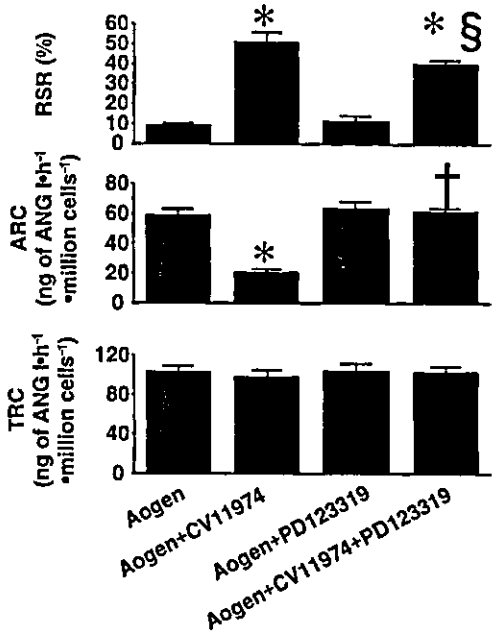

Fig. 3. *Effects of angiotensin II type 1 receptor blocker (CV11974, 10 μmol/l), angiotensin II type 2 receptor blocker (PD123319, 10 μmol/l), and both on renin secretion rate (RSR), active renin content (ARC), and total renin content (TRC) in juxtaglomerular cells incubated with buffer containing 100 nmol/l recombinant sheep angiotensinogen (Aogen). The experiment was performed with 6 primary cultures.* *$p<0.05$ vs. *Aogen alone.* †$p<0.05$ *for Aogen+CV11974+PD123319* vs. *Aogen+CV11974.* §$p<0.05$ *for Aogen+ARB+PD123319* vs. *Aogen+PD123319.*

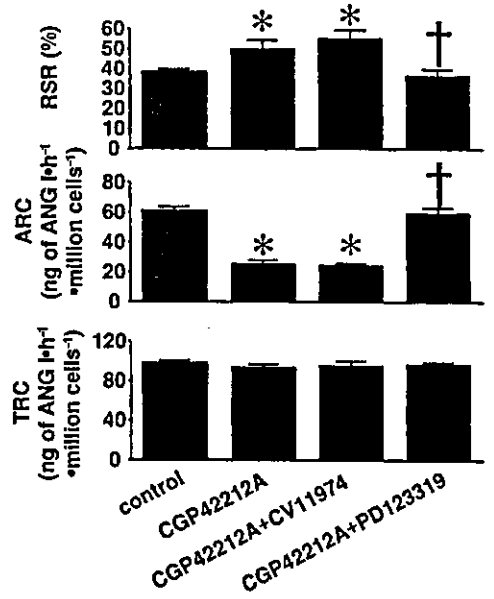

Fig. 4. *Effects of angiotensin II type 2 receptor agonist (CGP42212A, 0.1 μmol/l) on renin secretion rate (RSR), active renin content (ARC), and total renin content (TRC) in juxtaglomerular cells incubated with control buffer or buffer containing angiotensin II type 1 receptor blocker (CV11974, 10 μmol/l) and angiotensin II type 2 receptor blocker (PD123319, 10 μmol/l). The experiment was performed with 6 primary cultures.* *$p<0.05$ vs. *control.* †$p<0.05$ *for CGP42212A+PD123319* vs. *CGP42212A alone.*

Treatment with CGP42212A significantly increased RSR from 38.2±1.6% to 49.7±4.7% and significantly decreased ARC from 60.8±3.0 to 25.3±2.8 ng of Ang I·h^{-1}·million cells^{-1} without affecting TRC (97.7±2.8 and 93.2±3.6 ng of Ang I·h^{-1}·million cells^{-1} for control and CGP42212A, respectively). In the presence of CV11974, CGP42212A also increased RSR to 55.0±4.6%, decreased ARC to 24.3±1.6 ng of Ang I·h^{-1}·million cells^{-1}, and did not influence TRC in JG cells. These changes were similar to those observed in the JG cells treated with CGP42212A alone. In the presence of PD123319, however, CGP42212A did not influence RSR, ARC, or TRC of JG cells. The RSR of JG cells treated with CGP42212A+PD123319, averaging 36.3±3.6%, was similar to that of JG cells treated with control buffer and significantly lower than the RSR of JG cells treated with CGP42212A alone or CGP42212A+CV11974. The ARC of JG cells treated with CGP42212A+PD123319, averaging 59.0±4.1 ng of Ang I·h^{-1}·million cells^{-1}, was similar to that of the control buffer-treated JG cells and significantly greater than that of JG cells treated with CGP42212A alone or CGP42212A+CV11974.

Discussion

Addition of Aogen to culture medium significantly increased the medium Ang II concentration and significantly decreased RSR, suggesting that the Ang II generated from Aogen in the culture medium can inhibit renin secretion from JG cells. In addition, neither ARC nor TRC increased, despite the great decrease in RSR. It is therefore possible that the Ang II generated from Aogen also suppresses renin synthesis in JG cells. The ACEI, CV3317, significantly attenuated the conversion of Aogen to Ang II and inhibited the Aogen-induced decrease in RSR. The ARB, CV11974, also suppressed the Aogen-induced decrease in RSR, although the medium Ang II levels increased during ARB treatment. These results suggest that Ang II can inhibit renin secretion from JG cells through AT1 receptors, consistent with previous studies (*11, 23*).

Although suppression of the overall Ang II effects by ACEI treatment did not influence ARC or TRC of the Aogen-treated JG cells, the selective blockade of AT1 receptor with CV11974 significantly decreased the ARC of the Aogen-treated JG cells concomitant with a significant increase in the medium Ang II levels, suggesting that stimulation of

AT2 receptors may play an important role in this mechanism. Direct stimulation of AT2 receptors by CGP42212A also decreased the ARC of JG cells. Because selective blockade of AT2 receptors with PD123319 inhibited the decreases in ARC by either Aogen+CV11974 or CGP42212A, AT2 receptors appear to have been responsible for the ARB-induced decrease in ARC of the Aogen-treated JG cells. In addition, none of the treatments used in the present study induced any change in the TRC of JG cells. Based on the assumption that there is no intracellular degradation or inactivation of active renin after it is formed from the conversion prorenin, a decrease in ARC without a change in TRC suggests an inhibition of prorenin processing—that is, a conversion of inactive renin to active renin. Because intracellular active renin is extremely stable in secretary granules, AT2 receptors can have inhibitory effects on prorenin processing in JG cells. Therefore, in the presence of Aogen (or Ang II), ARB can suppress prorenin processing in JG cells through AT2 receptors. Although short-term treatment with an ARB increases plasma renin activity by stimulating renin secretion from JG cells *in vivo*, long-term treatment with ARB may decrease plasma renin activity by diminishing the stores of active renin in JG cells.

The stimulation of AT2 receptors by Aogen+CV11974 or CGP42212A significantly decreased ARC. Nevertheless, the blockade of AT2 receptors with PD123319 did not influence the ARC of JG cells treated with Aogen. These results suggest that the overall effects of Ang II generated from Aogen are dependent on AT1 receptors. Previous studies have reported that the affinity for Ang II and the number of AT1 receptors are greater than those of AT2 receptors under physiological conditions (*18, 20*). In the present study, confocal laser microscopy demonstrated abundant AT1 receptors and a low level of AT2 receptors in the JG cells. Therefore, the actions of AT2 receptors may be negligible during a simultaneous stimulation of AT1 receptors, and may be effective only when AT2 receptors are selectively stimulated.

Treatment with CGP42212A increased RSR with a significant decrease in the ARC of JG cells. In the JG cells treated with CGP42212A, however, the absolute value of the renin secreted to the culture medium averaged 25.6 ± 3.9 ng of Ang $I \cdot h^{-1} \cdot ml^{-1}$ and was significantly lower than that in control JG cells, averaging 38.2 ± 3.6 ng of Ang $I \cdot h^{-1} \cdot ml^{-1}$. These results suggest that CGP42212A significantly decreased ARC and thereby reduced the content of the renin secreted to the medium. Because of the great decrease in ARC, RSR increased only slightly despite a significant decrease in the absolute value of the renin secreted to the medium.

The culture medium concentrations of Ang II were significantly higher in JG cells treated with Aogen+CV11974 than in the cells treated with Aogen alone. Because Ang II generated from Aogen can be internalized to JG cells through Ang II receptors or degraded to Ang III in the culture medium, several possible mechanisms may be considered. First, CV11974 may enhance the processing from Aogen to Ang II. The rate-limiting enzyme of this processing is renin, and its concentrations in the culture medium were elevated by the increase in renin secretion induced by CV11974. Thus, treatment with CV11974 may increase the generation of Ang II by elevating the active renin concentration in the culture medium. Secondly, blockade of Ang II binding to AT1 receptors with CV11974 may inhibit the internalization of Ang II into cells, and thereby increase the medium Ang II concentration. Thirdly, CV11974 may inhibit the degradation of Ang II pharmacologically. In any case, the increased concentrations of medium Ang II contributed to the stimulation of AT2 receptors during treatment with Aogen+CV11974.

Treatment with CV3317 significantly decreased the medium Ang II concentration of JG cells treated with Aogen; however, the medium Ang II concentration of JG cells treated with Aogen+CV3317 was still higher than that of JG cells treated with control buffer alone. Nevertheless, CV3317 completely inhibited the Aogen-induced decrease in RSR. There may be a threshold concentration below which Ang II does not further inhibit renin secretion from JG cells. Alternatively, CV3317 itself might have a stimulating effect on renin secretion. However, our preliminary study demonstrated that the RSR of JG cells treated with CV3317 alone was similar to the RSR of untreated JG cells.

In conclusion, an ACEI significantly decreased the medium Ang II levels and increased RSR without affecting ARC or TRC in Aogen-treated cells in the present study. However, ARB significantly increased the medium Ang II levels and RSR and decreased ARC without affecting TRC. The decreases in ARC of the Aogen+ARB-treated cells were inhibited by the blockade of AT2 receptors. In addition, the stimulation of AT2 receptors significantly decreased ARC without affecting TRC. This decrease in ARC was inhibited by the AT2 receptor blocker but not by the AT1 receptor blocker. Therefore, unlike the ACEI, the ARB inhibited prorenin processing of JG cells through AT2 receptors.

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Induction of Glia Maturation Factor-β in Proximal Tubular Cells Leads to Vulnerability to Oxidative Injury through the p38 Pathway and Changes in Antioxidant Enzyme Activities*

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Proteinuria is an independent risk factor for progression of renal diseases. Glia maturation factor-β (GMF-β), a 17-kDa brain-specific protein originally purified as a neurotrophic factor from brain, was induced in renal proximal tubular (PT) cells by proteinuria. To examine the role of GMF-β in PT cells, we constructed PT cell lines continuously expressing GMF-β. The PT cells overexpressing GMF-β acquired susceptibility to cell death upon stimulation with tumor necrosis factor-α and angiotensin II, both of which are reported to cause oxidative stress. GMF-β overexpression also promoted oxidative insults by H_2O_2, leading to the reorganization of F-actin as well as apoptosis in non-brain cells (not only PT cells, but also NIH 3T3 cells). The measurement of intracellular reactive oxygen species in the GMF-β-overexpressing cells showed a sustained increase in H_2O_2 in response to tumor necrosis factor-α, angiotensin II, and H_2O_2 stimuli. The sustained increase in H_2O_2 was caused by an increase in the activity of the H_2O_2-producing enzyme copper/zinc-superoxide dismutase, a decrease in the activities of the H_2O_2-reducing enzymes catalase and glutathione peroxidase, and a depletion of the content of the cellular glutathione peroxidase substrate GSH. The p38 pathway was significantly involved in the sustained oxidative stress to the cells. Taken together, the alteration of the antioxidant enzyme activities, in particular the peroxide-scavenging deficit, underlies the susceptibility to cell death in GMF-β-overexpressing cells. In conclusion, we suggest that the proteinuria induction of GMF-β in renal PT cells may play a critical role in the progression of renal diseases by enhancing oxidative injuries.

