ERK5 activation enhances mesangial cell viability and collagen matrix accumulation in rat progressive glomerulonephritis

Maki Urushihara,1 Masanori Takamatsu,1 Maki Shimizu,1 Shuji Kondo,1 Yukiko Kinoshita,1 Kenichi Suga,1 Akiko Kitamura,1 Sato Matsuura,1 Masanori Yoshizumi,3 Toshiaki Tamaki,2 Hiroshi Kawachi,4 and Shoji Kagami1

Departments of 1Pediatrics and 2Pharmacology, Institute of Health Bioscience, University of Tokushima Graduate School, Tokushima; 3Department of Pharmacology, Nara Medical University School of Medicine, Nara; and 4Department of Cell Biology, Institute of Nephrology, University of Niigata Graduate School, Niigata, Japan

Submitted 3 March 2009; accepted in final form 20 October 2009

ERK5 activation enhances mesangial cell viability and collagen matrix accumulation in rat progressive glomerulonephritis. Am J Physiol Renal Physiol 298: F167–F176, 2010. First published October 21, 2009; doi:10.1152/ajprenal.00124.2009.—The mitogen-activated protein kinase (MAPK) cascade plays an important role in the regulation of various cellular functions in glomerulonephritis (GN). Here, we investigated whether extracellular signal-regulated kinase 5 (ERK5), a member of the MAPK family, is involved in the pathogenesis of chronic mesangiotrophogenic GN, using a rat model induced by uninephrectomy and anti-Thy-1 antibody injection. Immunostaining of kidneys obtained at different time points revealed that phospho-ERK5 was weakly expressed in control glomeruli but dramatically increased in a typical mesangial pattern after 28 and 56 days of GN. A semiquantitative assessment indicated that glomerular phospho-ERK5 expression closely paralleled the accumulation of extracellular matrix (ECM), collagen type I, as well as glomerular expression of reactive oxygen species (ROS) and ANG II. On the other hand, phospho-ERK1/2 expression increased on day 7 during the phase of enhanced mesangial cell (MC) proliferation and decreased thereafter. H2O2 and ANG II each induced ERK5 phosphorylation by cultured MCs. Costimulation with both H2O2 and ANG II synergistically increased ERK5 phosphorylation in MCs. Cultured MCs transfected with ERK5-specific small interference RNA showed a significant decrease in H2O2 or ANG II-induced cell viability and soluble collagen secretion compared with control cells. Treatment of GN rats with an ANG II type 1 receptor blocker resulted in significant decreases in phospho-ERK5 expression and collagen accumulation accompanied by remarkable histological improvement. Taken together, these results suggest that MC ERK5 phosphorylation by ANG II or H2O2 enhances cell viability and ECM accumulation in an experimental model of chronic GN.

extracellular signal-regulated kinase 5; angiotensin II; reactive oxygen species; extracellular matrix

PERSISTENT MESANGIAL CELL (MC)-mediated abnormal mesangial remodeling is a prominent biological feature of progressive glomerulonephritis (GN) and leads to glomerular dysfunction. This condition is pathologically characterized by increased numbers of MCs and the accumulation of fibronectin, laminin, and collagen types I, II, and IV in diseased glomeruli. Thus clarifying the molecular and cellular mechanisms responsible for pathological mesangial remodeling may help to elucidate the pathogenesis of progressive glomerular sclerosis.

Previous studies have suggested that several signaling pathways are involved in the pathophysiological mesangial remodeling. The mitogen-activated protein kinase (MAPK) signaling pathway is a highly conserved module involved in various cellular functions, including cell proliferation, cell survival, differentiation, and migration. Extracellular stimuli, such as growth factors and environmental stress, induce sequential activation of the MAPK cascade. At least four members of the MAPK family have been identified: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-amino-terminal kinase (JUN), p38, and ERK5 (22). ERK5, also known as big MAP kinase 1 (BMK1), is twice the size of other MAPKs (16, 32). Since it is activated by oxidative stress and hyperosmolarity, ERK5 was initially documented as a MAPK family member activated by stress stimuli (1). Subsequently, ERK5 was also shown to be activated in response to serum, one of the well-known activators of ERK1/2 (11). Nerve growth factor, ANG II, high glucose, and other stimulators of ERK1/2 can also increase ERK5 activity (9). The MEK-ERK5 pathway is blocked by MEK inhibitors, similar to the ERK1/2 pathway (9). Thus there are similarities between the activation modes and functions of the ERK5 and ERK1/2 pathways. However, recent studies have also identified some distinctive features of the ERK5 and ERK1/2 pathways (19, 28).

