Requirement of Cyclin D1 in Mesangial Cell Mitogenesis

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Abstract. Hyperplasia of mesangial cells (MC) is a frequent finding in glomerulonephritis. The control and function of cyclin D1, a regulator of cell cycle progression, in MC proliferation in vivo and in vitro were investigated. In a rat model of mesangioproliferative glomerulonephritis, increases in the number of cyclin D1-positive MC nuclei were prominent on day 5 of the disease, preceding the peak of MC hyperplasia. In growth-arrested rat MC in culture, mitogenic stimulation with serum or platelet-derived growth factor (PDGF) led to rapid increases in cyclin D1 protein expression. Transforming growth factor-β1 inhibited PDGF induction of cyclin D1 protein at 12 h. In an examination of the subcellular distribution of cyclin D1, it was observed that stimulation of MC with PDGF for 6 h caused translocation of cyclin D1 from the cytoplasm into the nucleus. Coincubation with PDGF and transforming growth factor-β1 completely inhibited this effect, without altering the cellular cyclin D1 protein abundance at that time point. To test whether reduction of cyclin D1 protein levels was sufficient to inhibit mitogenesis, MC were transfected with antisense oligonucleotides (ODN) complementary to rat cyclin D1 mRNA. Antisense ODN against cyclin D1 reduced the PDGF-induced increase in p21Waf-1 protein levels. The MC proliferation caused by serum or PDGF was markedly inhibited by antisense ODN against cyclin D1, as measured by [3H]thymidine uptake and cell counts. It is concluded that increased cyclin D1 protein expression of MC is required for MC proliferation. Targeting cyclin D1 expression may represent an effective means to inhibit MC proliferation in vitro and in vivo.

Increased proliferation of glomerular mesangial cells (MC), leading to mesangial hyperplasia, is a hallmark of many forms of glomerulonephritis (1). Although MC replication may reflect an early repair mechanism in the inflammatory response to glomerular injury, persistent MC hyperplasia is pathogenetically linked to progressive and irreversible glomerular scarring. Recently, several studies of experimental glomerular disease demonstrated that strategies to ameliorate MC mitogenesis could indeed preserve glomerular function by preventing glomerulosclerosis (2–4). Numerous factors that stimulate or inhibit MC proliferation, including various cytokines, autacoids, and hormones, have been identified. Many of these factors possess autocrine growth-modulating activities; one example is platelet-derived growth factor (PDGF), which is a potent MC mitogen in culture (5) and in vivo (6). PDGF and its receptor β-subunit are induced during mesangioproliferative glomerular changes, supporting the role of PDGF as an autocrine growth factor in glomerulonephritis (7). Moreover, blockade of PDGF activity, by infusion of neutralizing antibodies against PDGF, in anti-Thy1.1-treated nephritic rats prevents MC hyperplasia and improves renal function (2). Among the autocrine inhibitors of MC proliferation, transforming growth factor-β (TGF-β) has been demonstrated to play a major role in vitro and in vivo (8,9). Although TGF-β has profibrogenic effects in inflammatory glomerular diseases, it might also exert beneficial effects through its growth-inhibiting properties. Several studies have confirmed the antimitogenic potential of the TGF-β1 isoform (10,11). Moreover, Kitamura et al. (12) demonstrated that MC overexpression of active TGF-β1 in anti-Thy1.1-induced nephritis inhibits glomerular cell proliferation.

Cell cycle progression is controlled by cyclin-dependent kinases (cdk), whose catalytic activity is modulated by association with different cyclins, functioning as regulatory subunits. In mammalian cells, several classes of cyclins have been identified (13) and can associate with different cdk catalytic subunits (14). Three different D-type cyclins (D1, D2, and D3) and cyclin E are involved in controlling G1 phase progression and entry into S phase. D-type cyclins are growth factor-regulated genes. Depending on the cell type, D-type cyclins associate with either cdk4 or cdk6 to form active kinase complexes (15). Cyclin D activates cdk4 during G1 phase (16), which leads to phosphorylation of the retinoblastoma tumor suppressor protein (pRb) (17). This phosphorylation results in the inactivation of pRb and plays an essential role in the progression of cells from G1 to S, mainly through regulation of the E2F family of transcription factors (18). Because cdk targets such as pRb are localized to the nucleus, active cdk complexes must have access to the nuclear compartment. It has been observed that the activation of cyclin D1-cdk4 complexes that occurs in response to extracellular signals involves trans-
location of the complexes into the nucleus (19). Because no sequence identifiable as a basic nuclear localization sequence (NLS) is present in the primary sequence of vertebrate cdk or cyclins, it has been suggested that cyclin-cdk complexes bind substrates or regulators that contain recognizable NLS (20). Indeed, the cyclin kinase inhibitor (CKI) p21Waf-1, which contains diverse NLS motifs, was recently demonstrated to be important for the translocation of cdk4 and cyclin D1 to the nucleus (21).