In chronic nephropathies, proteinuria is reportedly one of the best predictors, independent of mean arterial blood pressure, for disease progression toward end-stage renal failure (1, 2). Microalbuminuria, which features a small quantity of albumin only (30–300 mg/24 h), is known as an important early sign of diabetic nephropathy (3, 4) and of progressive renal function loss in a non-diabetic population (5). In experimental models, proteinuria caused tubular insults accompanying infiltration of macrophages and T lymphocytes into the kidney (6). Interstitial inflammation can trigger fibroblast proliferation and accumulation of extracellular matrix proteins, which may facilitate tubulointerstitial fibrosis, which is a hallmark of progression of renal disease. In cultured proximal tubular (PT)[1] cells activated by administration of albumin, a number of genes encoding vasoactive and inflammatory molecules, which have potentially toxic effects on the kidney, were transactivated (7). These results strongly suggest that altering the disposition of PT cells by proteinuria must be involved in the process of renal damage. However, the mechanisms by which proteinuria accelerates renal disease progression remain largely unknown.

We recently found that the brain-specific glia maturation factor-β (GMF-β) gene is induced in PT cells by proteinuria by comparison of the gene expression profiles (8, 9)[2] of normal and proteinuria disease models (10, 11). GMF-β is a 17-kDa brain-specific protein that was isolated from bovine brain homogenate as a substance inducing the maturation of normal neurons as well as glial cells (12, 13); and at first, it was considered to be a neurotrophic factor. However, later intensive researches provided cumulative evidence that GMF-β is involved in cell signal transduction. This evidence comprised the following findings. 1) The GMF-β protein lacks a leader sequence and is not secreted by cells (14). 2) It contains consensus phosphorylation sites and is phosphorylated by protein kinase C, protein kinase A, casein kinase II, and ribosomal S6 kinase in *in vitro* studies (15, 16). 3) GMF-β inhibits the extracellular signal-regulated kinase-1/2 and enhances p38 activity (17). 4) Overexpressed GMF-β in primary astrocytes causes secretion of neurotrophic factors such as brain-derived neurotrophic factor and nerve growth factor through activation of the p38 pathway (18). The amino acid sequence of GMF-β is highly conserved among many species (19), suggesting that it plays basic roles across many species. The expression of GMF-β is largely limited to the brain (19), especially the glial cells and some neu-

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[1] The abbreviations used are: PT, proximal tubular; GMF-β, glia maturation factor-β; TNF-α, tumor necrosis factor-α; BSO, buthionine sulfoximine; PBS, phosphate-buffered saline; FCS, fetal calf serum; CuZn-SOD, copper/zinc-superoxide dismutase; Mn-SOD, manganese-superoxide dismutase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; TRITC, tetramethylrhodamine isothiocyanate; ROS, reactive oxygen species; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; NBT, nitro blue tetrazolium; GPX, glutathione peroxidase; ANOVA, analysis of variance.

[2] Available at bodymap.ims.u-tokyo.ac.jp/.

rons (20). Schwann cells of the distal segment of the transected nerve express GMF-β, and this induction of GMF-β coincides with the temporal expression of nerve growth factor receptors in the cells (21). These results suggest that GMF-β may play a protective role in the brain. However, its precise function in neurons and glial cells is still largely a matter of speculation.

It was thus of interest to determine what impact could be served by the induction of the brain-specific protein GMF-β in PT cells by proteinuria. In this study, we demonstrate that proteinuria induced GMF-β in PT cells in a time-dependent manner. Overexpression of GMF-β in a mouse PT cell line and NIH 3T3 cell lines resulted in susceptibility to cell death upon stimulation with tumor necrosis factor-α (TNF-α), angiotensin II, and other oxidative stress-inducing agents (buthionine sulfoximine (BSO) and H_2O_2). Further studies clarified that GMF-β overexpression caused enhancement of oxidative injury, leading to F-actin reorganization and apoptosis under oxidative stress. We demonstrate that GMF-β-overexpressing cells, as a mechanism of the vulnerability to oxidative stress, caused a prolonged increase in H_2O_2 through changes in several antioxidant enzyme activities and that p38 was significantly involved in this process. These results suggest that the induction of the brain-specific GMF-β gene in the kidney may play a key role in renal disease progression caused by proteinuria.

EXPERIMENTAL PROCEDURES

Protein-overloaded Proteinuria Murine Model—Five-week-old C57BL6 male and female mice weighing ~20 g were intraperitoneally given bovine serum albumin (10 mg/g of weight; Sigma) dissolved in saline for 5 days during a 1-week period. The final dose of 10 mg/g of weight was reached by incremental increases in the dose over the first week, beginning with 2.5 mg/g of weight. Control mice were treated with saline (7, 11). The load of bovine serum albumin was continued to 4 weeks. At 1, 2, 3, and 4 weeks, 24 h after loading of bovine serum albumin, kidneys were removed for investigations.

Tissue Preparation and Laser Capture Microdissection—The protein-overloaded proteinuria model mice were separated into three random groups. After 1, 2, 3, or 4 weeks of loading of bovine serum albumin, kidneys from the first group (n = three each) were removed after perfusion with phosphate-buffered saline (PBS) for Northern blot analysis, and those from the second group (n = one each) after perfusion first with PBS and then with 4% paraformaldehyde. They were made into specimens with the paraffin sectioning method after paraformaldehyde fixation and used for *in situ* hybridization. Kidneys from the third group (n = three each) were removed after perfusion first with PBS and then with 99.5% ethanol. Kidney tissue sections were prepared and subjected to laser microdissection using an LM200 Image Archiving workstation (Arcturus Engineering, Mountain View, CA) along with real-time PCR as described (25, 26). Total RNA was extracted from samples attached to LCM transfer film using TRIzol (Invitrogen) and reverse-transcribed with SuperScript™ II RNase H reverse transcriptase (Invitrogen). Quantitation of GMF-β mRNA/rRNA was performed with this real-time PCR system according to the manufacturer's instructions. The GMF-β TaqMan probe was 5′-TGGTTTCA-GTCTCTGCTAGTTCATACCGCA-3′. The GMF-β forward primer sequence was 5′-GAGGCTTGAAACATTGGTGGTT-3′, and its reverse primer sequence was 5′-CAAGCACCATGCTTACCAAAAG-3′. The rRNA TaqMan probe and the forward and reverse primers were obtained from TaqMan rRNA control reagents (Applied Biosystems, Foster City, CA).

Northern Blot Analysis—Total RNA from mouse kidney was extracted with TRIzol according to the manufacturer's instructions. Ten micrograms of the RNAs were fractionated on formaldehyde-agarose gels, transferred onto nylon membranes (Hybond-N$^+$, Amersham Biosciences), and subjected to Northern analyses. For the probes, we used the mouse GMF-β sequences (bp 3080–4130) obtained from the GenBank™/EBI Data Bank (accession number AF297220). Glyceraldehyde-3-phosphate dehydrogenase cDNA was used as an internal control.

In Situ Hybridization—GMF-β was subjected to *in situ* hybridization with the DNA nucleic acid detection kit (Roche Applied Science) according to the manufacturer's instructions. The GMF-β sense and antisense cRNA probes were prepared by PCReaction. The primers for PCR were 5′-GTCGCAGTAGAGTGGAGTGTGTTG-3′ and 5′-CAGGTCAGGGCC-ATTCACTCTATG-3′. The PCR product was then subcloned into pST-Blue-1, and the sequence was confirmed to be identical to that of mouse GMF-β (data not shown). The clone was digested by *Xho*I to produce the template of the antisense probe and by *Bam*HI to produce that of the sense probe. The digoxigenin-labeled antisense and sense cRNA probes were generated using 1 μg of template and T7 or SP6 RNA polymerase, respectively, in combination with the digoxigenin RNA labeling mixture (Roche Applied Science).

Cell Culture and Transfection—The mouse renal proximal tubular cell line mProx24 (60) and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum (FCS) (Invitrogen) in 5% CO_2 at 37 °C. The mProx24 cells were maintained in 0.1% gelatin-coated culture dishes. The mouse GMF-β coding region was amplified by PCR using forward primer 5′-CGGGATCCC-GCTGACGACCGGAAGGAAAATGAGTGAG-3′ and reverse primer 5′-CGGGATCCCGCCAGTACCCAGGAGTGGTCAGAGGAGG-3′, cut with *Bam*HI, and inserted into the *Bgl*II site of FLAG-linked pCMV-Taq1 (Stratagene, La Jolla, CA). Permanent transfection of cells with mammalian expression vectors was achieved with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The transfectants were maintained in Dulbecco's modified Eagle's medium containing 10% FCS and 200 μg/ml Geneticin (G418, Sigma).

Immunoblotting—Total cell lysates were separated by SDS-PAGE (12% acrylamide) and then electrotransferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences). FLAG-GMF-β was detected using mouse monoclonal anti-FLAG antibody M2 (Sigma) at a 5000:1 dilution. The blots were probed with individual primary antibodies as described above and then incubated with horseradish peroxidase-conjugated antibodies. Rabbit polyclonal antibody was raised against mouse GMF-β (the amino acid sequence of the epitope polypeptide was NH_2-YQHDDGRVSYPLC-COOH) and confirmed by immunoblotting to bind specifically the GMF-β protein band. Anti-CuZn-SOD and anti-Mn-SOD antibodies were purchased from Stressgen Biotech Corp. (Victoria, British Columbia, Canada). Proteins were visualized with an enhanced chemiluminescence reagent (Pierce).

Cell Viability Assay—Cell viability for mouse PT and NIH 3T3 cells was determined 84 and 48 h, respectively, after treatments by the addition of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) (inner salt; Promega) (27). The MTS tetrazolium compound is bioreduced by cells into a colored formation product that is soluble in culture medium. The solution of MTS mixed with the electron-coupling reagent phenazine methosulfate was added directly to the culture wells and incubated for 1 h, after which absorbance at 490 nm was recorded with a 96-well plate reader. Mouse TNF-α, angiotensin II, H_2O_2, and BSO were purchased from Sigma (Sigma).

Lactate Dehydrogenase Release Assay—The results obtained in the MTS assay were confirmed in a lactate dehydrogenase release assay. A cytotoxicity detection kit (Roche Applied Science) was used to quantitate cytotoxicity/cytolysis based on the measurement of lactate dehydrogenase activity released from damaged cells. The lactate dehydrogenase activities in the culture supernatants of mProx24 and NIH 3T3 cells were determined 48 and 24 h, respectively, after treatments according to the manufacturer's instructions.

Detection of Apoptotic Cells by Annexin V Flow Cytometer Analysis—Detection of apoptotic cells was performed with an annexin V-enhanced green fluorescent protein apoptosis detection kit (Medical & Biological Laboratories, Nagoya, Japan). The assay takes advantage of the properties of binding of annexin V to membrane phosphatidylserine, which is translocated from the inner face of the plasma membrane to the apoptotic cell surface to make early detection of apoptosis by flow cytometry possible. After the addition of H_2O_2 following one overnight FCS starvation, mProx24 cells were gently trypsinized and washed once with 10% FCS-containing Dulbecco's modified Eagle's medium before incubation with annexin V-enhanced green fluorescent protein at room temperature for 5 min in the dark. The analysis of annexin V-enhanced green fluorescent protein binding was performed with a FACSCalibur flow cytometry (excitation at 488 nm and emission at 530 nm; BD Biosciences) using a fluorescein isothiocyanate signal detector.

Activities of Caspase-3—Caspase-3 activities were detected using a colorimetric assay kit (Promega). Ac-DEVD-*p*-nitroaniline was used as the substrate for caspase-3. *p*-Nitroaniline was released from the substrate after cleavage with the DEVDase caspase-3. Free *p*-nitroaniline produced a yellow color that was monitored with a Emax microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. After this treatment, the adherent mProx24 cells, both the stable transfectants and

wild-type cells, were scraped off and washed with PBS by centrifugation. The cells were then resuspended in hypotonic cell lysis buffer (5 mM EDTA, 5 mM dithiothreitol, 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin) and lysed by three cycles of freezing and thawing. The cells were finally centrifuged at 16,000 × *g* for 20 min at 4 °C, and the supernatants were used as samples for caspase assay. The protein contents of the same supernatants were assayed with the Bio-Rad protein assay. Benzyloxycarbonyl-VAD-fluoromethyl ketone (50 μM) was included in the caspase-3 assay kit.

Detection of Nucleosomal Ladders in Apoptotic Cells—DNA was extracted using the DNAzol reagent (Invitrogen). To detect nucleosomal ladders for apoptotic cells, the PCR-based amplification kit for DNA ladders (Clontech, Palo Alto, CA) was used according to the manufacturer's instructions.

Staining of F-actin and Confocal Fluorescence Microscopy—Before the various treatments, dish-cultured cells were transferred to 0.1% gelatin-coated coverslips. After 24 h, the medium was removed, and the cells were starved in serum-free medium for 12 h, after which they were fixed with 3.7% formaldehyde and permeated with 0.5% Triton X-100 (Sigma) in PBS (pH 7.5). F-actin was detected using 0.165 μM TRITC-labeled phalloidin (Sigma). The cells were examined by confocal microscopy using a Radiance 2100 BLD (Bio-Rad).

