Glucocorticoids stimulate p21(CIP1) in mesangial cells and in anti-GBM glomerulonephritis

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Glucocorticoids stimulate p21(CIP1) in mesangial cells and in anti-GBM glomerulonephritis.

Background. Glucocorticoids are widely used for the treatment of glomerulonephritis, but the mechanism of cell cycle inhibition by glucocorticoids is poorly understood at a molecular level.

Methods. The effects of dexamethasone on cell cycle progression were examined in rat mesangial cells. To investigate the mechanisms of cell cycle inhibition by dexamethasone, we transfected the −2.3 kb p21(CIP1) promoter-CAT construct to mesangial cells using an electroporation method. We also examined whether glucocorticoids stimulate the expression of p21(CIP1) and inhibit cell proliferation in glomeruli of anti-glomerular basement membrane (GBM) glomerulonephritis in rats.

Results. Dexamethasone inhibited [H]-thymidine uptake and the percentages of S and G2/M phases in rat mesangial cells. Dexamethasone stimulated CAT activity of the p21(CIP1) promoter 4.5-fold. Deletion analysis of the p21(CIP1) promoter revealed that the glucocorticoid-responsive region (GRE) is present between −1.4 and −1.1 kb upstream of the transcription initiation site. Dexamethasone inducibility of p21(CIP1) promoter activity requires the presence of the C/EBPα DNA binding site in the GRE of the p21(CIP1) promoter and C/EBPα protein. Intravenous injection of anti-GBM antibody caused mesangial proliferation, crescent formation, and proteinuria in rats. Ten days of administration of prednisolone (1 mg/kg/day) reduced proteinuria and inhibited mesangial cell proliferation and crescent formation. The glomerular-sieving method revealed that prednisolone increased p21(CIP1) expression in glomeruli.

Conclusion. These data suggest that the cell cycle arrest of mesangial cells is mediated by a functional link between the glucocorticoid receptor and the transcriptional control of p21(CIP1) not only in vitro but also in vivo. Our observations provide new insights into the molecular mechanisms of glucocorticoid action in glomerulonephritis.

Key words: dexamethasone, cell cycle, mesangial proliferative glomerulonephritis, kinase inhibitory protein family, cyclin-dependent kinase.

Received for publication August 23, 2000
and in revised form November 22, 2000
Accepted for publication November 29, 2000
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Mesangial cell proliferation is an important feature of mesangial proliferative glomerulonephritis (GN), which progresses to end-stage renal disease [1, 2]. Mesangial cell proliferation accompanies a wide variety of renal diseases, especially proliferative forms of GN. The cell cycle of mesangial cells is under the control of a large number of humoral factors that either promote or suppress mitogenesis and cell proliferation [3, 4]. The precise mechanisms that lead to mesangial cell proliferation and cell cycle progression are incompletely understood.

The eukaryotic cell cycle is tightly regulated through a precious balance of positive and negative regulatory components that exert their effects during the first gap phase (G1) of the cell cycle [5, 6]. The most critical positive-acting components are G1 cyclins (cyclin D and cyclin E) [6, 7]. Cyclins assemble with cyclin-dependent kinase (CDK) and phosphorylate the key physiological activity requires the presence of the C/EBPα DNA binding site in the GRE of the p21(CIP1) promoter and C/EBPα protein. Intravenous injection of anti-GBM antibody caused mesangial proliferation, crescent formation, and proteinuria in rats. Ten days of administration of prednisolone (1 mg/kg/day) reduced proteinuria and inhibited mesangial cell proliferation and crescent formation. The glomerular-sieving method revealed that prednisolone increased p21(CIP1) expression in glomeruli.

Conclusion. These data suggest that the cell cycle arrest of mesangial cells is mediated by a functional link between the glucocorticoid receptor and the transcriptional control of p21(CIP1) not only in vitro but also in vivo. Our observations provide new insights into the molecular mechanisms of glucocorticoid action in glomerulonephritis.
Glucocorticoids are widely used for the treatment of GN. Glucocorticoid administration has been reported to ameliorate mesangial proliferative GN in humans [15, 16]. However, the mechanism of the antiproliferative effects of glucocorticoid is poorly understood at a molecular level in mesangial cells. The functional connection between the glucocorticoid-regulated transcriptional events and the cell cycle arrest of mesangial cells is not known.