It was recently reported that ERK1/2 activation occurs in the rat Thy-1 model of mesangiotrophic nephritis and that the blocking of the ERK1/2 pathway results in significant reduction in MC proliferation in this model (2, 3). In addition, ERK1/2 activation in human glomerulopathies is associated with cell proliferation, histological lesions, and renal dysfunction (18). ERK5-mediated MC growth has been reported to be involved in the pathogenesis of diabetic nephropathy (29). However, the significance of ERK5 signaling in GN is still poorly understood. To further elucidate whether ERK5 is involved in the pathogenesis of GN, we examined the expression and phosphorylation levels of glomerular ERK5 in progressive models of rat mesangiotrophic GN characterized by MC proliferation and extracellular matrix (ECM) accumulation. In addition, the potential role of ERK5 signaling in MC-mediated pathological mesangial remodeling was investigated in cultured MCs.

MATERIALS AND METHODS

Antibodies and reagents. A polyclonal rabbit anti-total-ERK5 antibody and a polyclonal rabbit anti-phospho-ERK5 antibody were purchased from Cell Signaling Technology (Beverly, MA). A polyc-
Fig. 1. Time course studies in glomerular histology and expression of phospho-extracellular signal-regulated kinase 5 (ERK5) in normal and glomerulonephritis (GN) rats. A: analysis of glomerular histology was performed with periodic acid-Schiff (PAS)-stained sections and immunostaining with either anti-phospho-ERK5 antibody or anti-collagen type I antibody. Magnification *H11003 200. B: double immunostaining of phospho-ERK5 (FITC green)- and mesangial cell-specific antigens [tetramethylrhodamine isothiocyanate (TRITC) in red] in nephritic glomeruli on day 56 of GN. Yellow staining in merged images indicates areas of simultaneous expression of phospho-ERK5 and anti-Thy-1 antigen (mesangial cell marker). Magnification *H11003 200. C: semiquantitative assessment of glomerular ECM accumulation in normal and GN rats. The level of ECM accumulation is expressed as a glomerulosclerosis score as described in MATERIALS AND METHODS. D: semiquantitative assessment of glomerular phospho-ERK5 and collagen I expression in normal and GN rats. The staining score is defined in MATERIALS AND METHODS. E: time course study of glomerular ERK5 phosphorylation by Western blotting in normal and GN rats. The level of ERK5 phosphorylation was determined by comparing the ratio of densitometric measurement of the phospho-ERK5 band divided by that of total-ERK5 band. Values are means ± SD. *P < 0.05 vs. rats at day 0.
Fig. 2. Glomerular expression of phospho-ERK1/2 in normal and GN rats. A: immunohistochemistry in kidney sections was performed with an anti-phospho-ERK1/2 antibody and subsequently with an ABC complex. Magnification ×200. B: semiquantitative assessment of phospho-ERK1/2-positive cells was performed during the course of these experiments. C: time course study of glomerular ERK1/2 phosphorylation by Western blotting in normal and GN rats. The level of ERK1/2 phosphorylation was determined by comparing the ratio of densitometric measurement of the phospho-ERK1/2 band divided by that of the total-ERK1/2 band. Values are means ± SD. *P < 0.05 vs. rats at day 0.