Determination of Reactive Oxygen Species (ROS) Production in PT Cells—Intracellular ROS generation was assessed using the oxidant-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Lambda Fluoreszenztechnologie, Graz, Austria) (28) in wild-type PT cells and those stably overexpressing GMF-β. One of the ROS, intracellular H_2O_2, could be assayed (29). PT cells were treated with 5 μg/ml DCFDA for 30 min at 37 °C in Krebs-Ringer bicarbonate solution (118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25.0 mM $NaHCO_3$, and 11.1 mM glucose). After DCFDA incubation, the attached PT cells were cooled and harvested by trypsinization at 4 °C, followed by flow cytometry analysis using a FACSCalibur. To examine the influence of intracellular esterase and probe efflux on the DCFDA assay, the oxidant-insensitive fluorescein diacetate derivative carboxyfluorescein diacetate (Molecular Probes, Inc., Eugene, OR) was used at 10 μM. The assay method using carboxyfluorescein diacetate was the same as that using DCFDA.

SOD Activity Assay—SOD activity was measured as described by Sutherland and Learmonth (30) using an SOD assay kit (Trevigen, Gaithersburg, MD). This method is based on the inhibition of the reduction of nitro blue tetrazolium (NBT) by SOD. Superoxide ions convert NBT into NBT-diformazan. NBT-diformazan absorbs light at 550 nm. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-formazan reflects the amount of SOD activity in a sample. CuZn-SOD was extracted with an ethanol/chloroform method, and its activity was measured. Mn-SOD activity was determined by subtracting CuZn-SOD activity from total SOD activity.

Catalase Activity Assay—Catalase activity was measured as described by Zhou *et al.* (31) using the Amplex® Red catalase assay kit (Molecular Probes, Inc.). Cells were lysed and processed in isotonic buffer (10 mM Tris-Cl (pH 7.4), 200 mM mannitol, 50 mM sucrose, and 1 mM EDTA). In the assay, catalase first reacts with H_2O_2 to produce water and oxygen. Next, the Amplex Red reacts, as calculated with a 1:1 stoichiometry, with any unreacted H_2O_2 in the presence of horseradish peroxidase to produce the highly fluorescent oxidized product resorufin. Since resorufin has also strong absorption of 563 nm, detection of this absorbance made it possible to determine the quantity of resorufin and therefore catalase activity.

Glutathione Peroxidase (GPX) Activity Assay—GPX activity was measured as described by Paglia and Valentine (32) using a GPX assay kit (Oxis Research, Portland, OR). This assay is an indirect measure of the activity of cellular GPX. Oxidized glutathione is recycled to its reduced form by glutathione reductase. The oxidation of NADPH resulting in NAD^+ is accompanied by a decrease in absorbance at 340 nm so that GPX activity can be monitored. To assay cellular GPX, a cell homogenate is added to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction is started by adding *t*-butyl hydroperoxide as a substrate, and the absorbance at 340 nm is recorded every 30 s for 3 min. The rate of decrease in the absorbance at 340 nm is directly proportionate to the GPX activity in the sample.

Cellular Glutathione Content Assay—Cellular glutathione content was measured using a glutathione content assay kit (GSH-400™, Oxis Research). The method used with this kit is based on a chemical reaction that proceeds in two steps. The first step is the formation of substitution products (thioethers) between 4-chloro-1-methyl-7-trifluromethylquinolinium methylsulfate and all mercaptans that are present in the sample. The second step is a β-elimination reaction under alkaline conditions, which transforms the substitution product (thioether) obtained with glutathione into a thione with a maximal absorbance at 400 nm. Cell pellets are resuspended in an ice-cold metaphosphoric acid working solution, and the cell lysate is produced by homogenizing the cell suspension.

Statistical Analyses—Values are expressed as means ± S.D. Except for the fluorescence-activated cell sorter analyses, all values were derived from measurements done in triplicate. Statistical analyses for multiple comparisons were performed with analysis of variance (ANOVA) and *post hoc* Bonferroni's correction. Unpaired Student's *t* test was used for the comparison of two groups in the studies with NIH 3T3 cells (Figs. 3, 4, and 8). A p value <0.05 was considered to indicate statistical significance. ANOVA and unpaired Student's *t* test were performed using a StatView software package (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Northern Blot Analysis—The gene expression profile of the proximal tubules of the proteinuria mouse model (11) confirmed that the expression of GMF-β was up-regulated by proteinuria in 1 week.[2,3] To examine the gene expression of GMF-β in the diseased mouse model kidney after >1 week of proteinuria, Northern blot analysis was performed using RNA from kidney tissues of control and diseased mouse models. The gene expression of GMF-β was found increase in a time-dependent manner (Fig. 1*A*), resulting in a significant increase in GMF-β gene expression after 2 weeks of proteinuria.

In Situ Hybridization of GMF-β in the Kidney—To examine the localization of GMF-β mRNA expression in the diseased mouse model kidney, *in situ* hybridization was performed. An increase in GMF-β gene expression was seen mainly in the proximal tubules after 3 weeks of proteinuria (Fig. 1*B*), whereas no staining was detected in the control and 1-week kidney specimens (data not shown).

Laser Microdissection and Real-time PCR Analysis—Laser microdissection and real-time PCR analysis (25, 26) were used to quantify the proximal tubule-specific gene expression of GMF-β induced by proteinuria. The laser microdissection enabled us to obtain a sufficient quantity of mRNA for the PT cells only (Fig. 1*C*, *left*). The increased expression of GMF-β mRNA in disease model PT cells was quantitatively confirmed in real-time PCR analysis (Fig. 1*C*, *right*). As shown in Fig. 1*C*, GMF-β mRNA in the PT cells from the proteinuria mouse model increased significantly (16.5 ± 3.7 times) compared with that in control PT cells and in parallel with the progression of proteinuria. These findings matched the results of Northern analyses using whole kidney mRNA (Fig. 1*A*).

Proximal Tubular Cell Lines (mProx24) That Overexpress GMF-β—To clarify the pathophysiological roles of GMF-β induction by proteinuria in PT cells, we constructed mouse proximal tubular cell lines (mProx24) that overexpress FLAG-tagged GMF-β as described under "Experimental Procedures." Immunoblotting with anti-FLAG and anti-GMF-β antibodies was employed to confirm expression of the GMF-β protein in stable transformants and mock-transfected wild-type PT cells. The results indicated that a high level of the 17-kDa GMF-β transgene product was present in the GMF-β stable transformant clones GMF0 and GMF1 (Fig. 2*A*). A basal level of endogenous GMF-β expression was detected in mock-transfected wild-type PT cells. Immunoblotting of the three types of cell lysates with anti-FLAG antibody detected FLAG-tagged GMF-β only in the GMF-β stable transformant (Fig. 2*A*). We also transfected the FLAG-GMF-β construct into mouse fibro-

[3] Available at www.med.osaka-u.ac.jp/pub/medone/kidney/array/index.html.

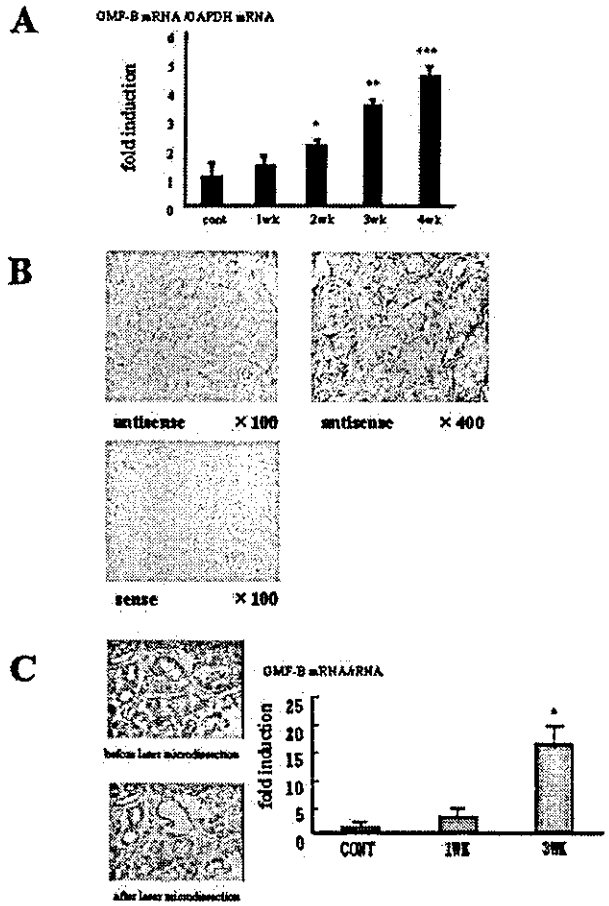

FIG. 1. **GMF-β mRNA induction by proteinuria in PT cells of the protein-overloaded proteinuria mouse model.** *A*, Northern blot analysis of GMF-β mRNA from proteinuria mouse model whole kidneys. The *bars* show the ratios of expression of GMF-β mRNA to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The *error bars* represent means ± S.D. of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* control mice (ANOVA). *cont* and *1w*, *2w*, *3w*, and *4w*, control mice and mice at 1, 2, 3, and 4 weeks of proteinuria, respectively. *B*, *in situ* hybridization of GMF-β. The data obtained with the antisense cRNA probe are shown (*left*, magnification ×100; *right*, magnification ×400) after a 3-week protein overload. Positive signals (*dark purple grains*) were observed mainly in the epithelial PT cells, but not in the specimen with the sense cRNA probe. *C*, expression of GMF-β mRNA in PT cells quantified by laser microdissection in combination with real-time PCR as described under "Experimental Procedures." As indicated by the *arrowheads* (*left*), only PT cells were isolated and collected by laser microdissection from 5-μm thick kidney section specimens. The GMF-β mRNA/rRNA ratio in the renal sections of the disease model PT cells resulting from 3-week proteinuria increased by a factor of 16.5 ± 3.7 (mean ± S.D., $n = 3$) compared with that in control PTs (*right*). *, $p < 0.05$ *versus* control mice (ANOVA).

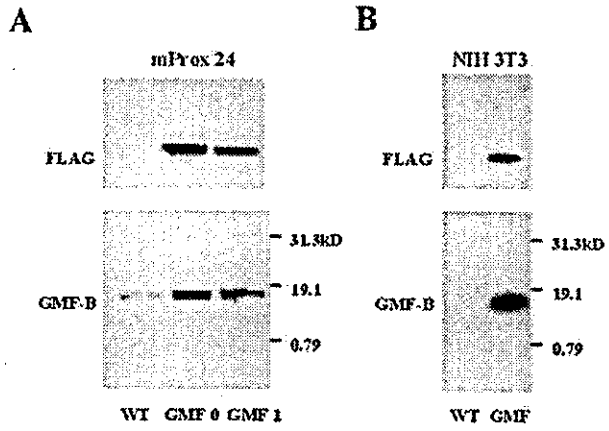

FIG. 2. **Overexpression of GMF-β in mouse PT cells and NIH 3T3 cells.** Shown are the results from immunoblotting of GMF-β using cell lysates of two clones of GMF-β (GMF0 and GMF1) and of mock-transfected wild-type (*WT*) PT cells (mProx24) (*A*) and NIH 3T3 cells (*B*). After overnight FCS starvation, total cell lysates of the five types of cell lines were immunoblotted with mouse monoclonal anti-FLAG antibody M2 and rabbit polyclonal anti-mouse GMF-β antibody.

blast NIH 3T3 cells to establish stable transformant cell lines. Immunoblotting confirmed the increased expression of GMF-β gene products (Fig. 2*B*).

Vulnerability of GMF-β-overexpressing Cells to Cell Death-inducing Stimuli—In the process of establishing GMF-β stable transformants, sudden and extensive cell death was observed as cells grew to confluence, with most cells becoming detached from the culture dish. The apoptosis-inducing stimuli TNF-α (100 ng/ml) and angiotensin II (1 μM) were added to the non-confluent PT cells to confirm the susceptibility to cell death of the GMF-β-overexpressing cells. The MTS cell viability assay showed that, with both stimuli, GMF-β-overexpressing PT cells (mProx24) were more vulnerable to cell death stimuli than were mock-transfected wild-type cells (Fig. 3, *A* and *B*). Similar results were obtained with experiments using NIH 3T3 cells stably transfected with GMF-β. The results for NIH 3T3 cells demonstrated that the vulnerability to cell death stimuli due to GMF-β overexpression was not restricted to the mouse PT cell line (mProx24). The results obtained with the MTS assay were confirmed by lactate dehydrogenase release assay (data not shown).