The purpose of this study was to investigate the mechanisms of glucocorticoid-induced cell cycle arrest in mesangial cells and in anti-glomerular basement membrane (GBM) GN. To determine whether the inhibitory mechanisms of glucocorticoid on mesangial cell proliferation are mediated by CDK inhibitors, we investigated the expression of CDK inhibitors (p27Kip1, p21CIP1) and activities of CDK2 and CDK4 in rat mesangial cells and in glomeruli of anti-GBM rats treated with glucocorticoids. We demonstrated that dexamethasone inhibited mesangial proliferation mainly via transcriptional up-regulation of p21CIP1. We also examined the effect of dexamethasone on a deletion mutant of p21CIP1 promoter-CAT activity and the effect of cotransfection of CCAAT/enhancer binding protein (C/EBPα) on p21CIP1 promoter-CAT activity. Finally, we demonstrated that glucocorticoid administration ameliorated cell proliferation and proteinuria in anti-GBM–induced rat proliferative GN at least partially by the induction of p21CIP1.

**METHODS**

**Mesangial cell culture**

Mesangial cell strains from male Wistar-Kyoto rats were isolated and characterized as previously reported [17]. Cells were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL of insulin, 5 μg/mL of transferrin, and 5 ng/mL selenite at 37°C in a 5% CO2 incubator. Cells used in experiments were from four to nine passages. After an initial incubation in medium plus 20% fetal calf serum (FCS) until approximately 80% confluence, the cells were incubated in medium containing dexamethasone (10−6 mol/L) plus 20 ng/mL platelet-derived growth factor (PDGF)-BB (Boehringer, Mannheim, Germany) for the indicated times.

**Animal model**

Female Wistar-Kyoto rats weighing 200 to 250 g were used in the present study. Experimental proliferative GN was induced by an intravenous administration of anti-GBM serum at a dose of 0.25 mL/kg body weight as described previously [18]. We divided the rats into three groups: the control group, the anti-GBM GN without prednisolone group, and the anti-GBM GN with prednisolone group. In the control group, we administered saline instead of anti-GBM antibody. In the glucocorticoid-treated anti-GBM GN group, prednisolone p.o. was started on the same day as the anti-GBM antibody injection and was continued for 10 days. In the anti-GBM GN without prednisolone group, saline p.o. was started on the same day as the anti-GBM antibody injection and was continued for 10 days. Rats were sacrificed on days 0, 5, and 10 (N = 5 at each time point) after anti-GBM antibody injection. Immediately after sacrifice, renal biopsies were taken from each animal for histologic studies, and glomeruli were isolated by differential sieving for protein extraction and mRNA detection as described previously [17].

**Expression vectors and reporter constructs**

C/EBPα human cDNAs were generous gifts of Dr. H. Baumann [19]. The p21CIP1 reporter construct used for the CAT assays contained human p21CIP1 promoter from residues −2280 to 139 cloned upstream of the CAT gene (generous gift of Dr. G.L. Firestone and Dr. B. Vogelstein) [20, 21]. The pCH110 reporter construct contains the SV40 promoter upstream of the β-galactosidase gene (Pharmacia Biotechnology Inc., Uppsala, Sweden) [22].

**Transient transfection and CAT assay**

Rat mesangial cells were transfected by the electroporation method. Data are representative of at least four independent experiments performed in duplicate and are expressed as “N-fold increase in luciferase activity” calculated relative to the indicated level of p21CIP1 promoter activity. Plasmid DNAs (10 μg) were transfected by the electroporation method. For experiments performed in exponentially growing cells, CAT activity was measured 48 hours after transfection using an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer). Normalization was achieved by cotransfecting 3.0 μg of pCH110, a β-galactosidase reporter construct, as an internal control for the transfection efficiency. For mitogen-stimulated p21CIP1 reporter expression, cells were stimulated with 20 ng/mL PDGF with or without dexamethasone for 24 hours from one day following the transfection. β-Galactosidase activities were measured according to the Promega (Madison, WI, USA) protocol.