Both serum and PDGF are known to induce cyclin D1 mRNA and protein expression in fibroblasts (22,23), and overexpression of the gene for cyclin D1 has been observed in some tumors (24). Terada et al. (25) recently reported that cyclin D1 is the predominant cyclin D isofrom in cultured MC. Protein expression of cyclin D1 in rat MC can be induced by mitogens such as endothelin-1 (25) and PDGF (26). The two other cyclin D isoforms, D2 and D3, are expressed in low abundance in cultured rat MC (25).

CKI, such as p21Waf-1, p27Kip-1, and members of the INK4 family of cdk inhibitors (p15, p16, p18, and p19), negatively regulate the cell cycle by inhibiting the formation or activation of cyclin-cdk complexes (27). The cdk inhibitor p21Waf-1, a 21-kD product of the WAF1/CIP1/MDA6 gene, can be induced by p53-dependent or -independent pathways (28). p21Waf-1 was originally identified as an inhibitor of cyclin-cdk complexes. However, it was also observed that p21Waf-1-containing cyclin-cdk complexes retained kinase activity, which was abolished by the addition of more p21Waf-1 molecules, indicating that p21Waf-1 can exert different functional activities (29). LaBaer et al. (30) reported that in vivo, endogenous cyclin D1 and p21Waf-1 bind concomitantly to cdk4 after release of the cells from G0 arrest. This complex formation occurs in parallel with an increase in cyclin D1-associated kinase activity. LaBaer et al. (30) also demonstrated that at lower concentrations, p21Waf-1 promotes the assembly of cdk4 with D-type cyclins, whereas at higher concentrations it inhibits cdk4 activity. Interestingly, a recent study indicated that the expression of p21Waf-1 mRNA is under the control of the transcription factor E2F (31). In glomeruli of normal rats, the CKI p27Kip-1 is highly expressed, whereas the levels of p21Waf-1 are low (32). The proliferation of MC in rat anti-Thy1.1-induced glomerulonephritis was associated with transient reduction of p27Kip-1 abundance, whereas the expression of p21Waf-1 was substantially increased. The glomerular abundance of p21Waf-1 remained elevated after the resolution of proliferation (32). However, the role of p21Waf-1 in the regulation of glomerular cell mitogenesis is presently unclear.

Gene therapy is emerging as a potential strategy for the treatment of proliferative disorders. It was demonstrated that a single intraluminal administration of antisense oligonucleotides (ODN) against cdk2 resulted in sustained inhibition of neointima formation after balloon angioplasty in a rat carotid injury model (33). In cultured MC, proliferation could be inhibited by using antisense ODN against the transcription factor early growth response gene 1 (34) or other cell cycle-associated proteins, such as proliferating cell nuclear antigen or Ki-67 (35). Recent findings by Terada et al. (25) suggested that transfection of antisense ODN against cyclin D1 reduced the proliferative responses of MC to endothelin-1, indicating that cyclin D1 plays a role in MC mitogenic signaling elicited by this vasopressor.

In this study, we investigated the expression of cyclin D1 protein during MC hyperplasia in rat glomerulonephritis and its involvement in cell cycle regulation of cultured rat MC. Specifically, we examined the effects of promitogenic and antimitogenic factors on the regulation of protein expression of cyclin D1 and cyclin D1-associated proteins and on the nuclear translocation of cyclin D1. In addition, we tested the hypothesis that antisense ODN against cyclin D1 inhibit MC proliferation induced by the potent MC mitogen PDGF.

Materials and Methods

Experimental Rat Anti-Thy1.1-Induced Glomerulonephritis

Inbred male Sprague Dawley rats (150 to 200 g) were obtained from Charles River Deutschland (Sulzfeld, Germany). The monoclonal antibody against Thy1.1 (ER4) was described by Bagchus et al. (36) and was kindly provided by Dr. E. de Heer (University of Leiden, The Netherlands). Nephritis was induced by a single intravenous injection of 1 mg/kg body wt anti-Thy1.1 antibody into the tail vein. Control animals received injections of isotonic saline solution. Kidneys were decapsulated and placed in methyl Carnoy’s solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) for fixation.