Oxidative Stress Enhanced by the Expression of GMF-β Induced Cell Death in PT Cells—Since both TNF-α and angiotensin II stimuli were reported to induce oxidative stress in cells and eventually cell apoptosis (33–35), we hypothesized that the cell lines could be susceptible to oxidative stress from other stimuli. The effects of oxidative stress on cells stably transfected with GMF-β were observed upon the addition of 250 μM H_2O_2 and 500 μM BSO, an inhibitor of glutathione synthesis. It was found that GMF-β stable transformants were more susceptible to oxidative stress caused by both agents than were mock-transfected wild-type cells (Fig. 4, *A* and *B*). These results were confirmed in the case of GMF-β-overexpressing NIH 3T3 cells (Fig. 4) and also using lactate dehydrogenase release assay in the two types of cells (data not shown), suggesting that GMF-β overexpression leads to an increase in cell death caused by oxidative stress.

GMF-β Overexpression Enhances Apoptosis Caused by H_2O_2—To further investigate the property of the cells with GMF-β overexpression, we studied the effect of GMF-β overexpression on apoptosis of PT cells (clone GMF0) under oxidative stress. At 250 μM H_2O_2, it took 84 h to detach the GMF0 cells. H_2O_2 at 8 mM was needed to cause apoptosis in this PT cell line in 24 h (data not shown). Binding of annexin V to phosphatidylserine to indicate early apoptotic events was also examined. There was no difference in apoptosis in the two cell types under control conditions and 1 mM H_2O_2 could not generate apoptotic change in either of the PT cell lines (Fig. 5*A*), whereas a clear increase in the numbers of apoptotic cells was observed only in GMF-β-overexpressing PT cells at 8 mM H_2O_2 (Fig. 5*B*). This increase in apoptotic cells was completely inhibited by the addition of the p38 inhibitor SB203580. Caspase-3 activity was significantly increased in GMF-β stable transformants after H_2O_2 treatment and was inhibited by SB203580 and the caspase-3 inhibitor benzyloxycarbonyl-VAD-fluoromethyl ke-

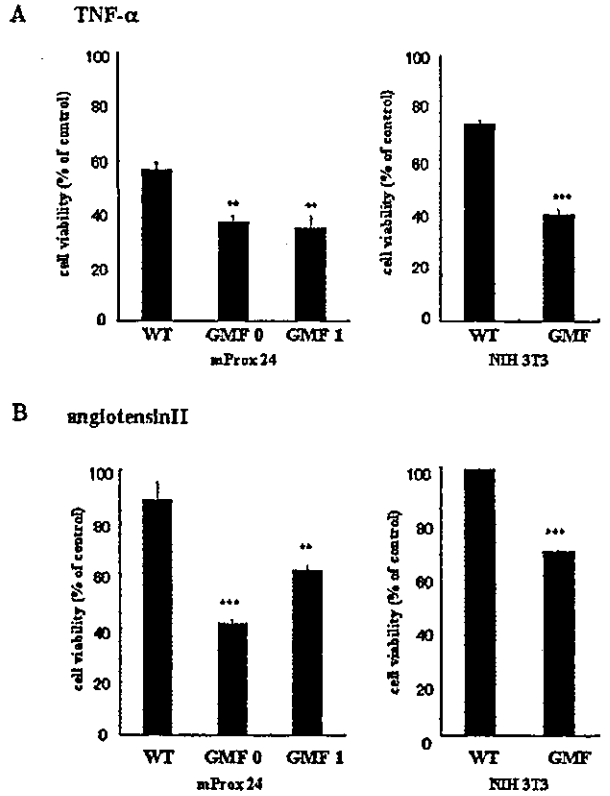

FIG. 3. **Cell viability of GMF-β-overexpressing and mock-transfected PT and NIH 3T3 cells after challenge with TNF-α or angiotensin II.** Each cell line was cultured on 96-well plates for 1 day and then treated with 100 ng/ml TNF-α or 1 μM angiotensin II for 84 h for PT cells and for 48 h for NIH 3T3 cells. Cell viability was determined with the MTS assay. Each graph shows the mean ± S.D. ($n = 3$). **, $p < 0.01$; ***, $p < 0.001$ *versus* wild-type cells (ANOVA (mProx24 cells) and Student's *t* test (NIH 3T3 cells)). *WT*, wild-type.

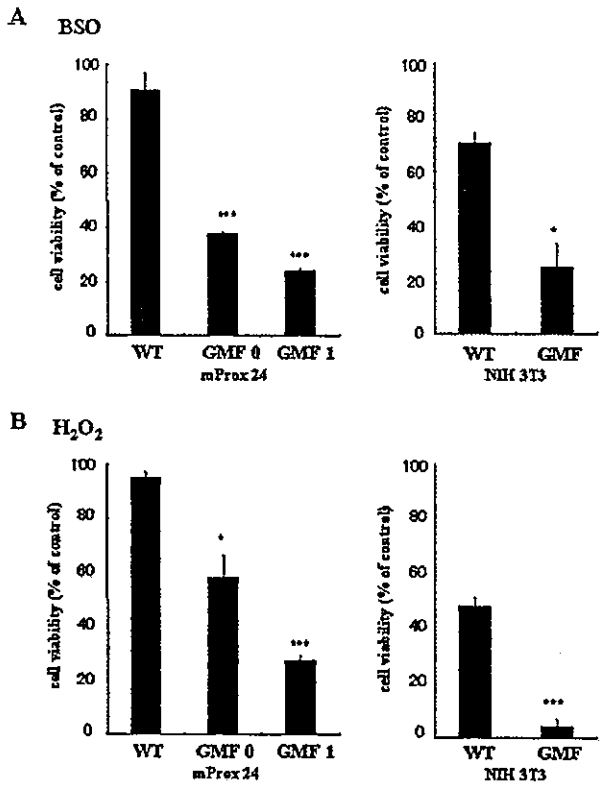

FIG. 4. **Cell viability of GMF-β-overexpressing and mock-transfected PT and NIH 3T3 cells after challenge with H_2O_2 and BSO.** Each cell line was cultured on 96-well plates for 1 day and treated with 250 μM H_2O_2 and 500 μM BSO for 84 h for PT cells and for 48 h for NIH 3T3 cells. Cell viability was determined with the MTS assay. Each graph shows the mean ± S.D. ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$ *versus* wild-type cells (ANOVA (mProx24 cells) and Student's *t* test (NIH 3T3 cells)). *WT*, wild-type.

tone (Fig. 5*C*). As shown in Fig. 5*D*, the GMF-β-overexpressing cell line showed ladder formation of DNA under H_2O_2 stress, whereas much less DNA ladder formation was seen in control cells and in the p38 inhibitor-treated GMF-β-overexpressing cells. These findings indicate that the expression of GMF-β in non-brain cells enhances the effect of oxidative stress and make the cells vulnerable to apoptotic changes governed by the p38 pathway.

F-actin Reorganization by Oxidative Stress Is Enhanced in GMF-β-overexpressing Cells—A change in F-actin organization is a well known hallmark of early oxidative cell injury in many cell types (36, 37), and this change is also found in renal diseases (22). To further examine the modification of oxidative stress by expression of GMF-β, we observed cytoplasmic F-actin reorganization under oxidative stress in GMF-β-overexpressing cells. As shown in Fig. 6*A*, it appears that cytoplasmic F-actin was disrupted and concentrated around the nuclei of wild-type PT cells 15 min after the addition of H_2O_2. In cells stably transfected with GMF-β (clone GMF0), this change in the organization of actin was more clearly observed, and the size of the cells was decreased by H_2O_2 treatment. Of interest, F-actin reorganization in GMF-β stable transformants was attenuated by the 1-h pretreatment with 10 μM SB203580 (Fig. 6*B*). In the case of the NIH 3T3 cell line, virtually no F-actin reorganization was observed in mock-transfected wild-type cells under oxidative stress for 15 min (Fig. 6*C*). However, in NIH 3T3 cells stably transfected with GMF-β, F-actin reorganization was clear, and cells contracted significantly, as seen in the case of the PT cell line (Fig. 6*D*). These results suggest that GMF-β overexpression aggravates early oxidative injury represented by the F-actin reorganization under stress in both PT and NIH 3T3 cells.

Increased and Prolonged Intracellular H_2O_2 Generation in Response to Various Stimuli as a Result of GMF-β Overexpression—Increase in intracellular H_2O_2, when converted into hydroxyl radicals, is thought to cause actin modification and cell killing. We therefore used the intracellular H_2O_2 marker DCFDA to examine the changes in intracellular H_2O_2 after the addition of H_2O_2 from outside the cells. Whereas the intracellular H_2O_2 level in wild-type cells returned to control levels ~30 min after reaching its peak at 15 min, the intracellular H_2O_2 level in GMF-β-overexpressing PT cells remained higher even 120 min following the addition of 8 mM H_2O_2 (Fig. 7*A*). Moreover, the increase in the intracellular H_2O_2 level in GMF-β-overexpressing cells was much higher (about twice as high 15 min after H_2O_2 treatment) than in wild-type cells (Fig. 7*B*). On the other hand, interestingly, pretreatment with the p38 inhibitor SB203580 was found to abolish the increase in the intracellular H_2O_2 level following the addition of 8 mM H_2O_2 (Fig. 7, *A* and *B*). Next, we examined the effects of GMF-β expression on intracellular H_2O_2 generation following treatment with 100 ng/ml TNF-α and 1 μM angiotensin II, both of which are reported to induce intracellular H_2O_2 generation and to use it as a second messenger (29, 38). As shown in Fig. 7 (*C* and *D*), the generation of intracellular H_2O_2 in GMF-β stable transfor-

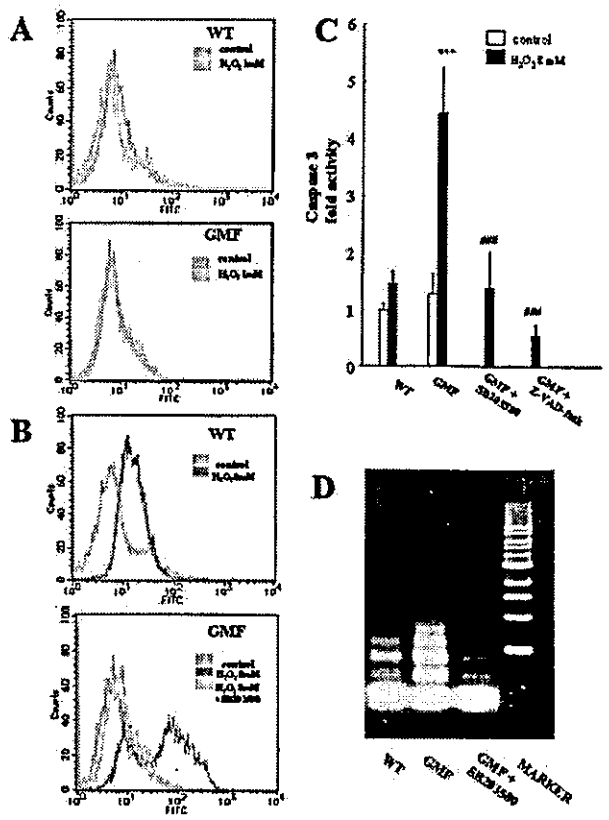

FIG. 5. **GMF-β expression aggravates the oxidative stress-induced apoptosis of PT cells.** *A*, shown are the results from flow cytometry analysis of annexin V binding to the cell-surface membrane of apoptotic PT cells upon oxidative stress. The two types of PT cell lines (GMF-β stable transformant (*GMF*) and mock-transfected wild-type (*WT*)) were treated with 1 mM H_2O_2 for 24 h after overnight FCS starvation. After the treatment, adherent cells were gently trypsinized and incubated with annexin V-enhanced green fluorescent protein. The incubated cells were then analyzed by flow cytometry using a fluorescein isothiocyanate signal detector. There was no difference in apoptosis between the two cell types under control conditions, and 1 mM H_2O_2 could not cause an apoptotic change in either PT cell line. *B*, a clear increase in the numbers of apoptotic cells was observed only in GMF-β-overexpressing PT cells at 8 mM H_2O_2. *C*, shown is the caspase-3 activity in wild-type PT cells and in PT cells stably transfected with GMF-β. The PT cells were treated with 8 mM H_2O_2 for 24 h after overnight FCS starvation. PT cells stably transfected with GMF-β were pretreated with 10 μM SB203580 or 50 μM benzyloxycarbonyl-VAD-fluoromethyl ketone (*Z-VAD-fmk*; a caspase-3 inhibitor) for 1 h before H_2O_2 treatment. Data are means ± S.D. ($n = 3$). ***, $p < 0.001$ *versus* wild-type PT cell H_2O_2; ###, $p < 0.001$ *versus* GMF-β stable transformant H_2O_2 (ANOVA). *D*, shown is the DNA ladder of H_2O_2-treated wild-type PT cells and PT cells stably transfected with GMF-β. The PT cells were treated with 8 mM H_2O_2 for 24 h after overnight FCS starvation.