**Antisense oligonucleotide**

Antisense and scrambled random sequence oligonucleotides (oligonucleoside phosphorothioates) against p21CIP1 were synthesized by a DNA synthesizer (Model 8909; Perseptive Biosystems, Cambridge, MA, USA) and purified by an high-performance liquid chromatography (HPLC). The p21CIP1 antisense oligonucleotide was designed around the start codon of rat p21CIP1, with the sequence 5′-GACATCACCAGGATCGGACAT-3′ and the scrambled random sequence control oligonucleotide p21CIP1 5′-TGGATCCGACATGTCAGA-3′, have been used previously to synthesize oligonucleotides to interfere with p21CIP1 expression in rat vascular smooth muscle.
cells [23]. For the liposome-mediated transfection procedure, 10^{-6} mol/L oligonucleotide was mixed with cationic liposome using a DOSPER liposomal transfection reagent (Boehringer Mannheim) in serum-free medium and incubated for 24 hours for 37°C [24]. We used fluorescein-isothiocyanate-labeled oligonucleotides (5'-GACATCACCAGGATCGGACAT-3') to show the efficiency of the oligonucleotide transfection. Proliferating mesangial cells were rinsed once in serum-free medium, incubated preliminarily with an oligonucleotideliposome solution, and then incubated in medium containing 0.5% serum for 24 hours in humidified incubators at 37°C with 5% CO2. The percentage of cells that were positive for uptake of fluorescein isothiocyanate-labeled oligonucleotides was determined by ultraviolet fluorescence microscopy. After incubation with antisense or scrambled random sequence control oligonucleotides (10^{-8} mol/L) for 24 hours, mesangial cells were incubated with 10^{-6} mol/L dexamethasone.

**Western blot analysis**

The proteins were extracted from mesangial cells or isolated glomeruli using an extraction buffer containing 50 mmol/L β-glycerophosphate, pH 7.3, 1.5 mmol/L EGTA, 0.1 mmol/L Na2VO3, 1 mmol/L dithiothreitol (DTT), 10 μg/mL leupeptine, 10 μg/mL aprotinin, 2 μg/mL pepstatin A, and 1 mmol/L benzamidine as described previously [25]. Fifty micrograms of protein were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were then transferred to an Immobilon P membrane (Daiichikagaku, Tokyo, Japan). To detect p27Kip1, p21CIP1, cyclin D1, cyclin E, CDK2, and CDK4, the membrane was incubated with antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000 in TBS containing 0.5% Tween 20 for two hours at 37°C and then visualized using the ECL chemiluminescence system (Amersham, Little Chalfont, UK). Films were scanned using an Epson scanner (Nagano, Japan). Signals on Western blots were quantitated by densitometry using an image analysis software application (NIH Image 1.47).

**Northern blot analysis**

The mRNA was extracted from isolated glomeruli using TRI-REAGENT™ (Life Technologies, Gaithersburg, MD, USA). Twenty micrograms of total RNA samples were separated on 1% agarose-formaldehyde-MOPS gels and transferred to Hybond-N hybridization membranes (Amersham). A series of 2.7 kb full-length murine p21CIP1 cDNAs [11] was labeled by the random priming method and used as probes for Northern hybridization. Following hybridization, the membranes were washed twice for five minutes at room temperature in 5 × SSPE and 0.5% SDS, twice at 37°C for 15 minutes in 1 × SSPE and 0.5% SDS, once at 37°C for 15 minutes in 0.1 × SSPE and 0.5% SDS, and then exposed to Fuji x-ray film (Fuji Photo Film, Kanagawa, Japan) for three to seven days at −80°C.

**[3H] thymidine incorporation**

Mesangial cells were plated in 24-well plates, incubated in medium containing 20% FCS for 24 hours, and then incubated in medium plus 20% FCS until approximately 70% confluence. Next, they were incubated in several mediums containing different concentrations of dexamethasone (10^{-5} to 10^{-10} mol/L) plus 20 ng/mL PDGF for 24 hours for indicated times. For the last four hours, the cells were pulsed with 1 μCi [3H] thymidine (Amersham) and counted in Aquasol-2 scintillation cocktail (NEN Research Products, Boston, MA, USA).

**Cell cycle analysis by flow cytometry**

Mesangial cells were cultured in a 75 cm² flask. Next, after an initial incubation in RPMI 1640 medium plus 20% FCS until approximately 80% confluence, the cells were incubated in medium with or without dexamethasone (10^{-6} mol/L) plus 20 ng/mL PDGF for 24 hours. The cells were stained with propidium iodide and analyzed by flow cytometry using a Coulter flow cytometer (Coulter Corporation, Hialeah, FL, USA), and the percentages of the cells in the G1, S, and G2/M phases were determined [26].