Fig. 3. Glomerular expression of superoxide (O$_2^-$) and ANG II in normal and GN rats. A: kidney sections were stained with either O$_2^-$-sensitive dye dihydroethidium (DHE), producing ethidium bromide (red fluorescence), or an anti-ANG II antibody (green fluorescence) Magnification ×200. B: semiquantitative assessment of fluorescence intensity to determine the level of glomerular O$_2^-$ content in normal and GN rats was performed as described in MATERIALS AND METHODS. C: semiquantitative assessment of glomerular ANG II expression in normal and GN rats was performed as described in MATERIALS AND METHODS and expressed as the staining score. Values are means ± SD. *P < 0.05 vs. rats at day 0.
clonal rabbit anti-phospho-ERK1/2 antibody was purchased from Cell Signaling Technology for immunohistochemistry. A polyclonal rabbit anti-total-ERK1/2 antibody and a monoclonal mouse anti-phospho-ERK1/2 antibody were purchased from Santa Cruz Biotechnology for Western blot analysis. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L; Bio-Rad, Hercules, CA) and HRP-conjugated horse anti-rabbit IgG (H+L; Cell Signaling Technology) were used for Western blot analysis. For immunohistochemistry, anti-collagen type I (collagen I) antibody and anti-Thy-1 antibody were obtained from Chemicon International (Temecula, CA) and Taiho Pharmaceutical (Tokushima, Japan), respectively. FITC-conjugated donkey anti-rabbit IgG antibody and tetramethylrhodamine isothiocyanate (TRITC)-coupled donkey anti-mouse antibody were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). An immunofluorescence staining kit (Peninsula Laboratories, San Carlos, CA) for ANG II was used to detect ANG II. A synthetic peptide of ANG II and diphenylene iodonium (DPI) were obtained from Sigma-Aldrich (St. Louis, MO). Propidium iodide (PI) and a 4-[3-(4-i odophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay kit were purchased from Dojindo (Kumamoto, Japan). ANG II type 1 receptor blockers (ARB) of candesartan (TCV-116 and CV11974) were kindly provided by Takeda Chemical Industries (Osaka, Japan).

Induction of progressive anti-Thy-1 GN and experimental design. All procedures and protocols used in this study were approved by the Institutional Animal Care and Committee of the University of Tokushima Graduate School. A progressive model of mesangio proliferative GN was induced as previously described (13). Briefly, rats (n = 36; SLC, Shizuoka, Japan) were uninephrectomized and 1 wk later received a single intravenous injection of 2 mg of the nephritogenic anti-Thy-1 mAb 1-22-3 (21). This mAb 1-22-3 recognizes a critical epitope of the Thy-1.1 molecule on the MC surface, the binding of which induces severe complement-dependent MC injury. The injection of mAb 1-22-3 into uninephrectomized rats induced

![Fig. 4. Effects of H2O2 and/or ANG II on ERK5 phosphorylation in cultured mesangial cells (MCs). A: MCs were stimulated with either H2O2 (A and B) or ANG II (C and D) for indicated times (A and C) and at indicated concentrations (B and D) and analyzed by Western blotting using either anti-phospho-ERK5 antibody or anti-total-ERK5 antibody. The level of ERK5 phosphorylation in MCs was determined by comparing the ratio of densitometric measurement of the phospho-ERK5 band divided by that of the total-ERK5 band. Values are means ± SD. *P < 0.05 vs. control.](http://ajprenal.physiology.org/)

**AJP-Renal Physiol** • VOL 298 • JANUARY 2010 • www.ajprenal.org
chronic progressive glomerulosclerosis with marked proteinuria. Control rats (n = 6) only received a vehicle. Six rats were killed at each time point (days 3, 7, 14, 28, and 56 after the mAb 1-22-3 injection). In addition, six rats were killed before the injection of mAb 1-22-3 as baseline controls (0 h), and five rats (Nx group) were injected with PBS 1 wk after uninephrectomy and killed on day 56 after the injection. Additional studies were performed to investigate whether ARB affects glomerular pathology and ERK5 signaling in GN by examining renal tissues from a previous study in which the same GN model in rats had been treated with 70 mg/ml candesartan in drinking water from disease induction (day 0) to day 56 (13).

Urine was collected from the rats over 24-h periods using metabolic cages at 1 wk after surgery and on days 3, 7, 14, 28, and 56 after mAb 1-22-3 or PBS injection. The amount of urinary protein excretion was measured by the Bradford method (Bio-Rad, Oakland, CA).

