HGF resulted in an increase in the phosphorylation of immunoprecipitated c-Met at 5 minutes.

Effects of HGF on phosphorylation of ERK1/2, p38 MAPK, and JNK in interstitial fibroblasts

To examine the effects of HGF on phosphorylation of different MAPKs, cells were treated with HGF, then activation of MAPK was assessed by examination of phosphorylated MAPK by Western blot analysis. As shown in Figure 2, treatment with HGF resulted in a marked (five- to tenfold) increase in phosphorylation of ERK1/2 and p38 MAPK, without a major change in JNK.

Effects of HGF on proteoglycan synthesis in interstitial fibroblasts

Next, the effects of HGF on proteoglycan synthesis were examined. As shown in Figure 3, treatment of NRK-49F cells with HGF resulted in a significant ($P < 0.01$) increase in proteoglycan synthesis. A time- and dose-dependent increase in both cell-associated and secreted proteoglycan synthesis was seen. In order to characterize the subclass of the proteoglycans in control and HGF-treated samples, conditioned media were treated with the enzymes chondroitinase ABC, chondroitinase AC, and heparitinase prior to CPC precipitation. Treatment of cells with HGF resulted predominantly in an

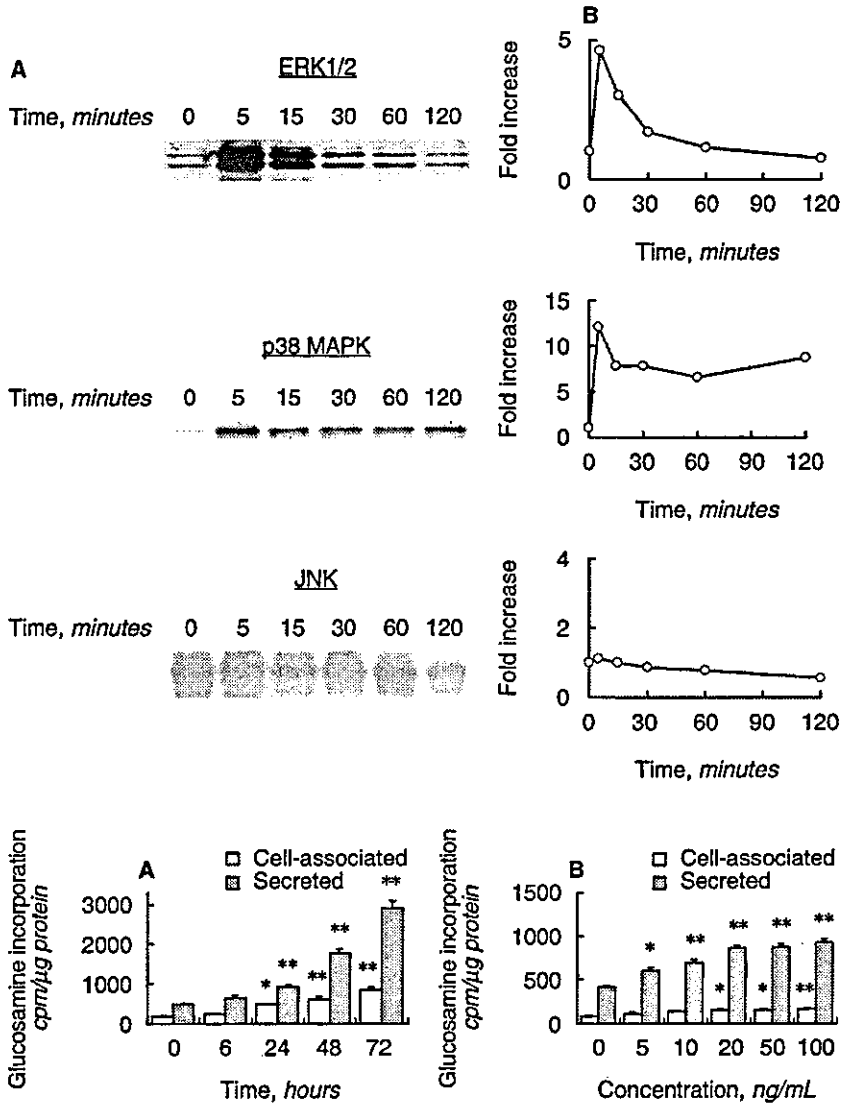

Fig. 2. Effects of hepatocyte growth factor (HGF) on phosphorylation of mitogen-activated protein kinase (MAPK) family proteins in NRK-49F cells. Quiescent cells were treated with HGF (20 ng/mL) for the indicated times, then phosphorylation of extracellular signal-related protein kinase (ERK) 1/2 and p38 MAPK, but not of c-Jun NH_2 terminal kinase (JNK) was assessed using specific antibodies as described in the **Methods** section. (*A*) Representative image of Western blot. (*B*) Results of densitometric analysis.

Fig. 3. Time course and dose dependency of hepatocyte growth factor (HGF)-induced changes in proteoglycan synthesis in NRK-49F cells. Quiescent cells were treated with 20 ng/mL HGF for various times (*A*) or various doses (*B*) of HGF for 48 hours, then proteoglycan synthesis in the cell layer and media was assayed as described in the **Methods** section. Results shown are the mean ± SEM (N = 4 per assay point). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control.