**Immunoprecipitation and CDK4, CDK2 kinase assay**

Immunocomplex kinase assay was performed using essentially the same methods described by Matsushima et al [27]. Cell lysates of mesangial cells and isolated glomeruli were incubated for two hours at 4°C with 20 μL of protein G-plus agarose (Oncogene Science, Uniondale, NY, USA) and 10 μL of anti-rat-CDK4 antibody or anti-rat-CDK2 antibody (Santa Cruz Biochemical Inc.), and immune complexes were recovered by centrifugation. Immunoprecipitated proteins were suspended in 30 μL of kinase buffer (50 mmol/L HEPES, pH 7.5, 10 mmol/L MgCl2, 1 mmol/L dithiothreitol) containing substrate (0.2 μg of soluble glutathione S-transferase-pRb fusion protein for the CDK4 kinase assay, and histone H1; Boehringer Mannheim for the CDK2 assay) and 2.5 mmol/L EGTA, 10 mmol/L β-glycerophosphate, 1 mmol/L NaF, 20 μmol/L ATP, and 10 μCi of [γ-32P] ATP (6000 Ci/mmol; NEN, DuPont, Boston, MA, USA). After incubation for 30 minutes at 30°C with occasional mixing, the samples were boiled in polyacrylamide gel sample buffer containing SDS and separated by electrophoresis. Phosphorylated proteins were visualized by autoradiography.

Immunoprecipitated protein was resolved in SDS-PAGE and analyzed by Western blotting with p21CIP1, CDK2, and CDK4 antibody.
Histologic examination

Renal tissues obtained from control, saline-treated, or prednisolone-treated anti-GBN GN rats were fixed in formalin overnight and then dehydrated and paraffin embedded. Thin sections were examined with periodic acid-Schiff staining as described previously [28].

Statistical analysis

The results were given as means ± SEM. The differences were tested using analysis of variance. P < 0.05 was considered significant.

RESULTS

Dexamethasone inhibited [3H]-thymidine incorporation and cell cycle progression induced by PDGF in rat mesangial cells

To determine the effects of dexamethasone on cell cycle progression, dexamethasone was added to asynchronous mesangial cells. Various concentrations of dexamethasone were added to mediums containing 20 ng/mL PDGF for 24 hours, and [3H]-thymidine incorporation was measured. As shown in Figure 1A, PDGF-stimulated [3H]-thymidine incorporation is inhibited dose dependently in the presence of dexamethasone. Cell cycle analysis by flow cytometry revealed that dexamethasone (10^{-6} mol/L) reduced the percentage of PDGF-stimulated increments of S phase and G2/M phase (Fig. 1B).

Effect of dexamethasone on CDK2 and CDK4 activities and protein levels of p21^CIP1, p27^KIP1, cyclin D1, cyclin E, CDK2, and CDK4 in rat mesangial cells

To determine the inhibitory mechanisms of dexamethasone on cell cycle progression, we examined the effects of dexamethasone on protein expression of the following G1/S-regulated genes: p21^CIP1, p27^KIP1, cyclin D1, cyclin E, CDK2, and CDK4. Rat mesangial cells were treated with or without (10^{-6} mol/L) dexamethasone for 24 hours in the presence of 20 μg/mL PDGF. Rat mesangial cells treated without dexamethasone or PDGF served as the control. Twenty micrograms of cell lysate protein were used for Western blot analysis. The protein expression of p21^CIP1, p27^KIP1, cyclin D1, cyclin E, CDK2, and CDK4 was determined by Western blot analysis using specific antibodies. Figure 2A demonstrates that dexamethasone significantly increased the expression of p21^CIP1 but not the expressions of p27^KIP1, cyclin D1, cyclin E, CDK2, and CDK4.