Immunohistochemistry

After overnight fixation in methyl Carnoy’s solution, tissues were dehydrated by bathing with increasing concentrations of methanol, followed by 100% isopropanol. After embedding in paraffin, 4-mm sections were cut with a Leitz SM 2000 R microtome (Leica Instruments, Nussloch, Germany). After deparaffinization, endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 20 min at room temperature. Sections were then layered with the primary antibody and incubated overnight at 4°C. After addition of the secondary antibody (biotin-conjugated goat anti-rabbit IgG, diluted 1:500), the sections were incubated with avidin-biotinylated horseradish peroxidase complex (Vectastain DAB kit; Vector Laboratories, Burlingame, CA) and exposed to 0.1% diaminobenzidine tetrahydrochloride and 0.02% H2O2 (as a source of peroxidase substrate). Each slide was counterstained with hematoxylin. As negative controls, equimolar concentrations of preimmune rabbit or goat IgG were used. Double immunostaining for cyclin D1 and Thy1.1 was detected using immunofluorescence microscopy. Primary antibodies were applied simultaneously, overnight at 4°C. After washing, sections were incubated with secondary antibodies (CY2-labeled goat anti-mouse IgG and CY3-labeled goat anti-rabbit IgG; both from Dianova, Hamburg, Germany) concurrently for 2 h. Washed sections were then covered with Tris-buffered mowiol, pH 8.6 (Hoechst, Frankfurt, Germany). Antibodies to cyclin D1 (diluted 1:50) and Thy1.1 (diluted 1:250) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and from Serotec (Biozol, Eching, Germany), respectively. Cyclin D1-positive nuclei were enumerated in glomerular profiles in renal sections from two rats for each time point. The total number of nuclei and the number of nuclei that were positive for cyclin D1 staining in each glomerular tuft were determined in a minimum of 30 glomeruli per animal.
**MC Isolation and Culture**

Glomeruli from rat kidneys were isolated, and glomerular outgrowth and subsequent subculturing of MC were performed as described previously (37). MC were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated (50°C for 30 min) fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 5 μg/ml insulin in a 95% air/5% CO₂ humidified atmosphere at 37°C. MC between passages 12 and 20 were used for experiments. Growth factors were used in the following concentrations: PDGF-BB (Sigma, Deisenhofen, Germany), 20 ng/ml; TGF-β1 (Life Technologies, Eggenstein, Germany), 5 ng/ml.

**Protein Extraction and Western Blot Analysis**

MC were grown to subconfluence in medium supplemented with 10% FCS and were growth-arrested for 72 h in medium containing 0.2% FCS. At the indicated time points, MC were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 10 μg/ml aprotinin, and 2 μg/ml leupeptin. MC lysates were incubated for 15 min on ice, and cellular debris was cleared by centrifugation (15,000 × g for 15 min at 4°C).

For detection of nuclear cyclin D1, MC nuclei were isolated. MC were washed twice with ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium vanadate and 1 mM sodium fluoride and were collected with a rubber policeman in 50 mM Tris, pH 7.5, 10 mM EDTA, pH 7.5, 1 mM sodium vanadate, 1 mM sodium fluoride. Cells were collected by centrifugation (1000 × g for 10 min at 4°C) and resuspended in 5 times the packed cell volume of ice-cold hypotonic buffer [10 mM Hepes, pH 7.5, 10 mM KCl, 0.1 mM EDTA, pH 7.5, 0.1 mM ethylene glycol bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA)], pH 7.5, 300 mM sucrose, 1 mM dithiothreitol (DTT), 1 mM sodium vanadate, 2.5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride]. After swelling on ice for 10 min, cells were disrupted by repeated aspiration through a 22-gauge needle. Cell disruption was confirmed by microscopic observation. Nuclei were separated from the cytosolic fraction by centrifugation (960 × g for 10 min at 4°C), washed once with hypotonic buffer, and solubilized in lysis buffer, as described above. Debris was cleared by centrifugation (15,000 × g for 15 min at 4°C). Equal amounts of either total MC protein or nuclear proteins (15 μg) were separated by SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose filters (Amersham Pharmacia, Freiburg, Germany); transfer was controlled by Ponceau-S staining. Membranes were blocked for 12 h at 4°C in PBS containing 0.06% Tween 20 and 5% nonfat dried milk. Proteins were observed using the ECL detection system (Amersham Pharmacia, Freiburg, Germany) after incubation for 2 h at room temperature with the indicated primary antibodies. Applied primary antibodies were directed against cyclin D1 (1:10,000), cdk4 (1:10,000), p21^{Waf1} (1:5000), p27^{kip1} (1:1000), and cdk2 (1:5000) (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA). The applied secondary antibody was peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Dianova).