Histology and immunohistochemistry. The right kidney of each rat was removed, immediately fixed in 10% buffered formalin embedded in paraffin, cut into 4-μm sections, and stained with periodic acid-Schiff reagent. The mean glomerular cell number was calculated based on the total glomerular cell count per glomerular cross section for 30 glomeruli/kidney after periodic acid-Schiff staining. A pathologist who was blinded to the other findings semiquantitatively analyzed the glomerulosclerosis score. The percentage of each glomerulus occupied by the mesangial matrix was estimated and assigned a code according to the following system: 0%, 0; 0.5–1.5%; 1, 1.5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. Frozen sections (3 μm) were fixed in acetone incubated with a rabbit anti-phospho-ERK5 antibody, anti-collagen I antibody, or anti-ANG II antibody overnight at 4°C and then incubated with a FITC-conjugated donkey anti-rabbit IgG antibody. Specificity of ANG II immunostaining on renal tissues was confirmed by preincubation of anti-ANG II antibody with excess ANG II synthetic peptide. This pretreatment prevented the positive staining with anti-ANG II antibody. In double staining experiments, sections were further incubated with an anti-Thy-1 antibody, followed by an appropriate TRITC-coupled donkey anti-mouse antibody. Negative controls included the omission of either of the primary antibodies for which no double staining was noted. To evaluate the level of glomerular staining with each antibody, we performed a semiquantitative analysis according to the following scoring system: 0, diffuse, very weak or absent mesangial staining; 1+, 1–25% of glomerular tufts exhibit strong mesangial staining; 2+, 25–50% of glomerular tufts exhibit strong mesangial staining; 3+, 50–75% of glomerular tufts exhibit strong mesangial staining; and 4+, >75% of glomerular tufts exhibit strong mesangial staining. For each kidney section, 30 glomeruli were selected at random and evaluated by the same blinded pathologist. The mean value per section was then calculated (31).

Formalin-fixed tissue sections (3 μm) were deparaffinized with xylene and rehydrated through a graded series of ethanol. Endogenous peroxidase was blocked by incubation with hydrogen peroxide, and the sections were heated at 121°C for 15 min in 0.01-mol/l citrate buffer (pH 6.0) for antigen retrieval. Next, the sections were incubated with an anti-phospho-ERK1/2 antibody and then diluted in PBS containing 1% BSA at 4°C for 24 h. After being washed with PBS, the sections were incubated with a biotinylated secondary antibody and avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, CA), before being developed with 3,3′-diaminobenzidine (DAB) (Dojindo, Kumamoto, Japan). Each section was counterstained with Mayer’s hematoxylin (Wako, Tokyo, Japan), dehydrated, and coverslipped. The number of phospho-ERK1/2-positive cells in 30 full-size glomeruli were counted, and the mean value was calculated (13).

Western blot analyses. For in vivo experiments, >12,000 isolated glomeruli were obtained from two rat kidneys using the sieving method as high purity (>90%) (7) and homogenized in lysis buffer. For in vitro experiments, MCs were treated with a lysis buffer and cells were scraped off the dish. Next, the samples were centrifuged and the protein concentrations were determined using the Bradford protein assay (Bio-Rad). Protein samples (20–40 μg) were separated by SDS-PAGE using a 12.5% gel and transferred to nitrocellulose membranes. The membranes were probed with either rabbit anti-phospho-ERK5 antibody or a mouse monoclonal anti-phospho-ERK1/2 antibody. Then, the membranes were incubated with an appropriate HRP-conjugated anti-rabbit secondary antibody or HRP-conjugated anti-mouse secondary antibody. Immune-reactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Blots were appropriately reprobed with either a rabbit anti-total ERK5 antibody or a rabbit anti-total ERK1/2 antibody as a loading control. Densitometric analysis was performed using an LKB Ultrascan XL apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden).