To determine the effects of dexamethasone on the CDK activities, CDK2 and CDK4 activities were measured in PDGF-stimulated mesangial cells with or without dexamethasone (10^{-6} mol/L). Equal amounts of cell extract (1 mg) were immunoprecipitated with antibodies against CDK2 or CDK4. The kinase activities of the immunoprecipitates were assayed using histone H1 for CDK2 and GST-Rb for CDK4. Figure 2B shows that the kinase activities associated with CDK2 and CDK4
Fig. 2. Effect of dexamethasone on CDK2 and CDK4 kinase activities and protein levels of p21CIP1, p27KIP1, cyclin D1, cyclin E, CDK2, and CDK4 in rat mesangial cells. (A) Western blot analysis. Rat mesangial cells were treated with or without (10^–6 mol/L) dexamethasone (Dex) in the presence or absence of 20 ng/mL PDGF for 24 hours. Twenty micrograms of cell lysate protein were used for Western blot analysis. The protein production of p21CIP1, p27KIP1, cyclin D1, cyclin E, CDK2, and CDK4 was determined by Western blot analysis using specific antibodies. (B) Kinase assay. CDK2 and CDK4 activities were measured in PDGF-stimulated mesangial cells with or without Dex (10^–6 mol/L). Equal amounts of cell extract (1 mg) were immunoprecipitated with antibodies against CDK2 or CDK4. The kinase activities of the immunoprecipitates were assayed using histone H1 for CDK2 and GST-Rb for CDK4. Western blot of p21CIP1 was performed after immunoprecipitation with CDK2 or CDK4.

were up-regulated in the presence of PDGF and repressed in dexamethasone-treated cells. To confirm whether or not the decrease of CDK2 and CDK4 activity was mediated by increment of p21CIP1 level, we performed Western blot analysis of p21CIP1 after immunoprecipitation with CDK2 or CDK4. The level of p21CIP1 associated with CDK2 was increased in the dexamethasone-treated mesangial cells (Fig. 2B). This suggested that the increment of p21CIP1 mediated the decrease of CDK2 kinase activity. The level of p21CIP1 associated with CDK4 was also increased in the dexamethasone-treated mesangial cell (Fig. 2B). This suggested that the increment of p21CIP1 mediated the decrease of CDK4 kinase activity.

Deletion analysis of the p21CIP1 promoter defines a 297 bp glucocorticoid-responsive element (GRE).

To determine which region within the p21CIP1 promoter was responsible for the glucocorticoid-mediated transcriptional activation, rat mesangial cells were transiently transfected with a series of p21CIP1-CAT reporter plasmids that contain indicated 5′ deletions of the p21CIP1 promoter and β-galactosidase plasmid. Cells were treated with (10^–6 mol/L) or without dexamethasone for 24 hours, and then the CAT-specific activity was determined using ELISA CAT assay kits. The CAT activity was normalized by β-galactosidase activity. The reported values are an average of four independent experiments (mean ± SEM). *P < 0.05 vs. CAT activity of −2280 plasmid.
containing the three largest p21<sup>CIP1</sup> promoter regions with deletions ending at −2280, −1846, and −1435 bp. Dexamethasone inducibility of the p21 promoter was lost in deletions beyond −1139 bp. These results indicated that the promoter sequence between nucleotides −1435 and −1139 of the p21<sup>CIP1</sup> promoter was the glucocorticoid-responsive region (GRE) in rat mesangial cells.

**Requirement of C/EBPα expression for the transcriptional stimulation of p21<sup>CIP1</sup> promoter activity by dexamethasone in rat mesangial cells**

The previous studies by Cram et al demonstrated first that the expression of C/EBPα transcription factor was required for the induction of p21<sup>CIP1</sup> protein in hepatoma cells, and second that a canonical C/EBPα DNA binding site was present at −1270 bp in the p21<sup>CIP1</sup> promoter [20]. In our current study, therefore, we examined the requirement of C/EBPα expression for induction of p21<sup>CIP1</sup>. At first, we examined the time course of dexamethasone effects on protein levels of p21<sup>CIP1</sup> and C/EBPα in rat mesangial cells. Cells were treated with or without (10<sup>−6</sup> mol/L) dexamethasone for indicated times. As shown in Figure 4, the protein level of C/EBPα was augmented as early as three hours after the addition of dexamethasone, while p21<sup>CIP1</sup> was augmented more slowly, and did not reach significant levels until 12 to 24 hours after the addition of dexamethasone. These results suggested that C/EBPα protein might be important for dexamethasone to induce p21<sup>CIP1</sup> promoter activity.