increase in chondroitinase ABC–sensitive proteoglycans (Table 1), without a major change in the heparitinase-sensitive proteoglycans.

In order to confirm these results, characterization of the proteoglycan subclass was also performed by ion exchange chromatography using DEAE-Sephacel (Fig. 4). In the supernatants from both control and HGF-treated samples, incorporated ^{35}S-sulfate radioactivity eluted from the ion exchange column predominantly at two peaks. The counts from peak I and II were attenuated by pretreatment with heparitinase and chondroitinase ABC, respectively, as reported previously [15], confirming that these peaks contained predominantly heparan sulfate proteoglycans (HSPG) and chondroitin sulfate proteoglycans (CSPG)/dermatan sulfate proteoglycans (DSPG). A clear increase in peak II was seen in the HGF-treated samples, whereas no major changes in peak I were observed, suggesting an increase in CSPG/DSPG consistent with the results of the enzyme digestion experiments.

Effects of MEK inhibitor and p38 MAPK inhibitor on the HGF-mediated increase in proteoglycan synthesis

To examine the signal transduction mechanisms involved in the increase in proteoglycan synthesis mediated by HGF, cells were pretreated with the MEK inhibitor PD98059 or the p38 MAPK inhibitor SB203580 in order to inhibit the ERK1/2 and p38 MAPK pathways, respectively. As shown in Figure 5, the HGF-mediated increases in proteoglycan synthesis in both the media and cell layers were attenuated by treatment with either the MEK or p38 MAPK inhibitors, suggesting that both

Table 1. Analysis of proteoglycan subtype synthesis in media from NRK-49F cells with and without HGF treatment

	Incorporation *cpm/μg protein*			
	HGF (−)		HGF (+)	
	^{3}H-glucosamine	^{35}S-sulfate	^{3}H-glucosamine	^{35}S-sulfate
Chondroitinase ABC-sensitive incorporation	331 ± 77	198 ± 44	949 ± 235*	405 ± 30*
Chondroitinase AC-sensitive incorporation	207 ± 71	94 ± 30	380 ± 116	141 ± 51
Heparitinase-sensitive incorporation	61 ± 29	29 ± 14	82 ± 20	42 ± 21

Abbreviations are: NRK-49F, rat renal interstitial fibroblasts; HGF, hepatocyte growth factor.
Results shown are mean ± SEM ($N = 6$).
* $P < 0.05$ vs. HGF (−)