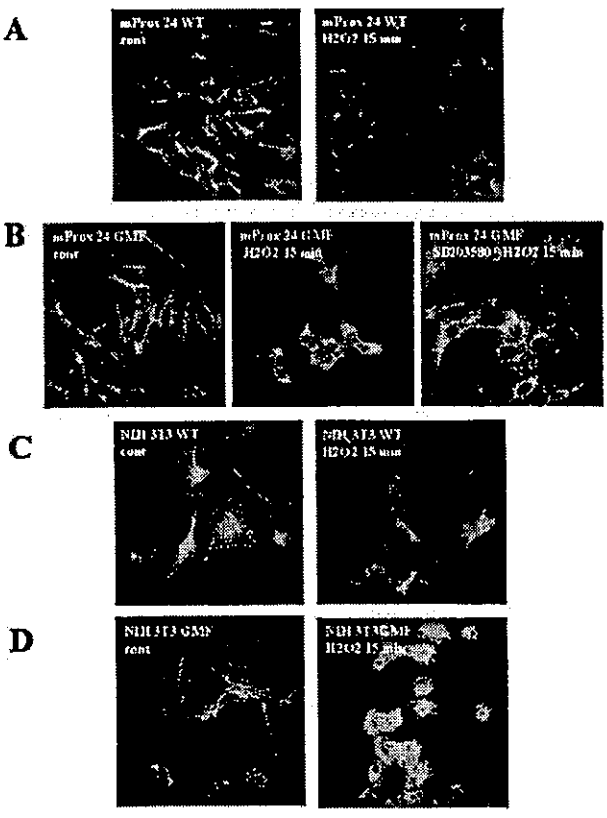

FIG. 6. **Enhanced F-actin reorganization in mouse GMF-β-overexpressing PT and NIH 3T3 cell lines.** *A*, shown are the results from confocal fluorescence microscopy of quiescent mouse mock-transfected wild-type (*WT*) PT cells (mProx24). After overnight FCS starvation, cells on the glass slides were fixed with 3.7% paraformaldehyde and permeabilized with 0.5% Triton X-100. F-actin was stained red with TRITC-labeled phalloidin (control (*cont*); *left*). The organization of cytoplasmic F-actin was slightly disrupted 15 min after the challenge with 1 mM H_2O_2 (*right*). *B*, F-actin organization in PT cells stably transfected with GMF-β (*GMF*) was virtually the same as that in wild-type PT cells under control conditions (*left*). Under oxidative stress (1 mM H_2O_2, 15 min), F-actin in GMF-β stable transformants was more clearly reorganized, and cells had contracted (*middle*). F-actin reorganization in GMF-β stable transformants was attenuated by the 1-h pretreatment with 10 μM SB203580 (a p38 inhibitor) (*right*). *C*, F-actin in mock-transfected NIH 3T3 cell was virtually the same under control (*left*) and oxidative stress (*right*) conditions. *D*, F-actin organization in NIH 3T3 cells stably transfected with GMF-β was similar to that in wild-type cells under control conditions (*left*). Under oxidative stress (1 mM H_2O_2, 15 min), F-actin in GMF-β stable transformants, as in PT cells, was more clearly reorganized, and cells had contracted (*right*).

mants was higher and retained longer than in wild-type PT cells. The prolongation of the increase in H_2O_2 was also observed in NIH 3T3 cells stably expressing GMF-β (data not shown). The assays using acetate esters of fluorescent probes, such as DCFDA, are influenced by intracellular esterase activities and cellular efflux. These two factors were checked in the two cell types using the oxidant-insensitive fluorescein diacetate derivative carboxyfluorescein diacetate. There was virtually no difference in fluorescence between GMF0 and control cells at 0 min as well as 120 min after the addition of 8 mM H_2O_2 (Fig. 7*E*). These findings indicate that DCFDA assay could detect the intracellular ROS levels equally in wild-type and GMF-β-expressing cells and that the intracellular esterase activity and cellular efflux were not changed. Collectively, the data obtained here suggest that GMF-β overexpression prolongs the generation of intracellular H_2O_2 under various stimuli, resulting in aggravated oxidative stress.

GMF-β Expression Causes Changes in the Expression and Activities of Intracellular Antioxidant Enzymes—The increase in intracellular H_2O_2 under various stimuli observed in GMF-β-overexpressing cells prompted us to examine the expression of intracellular antioxidant enzymes and their activities. The antioxidant enzymes include SODs, catalase, and peroxidase, with GPX being especially important. SOD catalyzes the dismutation of O_2^- to O_2 and H_2O_2, whereas catalase and GPX subsequently convert H_2O_2 into water to prevent the production of HO^-. In PT cells with GMF-β stable transformants (GMF0 and GMF1), an increase in the protein content and activity of CuZn-SOD was detected in immunoblot analyses

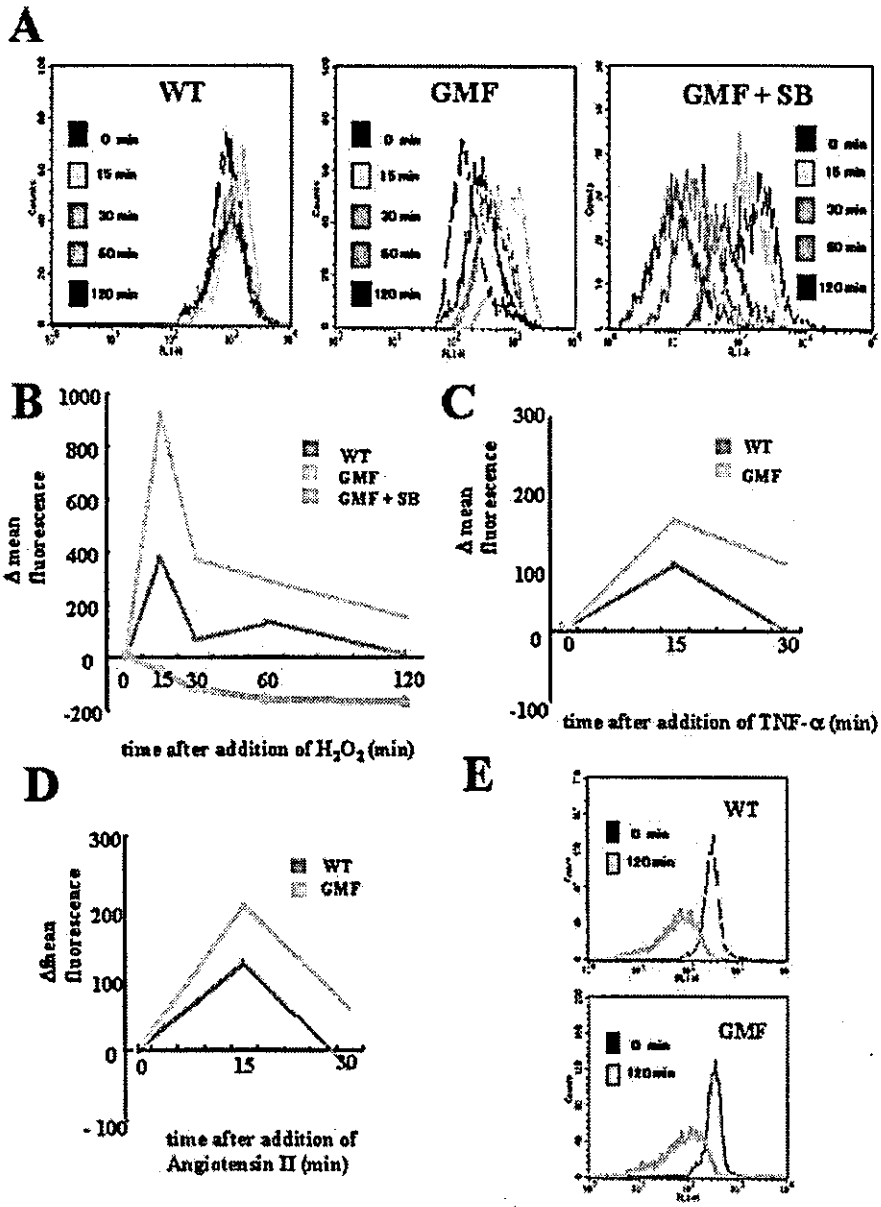

FIG. 7. **Time course of intracellular ROS levels in PT cell lines exposed to stimuli.** *A*, histograms showing the time courses of intracellular H_2O_2 increases after the addition of H_2O_2 to the culture medium in wild-type PT cells (*left*), GMF-β-overexpressing PT cells (*GMF*; *middle*), and GMF-β-overexpressing PT cells pretreated for 1 h with 10 μM SB203580 (*SB*; *right*). *B*, time course of the increase in mean relative fluorescence, which is equivalent to intracellular H_2O_2. Zero points represent the intracellular H_2O_2 levels before oxidative stress. The sustained increase in intracellular H_2O_2 was confirmed in cells stably transfected with GMF-β. On the other hand, the p38 inhibitor SB203580 suppressed the increase in intracellular H_2O_2 level. *C* and *D*, time courses of the increase in mean fluorescence, which represents intracellular H_2O_2, after the addition of 100 ng/ml TNF-α and 1 μM angiotensin II, respectively. The sustained increase in intracellular H_2O_2 in response to both stimuli was confirmed in PT cells stably transfected with GMF-β. *E*, histograms showing the time courses of the fluorescence of the oxidant-insensitive fluorescein diacetate derivative carboxyfluorescein diacetate incorporated into wild-type and GMF-β-overexpressing PT cells after the addition of 8 mM H_2O_2, respectively.

(Fig. 8*A*), whereas the protein content and activity of another type of SOD, Mn-SOD, did not change (Fig. 8*B*). These findings were also confirmed in the case of the NIH 3T3 cell line (Fig. 8, *A* and *B*). The activities of the peroxide-reducing enzymes catalase and GPX and the cellular GSH content were lower in cells with GMF-β stable transformants than in mock-transfected wild-type cells in the mouse mProx24 PT cell line and the NIH 3T3 cell line (Fig. 8, *C–E*). In particular, the GSH content and GPX activities were greatly reduced in the cells overexpressing GMF-β. These results suggest that GMF-β-overexpressing cells have a tendency toward aggravated and prolonged increases in intracellular H_2O_2.

DISCUSSION

It has been thought that the expression of GMF-β in normal tissues is largely limited to the brain (19), especially in the glial cells and some neurons (20). GMF-β is reportedly also induced in the developing retina (39). In this study, we showed for the first time that GMF-β is also induced in non-neuronal tissues (renal PT cells) by proteinuria (40, 41).