Next, we performed a mutation analysis of the C/EBPα DNA binding site in the stimulation of dexamethasone-induced p21<sup>CIP1</sup> promoter activity. Cotransfection of C/EBPα construct significantly increased dexamethasone-induced p21<sup>CIP1</sup> promoter activity. Rat mesangial cells were transiently cotransfected with a wild-type p21<sup>CIP1</sup> promoter (C) or mutated C/EBPα-CAT reporter (B) plasmid, a C/EBPα expression plasmid, and a β-galactosidase plasmid. Cells were treated with or without (10<sup>−6</sup> mol/L) dexamethasone for 24 hours, and CAT-specific activity was determined using ELISA-CAT assay kits. The CAT activity was normalized by β-galactosidase activity. The reported values are an average of four independent experiments (mean ± SEM). *P < 0.05 vs. CAT activity of wild-type p21<sup>CIP1</sup> promoter plasmid without dexamethasone or cotransfection of a C/EBPα expression plasmid.
Fig. 6. Glucocorticoid administration at least partially ameliorated cell proliferation and proteinuria in anti-GBM antibody-induced proliferative GN via the induction of p21CIP1. Wistar-Kyoto rats were divided into three groups: the control group, the anti-GBM GN without prednisolone group, and the anti-GBM GN with prednisolone group. In the anti-GBM GN with or without prednisolone group, we administered prednisolone (1 mg/kg/day) or saline for 5 or 10 days just after anti-GBM antibody injection to rats. In control rats, saline was administered instead of anti-GBM antibody. (A) Histologic examinations of periodic acid-Schiff staining (×400) at 10 days are shown: control group, anti-GBM GN without prednisolone group, and anti-GBM GN with prednisolone group. (B) Levels of proteinuria of the control group, anti-GBM GN without prednisolone group, and anti-GBM GN with prednisolone group. Administration of prednisolone significantly ameliorated proteinuria of anti-GBM GN at 5 and 10 days. Results are means ± SEM of five independent experiments (*P < 0.05). (C) Western blot analysis and Northern blot analysis from glomeruli of control group, anti-GBM GN without prednisolone group, and anti-GBM GN with prednisolone group. The level of p21CIP1 protein and mRNA expression increased in glomeruli of anti-GBM GN with prednisolone group compared with that of anti-GBM GN without prednisolone group, suggesting a transcriptional increment of p21CIP1 expression in vivo. (D) The protein levels of CDK2 and CDK4 were the same in the glomeruli of the anti-GBM GN with prednisolone group compared with that of the anti-GBM GN without prednisolone group. (E) CDK2 and CDK4 activities were measured in isolated glomeruli of control group, anti-GBM GN without prednisolone group, and anti-GBM GN with prednisolone group. Equal amounts of cell extract (1 mg) were immunoprecipitated with antibodies against CDK2 and CDK4. The kinase activities of the immunoprecipitates were assayed using histone H1 for CDK2 and GST-Rb for CDK4. Western blot analysis of p21CIP1 after immunoprecipitation with CDK2 or CDK4 revealed that protein levels of p21CIP1 associated with CDK2 and CDK4 were increased in the glomeruli of anti-GBM GN with prednisolone group.

previously for hepatoma cells [20]. These p21CIP1 promoter fragments containing either the wild-type or the C/EBP DNA-binding site mutation were linked upstream of the thymidine kinase (tk) minimal promoter sequence driving the bacterial CAT gene [20]. As shown in Figure 5, dexamethasone failed to induce CAT activity in the reporter-plasmid–mutated C/EBP DNA binding site in rat mesangial cells, whereas the reporter plasmid containing the wild-type C/EBP DNA-binding site was responsive to dexamethasone. Transient expression of C/EBPα increased the CAT activity significantly in the wild-type p21CIP1 promoter fragment but not in the cells transfected with the reporter plasmid mutated in the C/EBPα binding site.
Glucocorticoid administration ameliorated cell proliferation and proteinuria in anti-GBM antibody-induced rat proliferative GN at least partially via the induction of p21CIP1

To determine the inhibitory effect of glucocorticoid in mesangial cell proliferation in vivo, we established three groups of rats: a control group, an anti-GBM GN without prednisolone (saline-treated) group, and an anti-GBM GN with prednisolone group. In the anti-GBM GN with prednisolone group, prednisolone (1 mg/kg/day) was administered just after the anti-GBM antibody injection to rats for 5 or 10 consecutive days. As shown in Figure 6A, glomeruli of the anti-GBM GN without prednisolone group showed a marked increase in mesangial proliferation and crescent formation on day 10 compared with the control group. In contrast, the glomeruli of the anti-GBM GN with prednisolone group showed a significant amelioration in mesangial proliferation and crescent formation compared with the glomeruli of the anti-GBM GN without prednisolone group on day 10 (Fig. 6A). The level of proteinuria in the anti-GBM GN with prednisolone group was ameliorated compared with the level of the anti-GBM GN without prednisolone group at both 5 and 10 days (Fig. 6B).