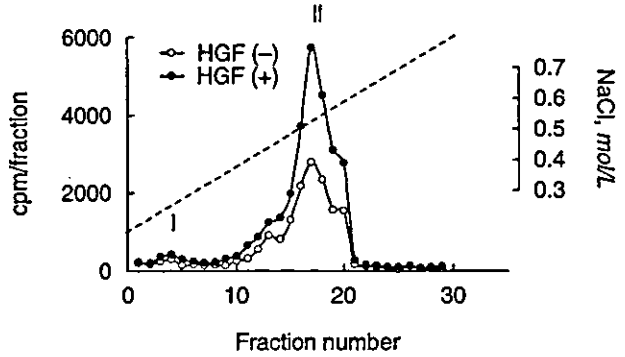

Fig. 4. Analysis of proteoglycans in media from control and hepatocyte growth factor (HGF)-treated NRK-49F cells by DEAE-Sephacel ion exchange chromatography.

ERK1/2 and p38 MAPK pathways were involved in the changes in proteoglycan synthesis mediated by HGF.

Effects of HGF on proteoglycan core protein expression

To examine if the changes in proteoglycan synthesis mediated by HGF involved changes in proteoglycan core protein mRNA, Northern blot analysis was performed to assess changes in the major proteoglycan core proteins. Since the signals obtained by Northern blot were low in the case of decorin, versican, and perlecan (data not shown), the mRNA for these core proteins were assessed by RT-PCR. Previous studies from our group have confirmed a close correlation between the results of RT-PCR and Northern blot analysis [16, 18]. Treatment of NRK-49F cells with HGF caused significant ($P < 0.05$) increases in biglycan and decorin mRNA, whereas no significant change was seen in the case of versican and perlecan. To confirm these findings, expression of the proteoglycan core proteins decorin and biglycan in control and HGF-treated samples were examined by Western blot analysis. These experiments were performed using NHF, since the antibodies used (LF-51 and LF-136) were raised against the human core proteins. The antibodies showed some cross-reactivity toward purified bovine biglycan and decorin, which were run in parallel as positive controls. Consistent with the result of Northern blot analysis and RT-PCR, expression of biglycan and decorin was increased in HGF-treated samples compared to control cells (Fig. 6B). Moreover, the increases in biglycan and decorin induced by HGF were attenuated by pretreatment with the MEK inhibitor PD98059 and the p38 MAPK inhibitor SB203580, suggesting that the MEK and p38 MAPK pathways affect both biglycan and decorin.

Effects of HGF on biglycan promoter activity

In order to examine the mechanisms of the HGF-induced increase in biglycan mRNA, NRK-49F cells were transfected with biglycan promoter constructs using lipofectamine, and promoter activity with or without HGF treatment was assessed by the dual luciferase assay. As shown in Figure 7, HGF caused a significant increase (~threefold) in the promoter activity of the biglycan construct Bgn (−1212, +42). In contrast, no significant increase in promoter activity was induced by HGF in the case of the truncated constructs Bgn (−686, +42), Bgn (−153, +42), Bgn (−78, +42), and Bgn (−46, +42). These results support the data from the Northern blot analysis, and suggest transcriptional control at a region between –686 and –1212 as a potential mechanism for the increased gene expression of biglycan mediated by HGF.

Effects of biglycan and basal and TGF-β–stimulated proliferation of interstitial fibroblasts

At present, the effects of biglycan on growth of fibroblasts are unclear. Therefore, in order to clarify these actions, cells were stimulated with biglycan with or without TGF-β1, and the effects on DNA synthesis were assessed by thymidine incorporation studies. As shown in Figure 8A, biglycan alone did not cause a significant change in thymidine incorporation by these cells. In contrast, biglycan pretreatment of TGF-β1 resulted in a significant reduction in thymidine incorporation compared to cells treated with TGF- β1 in the absence of biglycan. The effects of biglycan were dose-dependent, as shown in Figure 8B. In further studies, the activity of TGF-β1 (1 ng/mL) pretreated with or without biglycan (20 nmol/L)