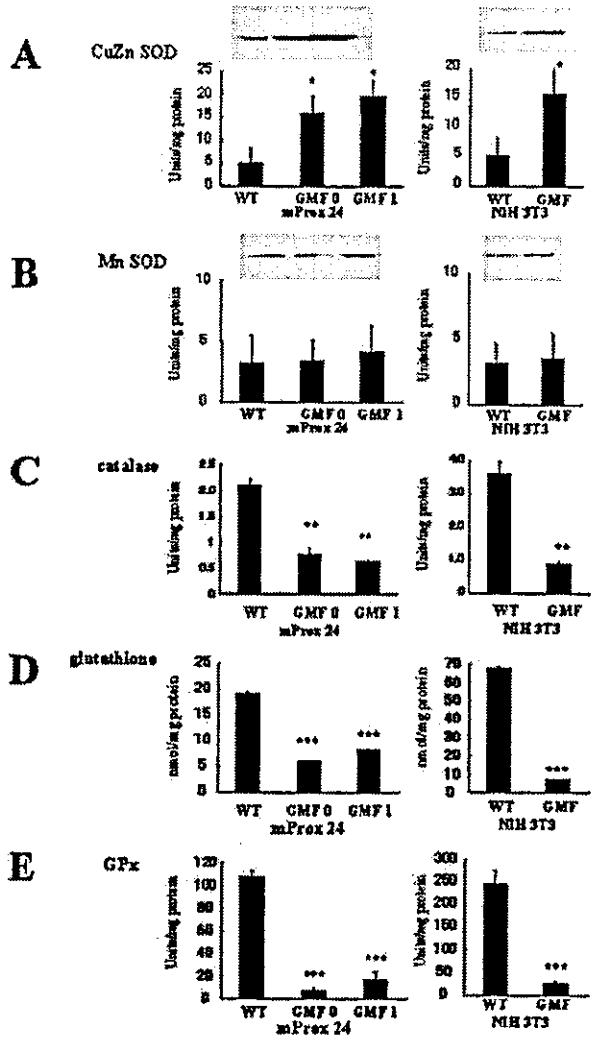

FIG. 8. **Antioxidant enzyme activity and GSH content in mouse GMF-β-overexpressing PT and NIH 3T3 cell lines.** *A*, CuZn-SOD immunoblotting (*upper*) and activity (*lower*) in GMF-β-overexpressing (*GMF*) and mock-transfected wild-type (*WT*) PT (*left*) and NIH 3T3 (*right*) cell lines. GMF-β stable transformants showed an increase in CuZn-SOD protein expression and activity. *B*, Mn-SOD immunoblotting (*upper*) and activity (*lower*) in GMF-β-overexpressing and mock-transfected wild-type PT (*left*) and NIH 3T3 (*right*) cell lines. Expression of the Mn-SOD protein and its activity were not different in the wild-type cells and GMF-β stable transformants. *C*, catalase activity in GMF-β-overexpressing and mock-transfected wild-type PT (*left*) and NIH 3T3 (*right*) cell lines. Catalase activity decreased in GMF-β-overexpressing cell lines. *D*, cellular glutathione content in GMF-β-overexpressing and mock-transfected wild-type PT (*left*) and NIH 3T3 (*right*) cell lines. Glutathione content was reduced in GMF-β-overexpressing cell lines. *E*, GPX activity in GMF-β-overexpressing and mock-transfected wild-type PT (*left*) and NIH 3T3 (*right*) cell lines. GPX activity in GMF-β-overexpressing cell lines was highly reduced compared with that in wild-type cells. Each graph shows the mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* wild-type cells (ANOVA (mProx24 cells) and Student's *t* test (NIH 3T3 cells)).

From the results obtained in Schwann cells (21) and primary astrocytes (18), GMF-β was considered to play a protective role in the cells at least in the brain since the overexpression of GMF-β results in secretion of neurotrophic factors and induction of its receptor (18, 21). In contrast, our study showed that GMF-β overexpression made PT and NIH 3T3 cells vulnerable to oxidative stress by the sustained increase in intracellular H_2O_2. This unique but critical phenomenon may be pathologically relevant to the progression of various diseases by exaggerating oxidative stress since the overexpression of GMF-β has a similar role in at least two different cell lines.

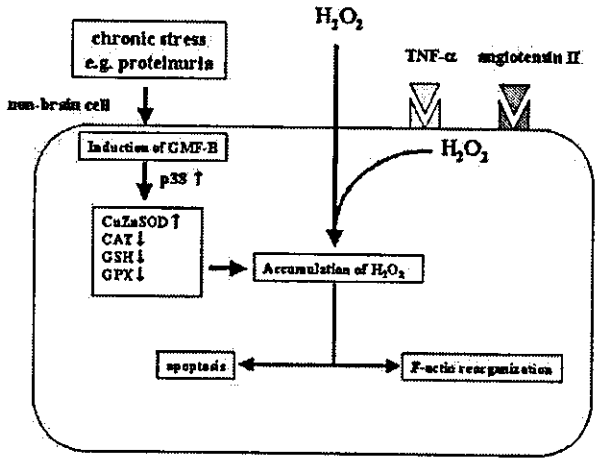

FIG. 9. **Schematic representation of the effect of proteinuria on PT cells.** Chronic stress, such as that produced by proteinuria, induces expression of GMF-β in PT cells. GMF-β expression causes a change in antioxidant enzyme activities, resulting in enhanced accumulation of H_2O_2 under oxidative stress. p38 possibly underlies the enhanced accumulation of H_2O_2. In this way, GMF-β expression results in aggravated oxidative injuries, F-actin reorganization, and apoptosis. *CAT*, catalase.

It has recently been well established that ROS play a key role as second messengers in a number of cellular events, for example, cell proliferation, differentiation, and death (29, 42, 43). ROS (particularly intracellular H_2O_2) generation is associated with TNF-α-, Fas-, and angiotensin II-mediated apoptosis and various other stimuli (44–46). In many cell types, including hepatocytes and fibroblasts, oxidative stress produces a severe F-actin reorganization (47–50), whereas in human intestinal cell monolayers (Caco-2), H_2O_2 enhances the chemical modification of actin and reorganizes F-actin (51, 52). These findings suggest that a sustained increase in intracellular H_2O_2 in GMF-β-overexpressing cells may result in their vulnerability to apoptosis and F-actin reorganization. The reorganization of F-actin is reportedly involved in several kidney disease models, whereas ischemic acute renal failure was found to induce F-actin reorganization, leading to loss of surface membrane polarity of tubules (22). Several lines of evidence have shown that apoptosis of epithelial PT cells is relevant to the progression of renal disease in hypertensive nephrosclerosis (24) and focal and segmental glomerulosclerosis (23). These findings suggest that induction of GMF-β may have an implication in the progression of renal diseases.

Such a high and prolonged increase in H_2O_2 resulting from GMF-β overexpression led us to hypothesize that GMF-β expression causes this change in antioxidant enzyme activity. Lim *et al.* (53) reported that GMF-β-overexpressing C6 glioma cells show a 3.5-fold increase in CuZn-SOD protein and dismutase activity. Our study showed that GMF-β expression caused not only an increase in the activity of the H_2O_2-producing enzyme CuZn-SOD, but also a decrease in the activity of the H_2O_2-reducing enzymes catalase and GPX as well as a reduction in cellular GSH content. Many studies have found that the balance between the activities of H_2O_2-producing enzymes (CuZn-SOD and Mn-SOD) and those of H_2O_2-reducing enzymes (catalase and GPX) governs the sensitivity of cells to oxidative stress (54–56). Therefore, PT and fibroblast cells stably transfected with GMF-β may become sensitive to oxidative stress through an imbalance between CuZn-SOD and catalase/GPX,

which causes accumulation of intracellular H_2O_2. Back *et al.* (57) reported that oligodendrocytes show a maturation-dependent vulnerability to oxidative stress because of low activity of the H_2O_2-reducing enzyme GPX and low GSH content (58, 59).

In GMF-β-overexpressing cells, DNA ladder formation, annexin V binding, and caspase-3 activation caused by H_2O_2 treatment were inhibited by the p38 inhibitor SB203580, suggesting that this apoptotic signal is related to the p38 pathway. Moreover F-actin reorganization in GMF-β-overexpressing cells was attenuated by pretreatment with SB203580. This observation shows that the p38 pathway is also involved in F-actin reorganization under oxidative stress in GMF-β-overexpressing cells. Finally, DCFDA assay showed that the prolonged accumulation of H_2O_2 was totally blocked by SB203580 (Fig. 7). These results suggest that p38, which significantly participated in the accumulation of intracellular H_2O_2, probably regulates the generation and degradation of ROS upstream of the antioxidant enzymes. It has reported that GMF-β is involved in cell signal transduction, particularly activation of the p38 pathway (17, 18). However, how p38 enhances the accumulation of H_2O_2 in GMF-β-overexpressing cells is largely unknown. The mechanisms of p38 action on ROS accumulation in GMF-β-overexpressing cells need to be clarified in future studies.

On the basis of our findings reported here, we propose a hypothetical model regarding the intracellular accumulation of H_2O_2 by induced GMF-β (Fig. 9). The brain-specific protein GMF-β induced by chronic stress, such as proteinuria, causes a change in the activities of antioxidant enzymes and promotes the accumulation of intracellular H_2O_2 possibly through the p38 pathway, rendering non-brain cells susceptible to oxidative injuries, leading to F-actin reorganization and apoptosis. Our discovery may direct attention to GMF-β, which is induced by proteinuria, as a new key factor in the progression of kidney diseases by aggravated oxidative stress.

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Stem Cell Gene Therapy for Chronic Renal Failure

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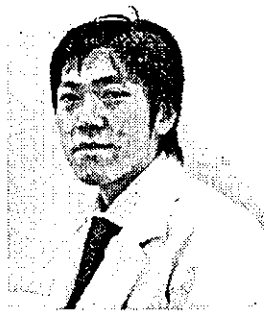

Abstract: Recently, stem cell research has attracted considerable attention because it could be used for the regeneration of damaged organs that are untreatable by conventional techniques, and several stem cells (or progenitor cells), such as endothelial stem cells and neural stem cells have been discovered. Following the progression of this field of research, the potential for stem cell gene therapy has increased and several therapeutic benefits have already been reported. Although this approach was originally investigated for fatal or hereditary diseases, chronic renal failure is also a candidate for stem cell gene therapy.

We have proposed two different therapeutic strategies for chronic renal failure depending on whether the bone marrow stem cells differentiate and commit into mesenchymal or hematopoietic stem cells. In the case of diseases, which need reconstitution of residential renal cells, such as congenital enzyme deficiency diseases, mesenchymal stem cells should be transplanted, and in contrast, hematopoietic stem cells may be used for gene delivery for diseases, which need foreign cytokines and growth factors, such as glomerulonephritis.

This article reviews the recent investigation on this tailor-made stem cell gene therapy for chronic renal failure and discusses the potential of this novel strategy and the major practical challenges of its clinical application.

Keywords: gene therapy, stem cell, regeneration, chronic renal failure, renotropic factor

INTRODUCTION

Recent clinical research has established that in patients with chronic renal failure, such as glomerulonephritis and diabetic nephropathy, once the glomerular filtration rate falls below approximately 25%, a relentless progression to end-stage renal failure inevitably ensues, even when the initial disease activity has abated [Mackenzie and Brenner, 1998]. Although long-term chronic replacement therapy with either hemodialysis or chronic ambulatory peritoneal dialysis has dramatically changed the prognosis of renal failure, it is not a complete replacement therapy and patients with end-stage renal diseases on dialysis continue to have major medical, social, and economic problems [U.S. Renal Data system, 1998]. In the United States, more than 200,000 patients receive dialysis each year, which constitutes a $15 billion industry [Garg *et al.*, 1999]. Kidneys may also be successfully transplanted from a donor individual to an autologous recipient patient; however, the lack of availability of suitable transplantable organs has prevented kidney transplantation from becoming a practical solution to most cases of chronic renal failure. This suggests the need for a next generation of therapeutic strategies against chronic renal failure that can overcome the conventional therapies. Although the application of gene therapy for non-lethal diseases is currently controversial [Robbins and Evans, 1996; Dalgleish, 1997], we speculated that such problems could be overcome, and proposed a transplantation-based gene therapy for renal diseases [Yokoo and Kawamura, 2002]. We previously used bone marrow-derived cells as a vehicle to deliver anti-inflammatory molecules into inflamed glomeruli [Yokoo *et al.*, 1999, 2001a]. In the *ex vivo* differentiation system, bone marrow cells were differentiated *ex vivo* to express ligands of adhesion molecules and acquire the potential to be recruited to the inflamed site [Yokoo *et al.*, 1998, 2001b], and were then transfected adenovirally with a foreign gene followed by transfusion to the affected mice. These cells may deliver anti-inflammatory cytokines into inflamed glomeruli [Yokoo *et al.*, 1999]. In the *in vivo* differentiation system, bone marrow cells were genetically modified using retrovirus and were transplanted to the affected mice before differentiation so that they may retain the potential for self-renewal, as well as differentiation *in vivo* [Yokoo *et al.*, 2001a]. Both the systems were able to demonstrate a therapeutic benefit in at least anti-glomerular basement membrane (anti-GBM) nephritis in mice [Yokoo *et al.*, 1999, 2001a]. These strategies have several advantages over the previous glomerulus-targeted gene delivery systems, i.e., use of peripheral vessels for administration, longer therapeutic time window, and possibly no immunoreaction due to autologous transfer, and suggest that bone marrow-derived cells can be utilized for therapeutic intervention to treat local inflammation.