Furthermore, we performed Western blot analysis, Northern blot analysis, and CDK2 and CDK4 assays from isolated glomeruli in the control group, anti-GBM GN without prednisolone group, and anti-GBM GN with prednisolone group. Glomeruli of anti-GBM GN with prednisolone group accumulated increased amounts of p21CIP1 protein and mRNA compared with glomeruli of the anti-GBM GN without prednisolone group (Fig. 6C). In contrast, Western blot analysis of CDK2 and CDK4 with total cell lysate of isolated glomeruli showed no changes in the protein levels of CDK2 and CDK4 in the glomeruli of the anti-GBM GN with prednisolone group (Fig. 6D).

To determine the effects of prednisolone on the CDK activities in glomeruli of the anti-GBM antibody-induced GN group, an immunocomplex kinase assay was performed from glomeruli of the control group, anti-GBM GN without prednisolone group, and anti-GBM GN with prednisolone group. Kinase activities of CDK2 and CDK4 of glomeruli from the anti-GBM GN with prednisolone group were significantly decreased compared with that of anti-GBM GN without prednisolone group, as shown in Figure 6E. Western blot analysis of p21CIP1 after immunoprecipitation with CDK2 or CDK4 revealed that protein levels of p21CIP1 associated with CDK2 and CDK4 were increased in the glomeruli of anti-GBM GN with prednisolone group.

Effect of p21CIP1 antisense and scrambled random sequence phosphorothioate oligonucleotide on p21CIP1 protein expression and dexamethasone-induced inhibition of [3H]-thymidine incorporation in rat mesangial cells

To test whether interference with p21CIP1 expression using an antisense oligonucleotide approach influenced
dexamethasone-induced cell cycle arrest, protein synthesis and [H]-thymidine incorporation were measured in rat mesangial cells transiently transfected with 10^-6 mol/L of p21CIP1 antisense and scrambled random sequence phosphorothioate oligonucleotides. At first, we used fluorescein-isothiocyanate-labeled oligonucleotides to show the efficiency of the oligonucleotide transfection. We observed that oligonucleotides were taken up and concentrated in the nuclei of 85 to 90% of cells.

As shown in Figure 7 (lower panel), transfection with p21CIP1 antisense phosphorothioate oligonucleotide significantly inhibited dexamethasone-induced p21CIP1 protein expression in rat mesangial cells. p21CIP1 protein expression was inhibited by p21CIP1 antisense phosphorothioate oligonucleotide but not by p21CIP1 scrambled random sequence phosphorothioate oligonucleotide. p21CIP1 antisense oligonucleotide abolished the inhibitory effects of dexamethasone on PDGF-stimulated [H]-thymidine incorporation in rat mesangial cells (Fig. 7, upper panel).

**DISCUSSION**

In the present study, we demonstrate that dexamethasone significantly increased the protein expression and promoter activity of p21CIP1 in rat mesangial cells. Deletion analysis revealed that dexamethasone inducibility of the p21CIP1 promoter was present between -1435 and -1139 bp. Dexamethasone inducibility of p21CIP1 promoter activity required the presence of the C/EBPα DNA binding site in the p21CIP1 promoter region and C/EBPα protein. We also demonstrated that glucocorticoid administration at least partially ameliorated cell proliferation and proteinuria in anti-GBM antibody-induced rat proliferative GN via the induction of p21CIP1.

Glucocorticoid has been widely used as a therapy for mesangial proliferative GN [15, 16]. Glucocorticoid is known to inhibit or stimulate the in vivo and in vitro growth of many types of normal and transformed cells [29–31]. However, the molecular mechanisms of the antimitogenic effects of glucocorticoid in mesangial proliferative disease have not been clarified. This report is the first, to our knowledge, to demonstrate the involvement of p21CIP1 in the inhibitory effects of dexamethasone on mesangial cell proliferation both in vitro and in vivo.

We believe that p21CIP1 may play a critical role in the glucocorticoid-induced cell cycle arrest in mesangial cells for the following reasons. First, the protein level of p21CIP1 was increased, and cell cycle progression was inhibited in the presence of glucocorticoid. Second, these inhibitory effects were accompanied by a reduction of CDK2 and CDK4 kinase activities, whereas the protein levels of CDK2, CDK4, p27kip1, cyclin D, and cyclin E were not changed in the presence of dexamethasone. Third, antisense oligonucleotide for p21CIP1 reversed the antiproliferative glucocorticoid action in cultured mesangial cells. Fourth, our previous report demonstrated that overexpression of p21CIP1 caused cell cycle arrest in rat mesangial cells [14].