In situ superoxide production. In situ production of O2− was determined using 30-μm sections of frozen tissue that had been incubated with dihydroethidium (DHE; 10 μmol/l) in PBS for 30 min at 37°C in a humidified chamber protected from light, as described previously. Upon reacting with O2−, DHE becomes oxidized to create ethidium bromide, which subsequently binds to DNA in the nucleus and emits red fluorescence. The ethidium bromide produced was detected with a 543-nm He-Ne laser combined with a 560-nm long-pass filter (13). The glomerular O2− content was estimated by analyzing the mean fluorescence intensity in glomeruli using the National Institute of Health’s imaging software, as previously described (13).

rat MC culture. Cultured rat MCs were isolated from glomeruli of Sprague-Dawley rats as previously described (7). MCs were serum-starved for 48 h in a serum-free RPMI medium Sigma) before stimulation with H2O2 and/or ANG II in the presence or absence of reagents.

Transfection. For small interfering (si)RNA assays, MCs were transiently transfected with ERK5-specific small interference (siRNA), comprising a mixture of three siRNAs designed by B-Bridge (Sunnyvale, CA), using Lipo lentamine 2000 (Invitrogen, Carlsbad, CA). After incubation in a low-serum-containing medium (5% FBS) for 18 h, the serum concentration was adjusted to that of a complete

![Fig. 5. Effects of H2O2 and/or ANG II on ERK5 phosphorylation in cultured MCs pretreated with ANG II type 1 receptor blocker (ARB) or diphenyle

iodoxamine (DPI). MCs were pretreated with the ARB candesartan or NAD(P)H oxidase inhibitor DPI for 60 min and subsequently stimulated with 250 μM H2O2 or 100 nM ANG II for 10 min. The level of ERK5 phosphorylation in MCs was determined by comparing the ratio of densitometric measurement of the phospho-ERK5 band divided by that of the total-ERK5 band. Values are means ± SD. *P < 0.05 vs. control.
medium and the cells were cultured. A nonsilencing siRNA (Ambion, Austin, TX) that does not target any known mammalian genes was used as a negative control.

Cell viability assay. The viability of siRNA-transfected cells cultured for 24 h in a serum-free medium was evaluated by two independent methods, combined staining with Hoechst 33342 and PI and the WST-1 assay determining cell survival rate.

Cells were suspended in PBS and stained with Hoechst 33342 and PI for 1 h. Next, fields of cells were photographed at ×400 magnification using appropriate filters to examine Hoechst 33342 (excitation wavelength, 348 nm; emission wavelength, 479 nm) and PI (excitation wavelength, 535 nm; emission wavelength, 617 nm) fluorescence. Hoechst 33342 freely enters both living and dead cells and stains their nuclei, whereas PI, a membrane-impairment dye, is excluded from viable cells and only taken up by dead cells. In a blind manner, a total of at least 400 cells/condition were counted. Cell mortality was quantified by determining the ratio of PI-positive cells to Hoechst 33342-positive cells (26). The WST-1 assay was performed using a cell counting kit according to the manufacturer’s protocol. In brief, siRNA-transfected cells grown in 96-well plates were washed with PBS, and 10 μl of WST-1 reagent was added to 100 μl of cell culture medium in each well. After incubation for 8 h, the absorbency of the samples was measured with a microplate reader at wavelengths of 450 (test) and 690 (reference) nm (12).

Collagen measurements. The soluble collagen levels in culture supernatants were measured by using a Sircol collagen assay (Bio-rad, Ltd., Belfast, UK) after incubation in serum-free medium for 24 h. This assay measured total secreted collagen from cultured cells. Briefly, cells were cultured for 24 h with or without treatment, and then supernatants were collected. One milliliter of Sirius red, an anionic dye that specifically reacts with basic side chain groups of collagens, was added to 200 μl of the supernatant and incubated with gentle rotation for 30 min at room temperature. After centrifugation, the collagen-bound dye was resolubilized in 1 ml of 0.5 M NaOH, and the absorbance at 540 nm was measured.