In the mean time, stem cell research, which was originally based on hematopoietic stem cells, has recently advanced progressively along with the discovery of other stem cells (or progenitor cells), such as endothelial stem cells [Asahara *et al.*, 1997] and neural stem cells [Bjornson *et al.*, 1999], and various therapeutic benefits have been reported; for example, endothelial progenitor cells were utilized to induce neovascularization for the treatment of ischemic diseases [Kalka *et al.*, 2000; Takahashi *et al.*, 1999], myogenic progenitor cells contributed to the regeneration of

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muscle fiber in damaged muscle [Ferrari *et al.*, 1998], and distrophin delivery was used for the treatment of Duchenne's muscular dystrophy [Partiridge, 1998; Gussoni *et al.*, 1999]. Following the progression of this field of research, the potential for stem cell gene therapy has expanded and it has been speculated that such an approach could also be used for chronic renal failure. A successful stem cell gene therapy for chronic renal failure will depend on a thorough knowledge of the fate of kidney cells, such as tubular cells, after birth. And until recently, it has been unclear whether renal residential stem cells exist in adults, and if so, where they originate from.

The other groups and we recently reported that bone marrow stem cells are capable of contributing to the formation of kidney cells, including mesangial cells [Imasawa *et al.*, 2001, Ito *et al.*, 2001], tubular epithelial cells [Gupta et al, 2002], and podocytes [Poulsom, R., *et al.*, 2001]. However, it is still unclear whether these cells are functional and can be utilized for therapeutic intervention. Furthermore, the possibility that renal stem cells already reside in the adult kidney cannot be precluded. To answer these questions, we have combined the technology of bone marrow transplantation and the recent expanded paradigm of renal stem cells, and proposed two different therapeutic strategies for chronic renal failure depending on whether the bone marrow stem cell differentiate and commit into mesenchymal or hematopoietic stem cells. In the case of diseases, which need reconstitution of residential renal cells, such as congenital enzyme deficiency diseases, mesenchymal stem cells should be transplanted, and in contrast, hematopoietic stem cells may be used for gene delivery for diseases, which need foreign cytokines and growth factors, such as glomerulonephritis.

In this article, we review the recent investigation on renal stem cells and possible application in therapeutic intervention, which may provide the next stage from "stem cell research" to "stem cell gene therapy", focusing on chronic renal failure as a model of mulifactorial disease.

STEM CELLS *IN SITU* THEORY

I. Identification of Tissue Stem Cells

It is possible that some renal stem cells migrate and populate the kidney during development and remain quiescent with the ability of self-renewal as well as differentiation, and then, in response to certain stimuli, regain their ability and differentiate into mature renal residential cells (stem cells *in situ* theory) (Fig. **1**). In this context, recent studies suggested that some growth factors may act as renotropic factors to activate resident stem cells. For example, hepatocyte growth factor (HGF) was shown to accelerate the regeneration of tubular parenchymal components after acute injury [Kawaida *et al.*, 1994; Miller *et al.*, 1994] and chronic renal disease [Mizuno *et al.*, 1998]. Although it is not clear where the responders to HGF during tubulointerstitial damage are derived from - by regression of mature surviving epithelial cells or by the activation of a resident, but quiescent, renal stem cell - the fact that newly acquired cells that reline the injured tubule appear immature and express the mesenchymal cell marker vimentin, which is not normally expressed by mature epithelial cells [Witzgall

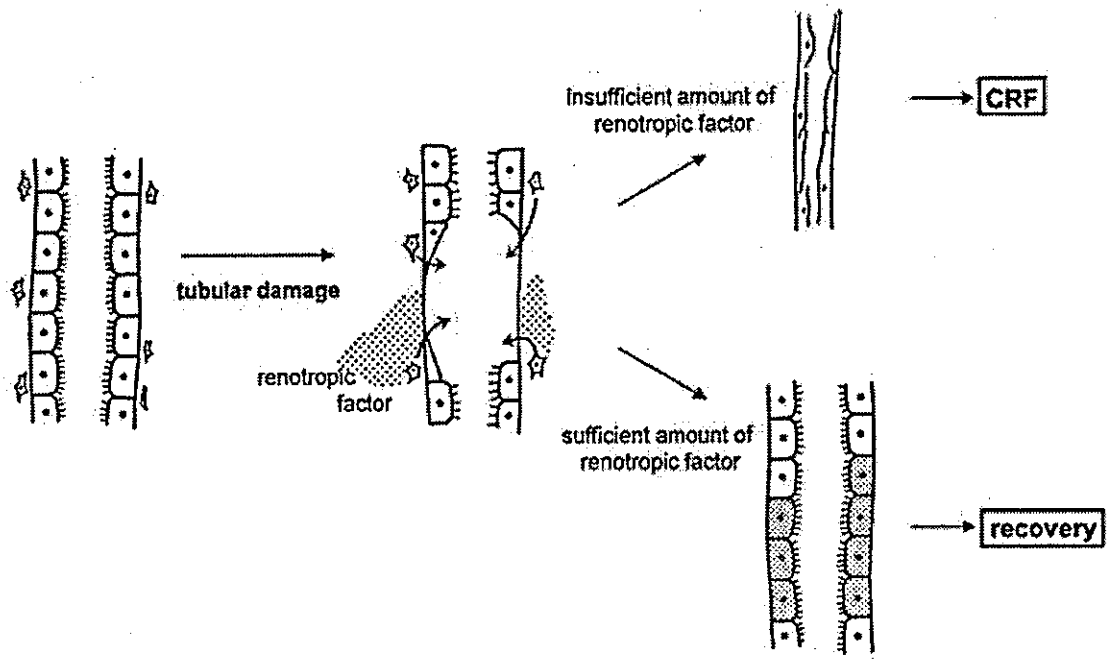

Fig. (1). Putative tubular regeneration based on stem cells *in situ* theory.

et al 1994] but is expressed during mesenchymal-to-epithelial progression of the S-shaped body in renal development [Saxen, 1987], suggested that immature mesenchymal cells populate the adult kidney and might be renal tubular stem (or progenitor) cells. On the basis of this notion, several attempts to identify the renal stem cells in the adult kidney have been performed; however, it is still a mystery. Recently, side-population (SP) cells, which are able to efflux the fluorescent dye Hoechst 33342, were isolated from bone marrow cells [Goodell *et al.*, 1996, 1997]. This property is due to the expression of efflux pumps that belong to the ATP-binding cassette superfamily of membrane transporters [Zhou *et al.*, 2001] and confer a survival advantage. Although they were originally investigated as uncommitted hematopoietic stem cells, recent studies revealed that SP cells might populate other organs [Jackson *et al.*, 1999], while maintaining the potential as tissue stem cells [Gussoni *et al.*, 1999]. It is not clear whether there are SP cells in the kidney or not, although some researchers have attempted to identify renal stem cells by means of SP cell isolation from kidney tissue specimens.

II. Discovery of Renotropic Factors

Specific renotropic factors, which may activate quiescent renal stem cells in response to renal damage, should be identified. For this purpose, growth factors, which mediate renal development, have been intensively examined because it has been suggested that renal regeneration processes may recapitulate developmental paradigms, and common factors might be involved [Bacallao and Fine, 1989; Wallin *et al.*, 1992]. In fact, during perinatal kidney development, HGF induces branching morphogenesis of the ureteric bud [Woolf *et al.*, 1995; Santos and Nigam, 1993] and stimulates epithelial differentiation of metanephric mesenchymal cells [Karp *et al.*, 1994]. *bcl*-2, *bax*, and Pax-2 play important roles in metanephric and urogenital development [Veis *et al.*, 1993, Knudson *et al.*, 1995, Torres *et al.*, 1995], and have been shown to be re-expressed in proximal tubular cells after acute tubular damage [Basile *et al.*, 1997; Imgrund *et al.*, 1999]. These examples are consistent with the hypothesis that during tissue regeneration, a cascade of developmental gene pathways may be reactivated. Currently a number of growth factors have been reported to contribute to renal development [Kuure *et al.*, 2000], and several of those were further investigated and found to play roles as renotropic factors. Not only HGF, but also the other growth factors, such as epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and bone morphogenetic protein-7 (BMP-7), have been shown to be involved in tubular regeneration of the kidney [Nigam and Lieberthal, 2000]. These factors are potent regulators of kidney organogenesis [Kuure *et al.*, 2000; Davies and Bard, 1998], and it has been reported that administration of these growth factors promotes tubular regeneration after a variety of insults [Nigam and Lieberthal, 2000]. Reciprocally, inhibition of activin A, which acts as a negative regulator of branching morphogenesis during kidney development, by follistatin accelerates renal regeneration after renal injury [Maeshima *et al.*, 2001; 2002]. Furthermore, the discovery of novel renotropic regulators is also continuing. Nishinakamura *et al.* recently reported that homozygous deletion of *Sall1* causes an incomplete ureteric bud outgrowth and a failure of tubule formation in the mesenchyme, suggesting *Sall1* may regulate the initial step in mesenchymal-to-epithelial conversion [Nishinakamura *et al.*, 2001], which is distinct from the known regulators, such as BMP-7 [Dudley *et al.*, 1995, Luo *et al.*, 1995] and leukemia inhibitory factor [Barasch *et al.*, 1999]. Elucidation of these molecules may lead to the discovery novel renotropic factors, which specifically activate tissue stem cells to differentiate into mature cells.

III. Gene Delivery of Renotropic Factors

Another issue regarding *in situ* stem cell therapeutic intervention is whether the renotropic factor may be continuously delivered into the temporally and spatially optimal site of renal injury, since the renotropic factor may have a diverse effect and could have an effect on un-targeted organs. Therefore, for the clinical application of these renotropic factors for the treatment of chronic renal injury, a gene delivery system is required that can continuously and site-specifically supply these factors. For this purpose, we established an *in vivo* differentiation system of transplantation-based gene therapy [Yokoo and Kawamura, 2002]. To confirm that this system can achieve a sufficient amount of gene delivery, an anti-inflammatory cytokine was firstly delivered to inflamed site and the therapeutic utility was estimated [Yokoo *et al.*, 1999; 2001a]. Before differentiation, hematopoietic stem cells are genetically modified to possess an anti-inflammatory cytokine via transfection with retrovirus followed by transplantation, so that anti-inflammatory mononuclear cells are continuously supplied from the reconstituted bone marrow, and long lasting suppression of local inflammation is achieved. The bone marrow of female recipient mice was reconstituted with that of male mice, which had been genetically modified to possess the interleukin-1 receptor antagonist (IL-1Ra) or mock gene using modified retroviral transduction methods [Yokoo *et al.*, 2001a]. Glomerulonephritis was induced in the IL-1Ra chimera and mock chimera using anti-glomerular basement membrane serum 8 weeks after the primary transplantation. Serum creatinine and urea nitrogen levels as well as urine albumin excretion levels rose progressively in the mock chimera, whereas these increases were suppressed significantly in the IL-1Ra chimera. This therapeutic effect was confirmed to last for 4 months after the primary bone marrow reconstitution, demonstrating that the donor cells were continuously supplied from the reconstituted bone marrow and secreted anti-inflammatory cytokine [Yokoo *et al.*, 2001a]. This success shows that bone marrow reconstitution with anti-inflammatory stem cells confers long-lasting resistance against glomerular inflammation and suggests its application for the continuous delivery of renotropic factors into the kidney with chronic renal failure.

As the next step, we attempted to modify this system by seeking another source of hematopoietic stem cells, since taking stem cells from the bone marrow is highly inconvenient for clinical use, especially when it is for the treatment of non-lethal and symptom-free (if any) diseases, like chronic renal failure. In this regard, umbilical cord blood cells may be a useful alternative since i) a substantial amount of blood (about 100ml from each delivery [Broxmeyer *et al.*,

1990]) may be collected without pain or risk to the mother or infant, ii) they contain a significantly higher number of hematopoietic progenitor cells when compared with adult peripheral blood [Cairo and Wagner, 1997], iii) a low incidence and severity of GVHD after transplantation occurred due to the immature immune system [Cairo and Wagner, 1997], and iv) recently, large-scale banks of cord blood have been set up or are being considered throughout the world. We therefore examined their suitability as a source of hematopoietic stem cells for transplantation-based gene therapy for inflammatory diseases. Since $CD34^+$ cells from human cord blood have already been transduced with a foreign gene using retroviral vector [Ohashi *et al.*, 1998], they were further examined as a source of hematopoietic stem cells for this gene delivery system. After transfection with the human β-glucuronidase (HBG) gene as a reporter using retrovirus, human cord blood-derived $CD34^+$ cells were transplanted into the non-obese diabetic/severe combined immunodeficiency mice since this strain is characterized by a functional deficit of natural killer cells, absence of circulating complement, and defects in the differentiation and function of antigen presenting cells, as well as an absence of T and B cell function, facilitating reconstitution with human hematopoietic cells [Schultz *et al.*, 1995; Larochelle *et al.*, 1996]. Flow cytometric analysis revealed that 24.1±14.5% of bone marrow cells in these chimera mice expressed human HLA 8 weeks after transplantation. Also, clonogenic assay showed a sustained engraftment of human hematopoietic cells expressing HBG. CD14-positive cells were recruited into the glomeruli upon LPS treatment and they secreted bioactive HBG, suggesting that cord blood-derived $CD34^+$cells may differentiate into monocyte lineage while maintaining the expression of the transgene [Yokoo *et al.*, 2003]. These data demonstrate that human cord blood cells can be utilized as a source of hematopoietic stem cells for transplantation-based gene delivery systems. This option would allow the next step aimed at clinical application of stem cell gene therapy to proceed.