To our knowledge, this is the first report showing that prednisolone administration increases p21CIP1 protein levels in the glomerulus in vivo. Furthermore, this report is the first to propose a mechanism of glucocorticoid inhibition of mesangial cell proliferation and proteinuria in proliferative GN rats. Our data from immunoblot, Northern blot, and immunohistological analyses clearly demonstrate that glucocorticoid increases p21CIP1 mRNA and protein levels in glomeruli of rat anti-GBM GN. The evidence from the present in vitro and in vivo experiments strongly suggests that glucocorticoid at least partly inhibited mesangial proliferation via a transcriptional induction of p21CIP1 in anti-GBM GN. However, in our experiments, p21CIP1 actually was demonstrated only in isolated glomeruli and not in mesangial cells in vivo (Fig. 6). It is possible that prednisolone might induce p21CIP1 in other glomerular cells, particularly in the epithelial cells.

Many glomerular diseases are characterized by mesangial cell proliferation and accumulation of mesangial extracellular matrix [1, 2]. The mechanisms of regulation of the mesangial cell cycle are largely unknown. Shankland et al recently showed that the normal glomerulus has a high endogenous expression of p27kip1 that decreases with the initiation of the mesangial cells' proliferative response and then normalizes with the resolution of the mesangial cell proliferation in the Thy-1.1 model of experimental mesangial proliferative GN [13]. Furthermore, they found that p21CIP1 has low levels of expression in the normal rat glomerulus [13]. Recently, in our own investigation of the mechanisms of the cell cycle regulation of rat mesangial cells, we demonstrated that FCS and PDGF stimulated CDK2 and CDK4 kinase activity and that these stimulatory activities were inhibited by overexpressions of CDK inhibitor (p16INK4a and p21CIP1) [14]. These studies indicated that mesangial cell proliferation is regulated by a precise balance of CDK kinase and CDK inhibitors. The present study demonstrates that among these CDK inhibitors, p21CIP1 plays a key role in the mesangial cell cycle not only in vitro but also in vivo. Furthermore, other recent studies demonstrated that p21CIP1 induces cell cycle-independent effects such as cell differentiation and apoptosis in myocytes and vascular smooth muscle cells [23, 32, 33]. The effects may influence mesangial cell transformation and anti-inflammatory mechanisms in the rat GN model.

This study demonstrates that dexamethasone stimulates the promoter activity of p21CIP1 and increases the p21CIP1 protein expression in mesangial cells. We define the GRE as bases -1435 to -1139 of the p21CIP1 promoter sequence. This result is in agreement with the Firestone group’s reports on hepatoma cell lines [20, 34].
We also demonstrated that dexamethasone stimulated the expression of C/EBPα before it stimulated that of p21CIP1. For two reasons, we conclude that this induction of C/EBPα may activate the transcriptional stimulation of p21CIP1 promoter in mesangial cells. First, the mutation of C/EBP binding site eliminated the stimulatory effects of dexamethasone, and second, the cotransfection of C/EBPα enhanced the stimulatory effects of dexamethasone on p21CIP1-promoter activity. Our results compare well with the Firestone group's reports on hepatoma cell lines [20, 35]. It would be interesting to see whether C/EBPα elements could be found in other promoters involved in mesangial hypercellularity, such as smooth muscle α-actin, PDGF and transforming growth factor β because, as far as we could find, there are no C/EBPα elements in those promoters. The precise function of C/EBPα in dexamethasone-induced p21CIP1 up-regulation should be analyzed in future studies.

In conclusion, the present study provides new insights into the pathophysiological role of glucocorticoid in proliferative GN. These data indicate a functional link between the glucocorticoid receptor signaling pathway and the transcriptional control of p21CIP1 that mediates cell cycle arrest, as well as an enhancement of this linkage by C/EBPα, in mesangial cells in vitro and in rat proliferative GN.

ACKNOWLEDGMENTS

We thank Drs. A. Noda, H. Baumann, G.L. Firestone, and B. Vogelstein for kindly providing the plasmids used in this study.

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