Quantitative real-time PCR. Total RNA was isolated using RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol, and 500 ng of the isolated total RNA was reverse-transcribed using an ExScript RT Reagent Kit (Takara, Shiga, Japan). The mRNA levels of ERK5 were quantified using real-time PCR (iCycler; Bio-Rad). The forward and reverse primers used were ERK5, 5'-CTCCTTCGACGTGACCTTTG-3' and 5'-ATGACACCATGATGTTCTGG-3'; and GAPDH, 5'-GACAACTTGGCATCGTGGA-3' and 5'-ATGCAGGGATGATGTTCTGG-3'. The PCR conditions were 40 cycles of denaturation (95°C for 15 s), annealing (55°C for 30 s), and elongation (72°C for 30 s). The reactions were characterized by comparing the threshold cycle (Ct) values. Samples with higher starting copy numbers showed increases in fluorescence early in the PCR process resulting in lower Ct numbers, whereas those with lower starting copy numbers resulted in higher Ct numbers. Initial characterization of GAPDH expression using RT-PCR, followed by agarose gel electrophoresis and real-time PCR, indicated no signifi.
cant change in the intensity of the GAPDH signals in rat MCs after siRNA. Therefore, the mRNA levels were normalized relative to the corresponding GAPDH values.

Statistical analysis. All experiments reported in this paper were repeated at least three times with similar results and are presented as means ± SD. P values <0.05 were considered to indicate significant differences (Student’s t-test).

RESULTS

Proteinuria and light microscopic findings. In uninephrectomized rats (nephritic rats) that received mAb 1-22-3, urinary protein excretion increased on day 3 (39.4 ± 11.8 mg/24 h) and day 7 (78.0 ± 12.9 mg/24 h). The urinary protein extraction value was 74.0 ± 17.1 mg/24 h on day 14, but subsequently rose again on day 28 (170 ± 64.5 mg/24 h) and reached 273 ± 106 mg/24 h on day 56. No abnormal proteinuria was detected in control uninephrectomized rats that did not receive mAb 1-22-3 (21).

Expression of phospho-ERK5 and phospho-ERK1/2 in normal and nephritic rat glomeruli. On days 0 and 3, glomerular phospho-ERK5 expression was very weak. However, the level of this expression increased in the expanded mesangial area on day 7 and dramatically increased in a typical mesangial pattern on days 28 and 56 (Fig. 1A). Double immunostaining with an MC marker revealed that phospho-ERK5 was colocalized with most of the MCs that stained positive for Thy-1 (Fig. 1B). A semiquantitative assessment indicated that the level of glomerular phospho-ERK5 expression closely paralleled the level of collagen I accumulation as well as the glomerulosclerosis score during the course of chronic anti-Thy-1 GN (P < 0.05) (Fig. 1, C and D). The anti-phospho-ERK5 Western blot analysis revealed that phosphorylation of glomerular ERK5 was slightly increased, followed by a marked increase on day 14 that lasted until at least day 56 of GN (Fig. 1E). These findings were closely associated with the time course of glomerular phospho-ERK5 expression evaluated by immunofluorescence staining.

In contrast to the time course of glomerular ERK5 phosphorylation, the early induction of ERK1/2 phosphorylation was detected on days 3 and 7 of GN by both immunohistochemical and Western blot analysis. A significant increase in phosphorylated ERK1/2 was not seen in the late phase of GN (Fig. 2).

Fig. 7. Cell viability and collagen secretion in ERK5 siRNA-transfected MCs. Cells were transfected with ERK5-specific siRNA (ERK5 siRNA) at indicated concentration or nonsilencing siRNA (50 nM; control siRNA) by the lipofection method. Native control cells were not transfected (no siRNA). Cell viability and collagen secretion were examined after incubation in serum-free medium for 24 h. A: evaluation of cell viability was performed using the WST-1 assay (8 h). B: the level of collagen secretion from MCs in supernatants was determined by Sircol assay. Values are means ± SD. *P < 0.05 vs. control siRNA.