Currently, the utility of this system for renal regeneration is being examined in our laboratory by delivering HGF into mouse model of chronic renal failure, and as novel and more specific renotropic factors are discovered, we will attempt to deliver these genes and affirm the most effective molecule for each damaged condition.

STEM CELLS IN BONE MARROW THEORY

Sakai *et al.* previously reported a patient with IgA nephropathy, which is the most frequent form of glomerulonephritis [D' Amico, 1988], associated with chronic myelobastic leukemia, in which mesangial deposits disappeared after allogeneic bone marrow transplantation [Sakai, 1997]. Furthermore, bone marrow exchange between normal mouse and the IgA nephropathy model mouse altered serum IgA level as well as glomerular damage [Imasawa *et al.*, 1998, 1999]. These findings provided the first evidence that abnormalities of bone marrow stem cells may be involved in the pathogenesis of some renal diseases and gave rise to the hypothesis that some renal progenitor cells are resident in and mobilized from bone marrow (stem cells in bone marrow theory) (Fig. 2), and therefore reconstitution of bone marrow might be a therapeutic strategy independent of differentiation to inflammatory cells. In this regard the other groups and we attempted to clarify the existence of renal

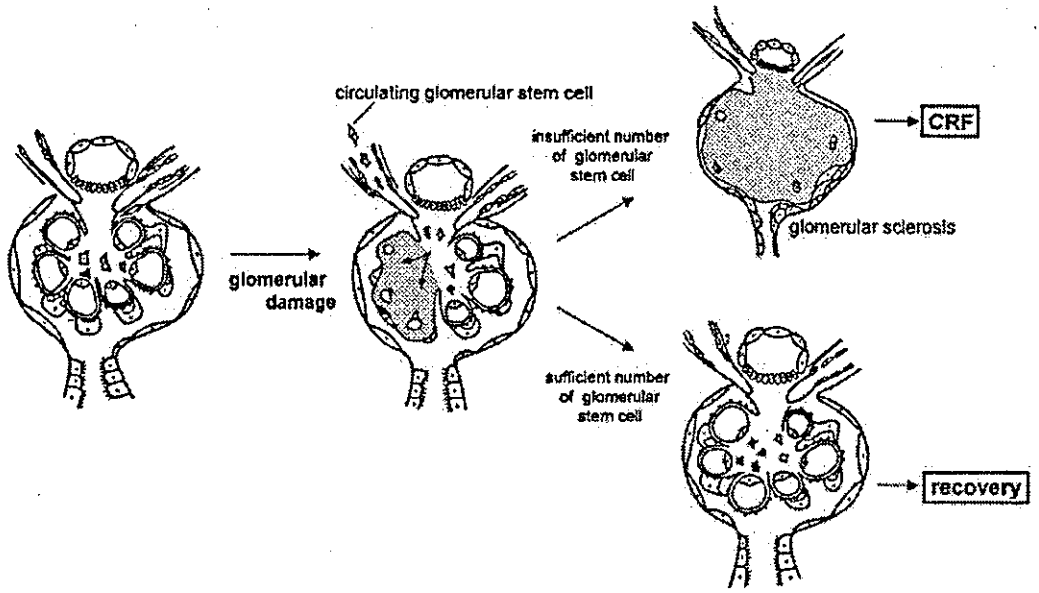

Fig. (2). Putative glomerular regeneration based on stem cells in bone marrow theory.

progenitor cells in bone marrow using transplantation technology. In our study, C57BL/6j mice were transplanted with crude bone marrow cells from green fluorescent protein (GFP)-transgenic mice [Okabe, 1997], and were examined for the development of donor cells into glomerular residential cells [Imasawa *et al.*, 2001]. The number of green cells in the glomeruli increased markedly in a time-dependent manner from 2 weeks until 24 weeks after transplantation and these cells possessed properties of mesangial cells, such as positivity for desmin and potential to contract in response to angiotensin II, suggesting that bone marrow cells contain mesangial stem (or progenitor) cells. In contrast, Ito *et al.*, using a similar technique with a GFP transgenic rat, reported that very few transplanted donor cells are able to differentiate into mesangial cells in normal conditions but the numbers increase during glomerular remodeling after experimental nephritis [Ito *et al.*, 2001]. Furthermore, Poulsom *et al.* reported that tubular epithelial cells and interstitial cells as well as podocytes might be borne from bone marrow cells by detection of the Y chromosome in the female mice that have received male whole bone marrow transplants [Poulsom *et al.*, 2001]. These data suggested that renal stem cells may be resident in the bone marrow but the signals for migration, homing, and differentiation vary between species and even genetic backgrounds. In all these reports on transdifferentiation, detection of the donor-originated genes, such as GFP and the Y chromosome, was employed to identify the donor-derived cells, however, two recent papers have shown that bone marrow cells and CNS stem cells may be fused with embryonic stem (ES) cells *in vitro* and subsequently adopt some of the phenotypes typical of ES cell differentiation, warning of the possibility that the development of a new marker might simply be due to the fusion of bone marrow-derived cells with pre-existing differentiated cells in the host's organs [Terada *et al.*, 2002; Ying *et al.*, 2002]. Cell fusion events have not been shown in an *in vivo* situation and several observations denying the possibility that fusions account for all instances of transdifferentiation were reported [Poulsom *et al.*, 2002]. Further study is needed to clarify this controversy.

Another question yet to be answered to date is which fraction of the bone marrow cells has the potential to be renal stem cells. We have recently been focusing on the mesenchymal stem cells because of our evidence showing that donor cells could differentiate into mesangial cells after transplantation of crude bone marrow cells whereas donor-derived mesangial cells were not detected when bone marrow cells were transplanted after the selection of hematopoietic stem cells by culture in IL-3, IL-6, and stem cell factor for 72 hours [Yokoo *et al.*, 2001]. This is consistent with a recent finding that no donor-derived renal cells were seen in the kidney if purified hematopoietic stem cells were engrafted [Krause *et al.*, 2001] and mesenchymal cells of host origin were found in the vascular and interstitial compartment of sex-mismatched renal allografts [Grimm *et al.*, 2001]. As the next step, we established mouse mesenchymal cells, stably expressing the LacZ gene by retroviral infection and transplanted them into the mice of same genetic background without any myeloablation. Unexpectedly, our preliminary data showed that some of the tubular epithelial cells expressed LacZ activity and this increased after ischemia/reperfusion insult (unpublished data), suggesting that mesenchymal stem cells may integrate in the kidney and transdifferentiate into tubular epithelial cells even in an undamaged situation. This evidence is consistent with previous data that up to 8% of the renal tubular epithelial cells were found to be of bone marrow origin in mice without any evidence of renal damage, representing the baseline contribution of bone marrow on the normal wear-and-tear renewal of the tubular epithelium [Forbes *et al.*, 2002], thus making mesenchymal cells attractive for stem cell gene therapy strategies. We therefore applied this cell replacement strategy to the treatment of inherited metabolic disease, in which a single gene product is deficient causing an abnormal accumulation of metabolites in the kidney, which may affect renal function. We focused on a model of lysosomal storage disease, mucopolysaccharidosis type VII (MPSVII), a model of inherited metabolic disease, to affirm the feasibility of cell replacement gene therapy using mesenchymal stem cells. Mice homozygous for a frameshift mutation in the β-glucuronidase gene are devoid of the secreted β-glucuronidase enzyme. This enzymatic deficiency results in lysosomal accumulation of undegraded glycosaminoglycans in various tissues [Neufeld *et al.*, 1995]. We previously reported that lysosomal storage in the liver and spleen in murine models of MPSVII might be reduced by transplantation of terminally-differentiated mononuclear cells with the β-glucuronidase gene [Ohashi *et al.*, 2000]. Although lysosome was also accumulated in renal tubular cells in this model (unpublished data), it could not be reduced by this manipulation. Therefore, we are now attempting to confirm whether it is possible to replace the affected tubular cells with those bearing the missing gene that can reline and restore tubule epithelial integrity either by transplantation of wild type or by retrovirally transfected mesenchymal stem cells. It is tempting to speculate that once transplanted mesenchymal stem cells reside within the kidney, transdifferentiation to tubular cells may be enhanced due to strong positive selection pressure and could rescue the renal function. Although we have just started down a long avenue of research, this stem cell transplantation-based cell replacement therapy may provide a possible new treatment for inherited chronic renal diseases, for which there has been no curative treatment available until recently.

CONCLUSION

Despite recent progress in stem cell biology, the question of whether renal stem cells exist, and if so, where they originate from still remains undetermined. One possibility is that stem cells reside in the adult kidney (stem cells *in situ* theory) and the other is in the bone marrow (stem cells in bone marrow theory). In the liver, recent evidence has suggested that liver progenitor cells, called oval cells, which transdifferentiate into hepatocytes or biliary epithelial cells, may originate not only from the point where the terminal bile ducts meet the periportal hepatocytes, but also from the bone marrow [Forbes *et al.*, 2002]. It is worth speculating that the origin of renal stem cells is not restricted to a single place and they may be supplied from different places depending on the severity, location, and duration of damage. In fact,

resident tissue stem cells are a small percentage of the total cellularity, for instance, in the mouse small intestine, there are 4-5 stem cells out of a total crypt population of about 250 cells [Alison *et al.*, 2002], suggesting tissue-specific stem cells are not sufficient in number and stem cells may be supplied from other organs (presumably from the bone marrow) on demand.

This forces us to investigate both possibilities for the kidney stem cells. In this review, we focused on the recent progress in research on these hypotheses and proposed possible therapeutic applications for chronic renal failure in combination with the emerging knowledge of kidney stem cell biology, as a stem cell gene therapy. In the case of diseases which need reconstitution of residential renal cells, such as renal failure due to congenital metabolic diseases, mesenchymal stem cells should be transplanted on the basis of the stem cells in bone marrow theory, to replace the affected cells with wild-type, and in contrast, hematopoietic stem cells may be used as a vehicle for gene delivery for diseases which need foreign cytokines and growth factors as renotropic factors to regain the resident stem cells, such as glomerulonephritis. We realize that stem cell gene therapy may not be simply applied for multifactorial diseases and differently committed bone marrow stem cells should be selectively used depending upon the situation of renal injury. Such "tailor-made" stem cell gene therapy may be required for chronic renal failure.

Finally, we will need another option in case renal stem cells are damaged or disappeared, which could happen in patients with end-stage renal damage undergoing long-term dialysis. For such patients, the ultimate stem cell therapy might be the development of a functional whole kidney from renal stem cells. In this regard, it has previously been reported that the pronephros, the earliest form of the embryonic kidney, may be established from the undifferentiated animal pole ectoderm of *Xenopus laevis* treated with activin A and retinoic acid *in vitro*, and has renal function *in vivo* after transplantation into other individuals [Uochi and Asashima, 1996]. More recently, Dekel *et al.* reported that kidney precursor cells derived from early embryos and fetal tissue might form a functional miniature kidney [Dekel *et al.*, 2003]. These findings may suggest the possibility that a whole kidney could be established *ex vivo*, indicating a new direction of stem cell research for renal disease.

Although stem cell gene therapy is still in the learning phase and still a long way from being established, stem cell gene therapy, the combination of gene therapy technology with emerging stem cell knowledge may allow us to proceed to the next generation of therapeutic strategies aimed at "reset" the abolished function by replacing or regaining damaged components for the treatment of chronic renal failure, which affects a large number of patients physically and even financially.

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