Fig. 8. Effects of ANG II or H2O2 in cell viability and collagen secretion in culture supernatants in ERK5 siRNA-transfected MCs. MCs were transfected with nonsilencing siRNA (control siRNA) or ERK5-specific siRNA (ERK5 siRNA) and then stimulated with ANG II (100 nM) or H2O2 (50 μM) after incubation in serum-free medium for 24 h. Cell viability was evaluated by WST-1 assay (8 h; A), and collagen secretion in culture supernatants was evaluated by Sircol assay (24 h; B). Values are means ± SD. *P < 0.05 vs. control siRNA.
Effects of H₂O₂ and/or ANG II on ERK5 phosphorylation in cultured MCs. Glomerular expression of O₂⁻ and ANG II were significantly increased from day 7 to day 56 of GN in rats (Fig. 3). Considering these findings in Figs. 1 and 3 together, those levels seemed to parallel the expression level of phospho-ERK5 in nephritic glomeruli. Thus the effects of H₂O₂ or ANG II on ERK5 phosphorylation in cultured MCs was examined by Western blot analysis. As shown in Fig. 4A, H₂O₂ (250 μM) significantly elevated ERK5 phosphorylation in MCs at 5 min and maximally at 10 min, which was sustained for 60 min. In the examined concentration of H₂O₂ from 50 μM to 1 mM, ERK5 phosphorylation was increased in a dose-dependent manner (Fig. 4B). On the other hand, the maximum ERK5 phosphorylation of MCs by ANG II (100 nM) was observed at 2.5–5 min (Fig. 4C). ANG II concentration from 1 nM to 1 μM induced ERK5 phosphorylation in a dose-dependent manner (Fig. 4D). It is well known that ANG II induces activation of NAD(P)H oxidase and the development of oxidative stress by MCs (5). Thus we examined whether the oxidative stress mediates the effects of ANG II on ERK5 phosphorylation in MCs. MCs were pretreated with ARB or DPI before stimulation with ANG II or H₂O₂. As shown in Fig. 5, ANG II-induced ERK5 phosphorylation in MCs was blocked by the pretreatment with ARB but not DPI, indicating that ANG II-induced ERK5 phosphorylation by MCs was mediated by the ANG II type 1 receptor but not ANG II-induced NAD(P)H oxidase-dependent ROS production. Furthermore, the costimulation of MCs with ANG II and H₂O₂ resulted in synergistic increase in ERK5 phosphorylation compared with the stimulation of MCs with either ANG II or H₂O₂. These findings suggest that ANG II and H₂O₂ stimulate ERK5 phosphorylation in MCs through different pathways.

Effect of ERK5 inhibition on cell viability and collagen secretion in cell culture supernatants. To examine the endogenous function of ERK5 in MCs, we used the siRNA technique for ERK5 gene silencing. The transfection efficiency was determined under the same experimental conditions by counting the number of fluorescently labeled siRNA-transfected
cells under a fluorescence microscope, and it was ~80% (data not shown). Inhibition of ERK5 expression was verified by quantitative real-time PCR (Fig. 6A) and Western blot analysis (Fig. 6B). Representative fluorescent staining patterns of nuclei with Hoechst 33342 and PI are shown in Fig. 6C. Control siRNA-transfected MCs showed a normal nuclear morphology and were negative on PI staining. However, ERK5 siRNA-transfected MCs showed significant increases in shrunken and condensed nuclei that were positive for PI staining (Fig. 6D). In WST-1 and Sircol assays, the significant decreases in both cell viability and soluble collagen secretion in culture supernatants were observed in ERK5 siRNA-transfected MCs compared with control cells in a concentration-dependent manner (Fig. 7). Next, the effect of oxidative stress and ANG II on ECM accumulation and cell viability and the involvement of ERK5 expression in those effects were examined. As shown in Fig. 8, A and B, ANG II or H2O2 significantly increased both cell viability and collagen secretion in the supernatants in control siRNA-transfected MCs (P < 0.05). Transfection of ERK5 siRNA significantly reduced ANG II- or H2O2-induced MC viability and collagen secretion compared with control cells, respectively (P < 0.05).

**Effect of ARB treatment on histology and phospho-ERK5 expression in nephritic rat glomeruli.** As we reported previously (13), ARB treatment significantly inhibited MC proliferation and ECM accumulation, such as collagen I, on day 56 of GN (Fig. 9, A–C). ARB also could significantly prevent the increased glomerular phospho-ERK5 expression in GN (Fig. 9, A and C).

**DISCUSSION**

A crucial role of MAPK pathways in renal diseases has been reported in a number of recent studies (2, 3, 17, 18, 20, 23). In the present study using a progressive rat model of GN, we showed that a strong induction of ERK1/2 phosphorylation was detected on day 7 in the phase of active MC proliferation whereas phospho-ERK5 expression peaked on days 28 and 56 in the phase of progressive glomerular lesions characterized by sustained MC proliferation and massive ECM accumulation. Our previous study also reported that increased ERK5 phosphorylation is not only found but also associated with MC proliferation and ECM accumulation in the glomeruli of 52-wk-old OLETE rats, a model of type 2 diabetic mellitus (DM) (29). Bokemeyer et al. (2, 3) demonstrated that glomerular ERK1/2 was maximally activated on day 6 and represented a putative mediator of the proliferative response in mesangio-proliferative GN. Thus those studies with respect to ERK activation in damaged glomeruli raise the possibility that ERK1/2 is an important signal molecule for acute inflammation-induced cellular proliferation, and ERK5 for the development of chronic glomerular lesions in GN.

ERK5 was originally shown to be activated by stress stimuli or serum (9, 11). Recent studies including ours reported that other stimuli, such as ROS, ANG II, and high glucose, can also activate ERK5 in various cell types (10). ROS and ANG II have been proposed as potential progressive mediators for human and rat glomerulopathies (13, 25, 30). The present study indicated that the level of glomerular ERK5 phosphorylation parallels those of glomerular ROS and ANG II expression in the rat progressive GN model. Furthermore, we found that H2O2 and ANG II could each strongly induce ERK5 phosphorylation by cultured MCs, suggesting that those mediators are involved in the activation of ERK5 observed in chronic glomerular lesions. Since ANG II stimulates ERK1/2 activation via NAD(P)H oxidase-dependent ROS production in cultured rat MCs (5), ANG II-induced MCs-ERK5 phosphorylation might be mediated with NAD(P)H oxidase-dependent ROS. However, our cell culture studies in pretreatment of MCs with DPI demonstrated that ANG II directly induces ERK5 phosphorylation via NAD(P)H in an oxidase-independent manner. A synergistic effect of H2O2 and ANG II on the enhancement of MC-ERK5 phosphorylation also suggests that ROS and ANG II could each induce ERK5 phosphorylation in MCs through different signal pathways.

Many studies demonstrated that the ERK5 pathway controls cell behaviors such as cell proliferation, survival, differentiation, and ECM metabolism under pathophysiological conditions (4, 15, 22). A substantial role of ERK5 expression on MCs in chronic progressive GN was investigated in our in vitro and in vivo studies. We found that the enhancement of both cell viability and collagen secretion in the culture supernatants was induced in control MCs by ANG II or H2O2. Those changes were significantly prevented in ERK5 siRNA-transfected MCs, suggesting a possible role of ERK5 expression in the phase of sustained MC proliferation and pathological ECM accumulation seen in progressive GN. Furthermore, the data obtained from the treatment of GN rats with ARB indicated that ANG II is an activator for MCs-ERK5 phosphorylation in vivo and supported the involvement of ANG II-induced ERK5 phosphorylation in progressive glomerular lesions. In general, the repair of the damage in proliferative GN requires regression of the proliferated glomerular cells by apoptosis and appropriate removal of accumulated pathological ECM (27). Based on our results, the activation of an ERK5 signal may not only induce prolonged MC survival but also enhance the secretion of pathological collagen I within damaged glomeruli and thereby contribute to the development of progressive GN.

In conclusion, the present study has revealed that glomerular ERK5 expression and phosphorylation are markedly increased in an experimental model of progressive GN. Furthermore, the enhancement of ERK5 phosphorylation appears to be linked to the increased MC viability and pathological ECM accumulation. We therefore propose that controlled regulation of glomerular ERK5 activation could provide the basis for an effective therapeutic strategy for preventing the progression of GN.

**ACKNOWLEDGMENTS**

We thank Naomi Okamoto, Chizuko Yamamoto, Keita Osumi, Junki Yamajo, and Hiroki Matsumoto for excellent technical assistance.

**GRANTS**

This work was supported by grants from the Japanese Ministry of Welfare (18591189, 18790723, 20591277 and 2059127) and Morinaga Foundation for Health and Nutrition.

**DISCLOSURES**

No conflicts of interests are declared by the authors.

**REFERENCES**