**Immunocytochemistry**

For cyclin D1 staining, MC grown on 8-well glass chamber slides, with or without stimulation with soluble agonists, were fixed in 3% paraformaldehyde/PBS and permeabilized for 10 min at 4°C with 1% Triton X-100 in PBS. Nonspecific binding was blocked using 100% FCS. Primary antibody against cyclin D1 (diluted 1:50; Santa Cruz Biotechnology) was applied overnight at 4°C. As a negative control, equal concentrations of polyclonal rabbit IgG were used. After washing, slides were incubated for 2 h with CY3-labeled goat anti-rabbit IgG secondary antibody (Dianova). Washed slides were then covered with Tris-buffered mowiol, pH 8.6 (Hoechst).

**Transfection of Antisense ODN**

Phosphothioate-modified, 20-nucleotide, antisense, sense control, or mismatch control ODN were purchased from MWG Biotech (Ebersberg, Germany) and dissolved in 10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8. The ODN had the following sequences and positions, as derived from the rat cyclin D1 sequence (38): AS2 (positions 234 to 253), 5′-GTCTTAAGCATGGGCTCGAC-3′; S2, 5′-CTCGAGACCATGCTTAAGAC-3′; AS3 (positions 148 to 167), 5′-GAGCTGTTTCCATGCGCG-3′; MM3, 5′-GCCATGTTGGTTACCT-GCGGC-3′.

For assessment of transfection efficiency, fluorescein-labeled ODN AS2 was used (MWG Biotech). For transfection of ODN, two different methods were used (1). Polycationic transfection reagent (Superfect; Qiagen, Hilden, Germany) was used to facilitate uptake of ODN, according to the protocol recommended by the manufacturer. A mixture of ODN with polycationic transfection reagent was preincubated for 3 h before mitogenic stimulation of MC. At that time, ODN and polycationic transfection reagent were removed by washing with PBS, and Dulbecco’s modified Eagle’s medium with 2% FCS was added (2). When no polycationic transfection reagent was used, MC were preincubated with ODN for 16 h. After preincubation, medium was removed and MC were exposed to fresh medium containing ODN (at the same concentration as during the preincubation) and mitogenic factors as indicated. To achieve equal transfection efficiencies with the two methods, different concentrations of ODN had to be used. Tenfold higher concentrations of ODN were used for treatment without polycationic transfection reagent, compared to treatment with polycationic transfection reagent.

**Determination of [3H]Thymidine Uptake and Cell Numbers**

In 96-well plates, MC were subcultured until subconfluent in medium supplemented with 10% FCS and were growth-arrested for 48 to 72 h in medium containing 0.2% FCS. During serum deprivation, MC were exposed for 16 h to fresh medium without FCS, containing ODN in the indicated concentrations; cells were then stimulated with 2% FCS or PDGF (20 ng/ml). For determination of [3H]thymidine uptake, MC were pulsed with 1 μCi/ml [methyl-3H]thymidine (specific activity, 5 mCi/mmol; Amersham, Braunschweig, Germany) for 24 h. MC were then washed twice with PBS, trypsinized, and collected onto filter paper using an automated cell harvester (Bibby Dunn, Asbach, Germany). Incorporated counts were measured in a liquid scintillation counter (Beckmann, Munich, Germany). Cell numbers were determined 72 h after growth stimulation. Monolayers of MC were washed twice in PBS, and MC were trypsinized and transferred into 10 ml of Isoton solution (Coulter, Luton, United Kingdom) for counting in a cell counter (Coulter).

**Assay of Cdk4-Associated Kinase Activity**

MC were lysed in cdk4 immunoprecipitation buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM DTT, 0.1% Tween 20, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cellular debris was removed by centrifugation (16,000 × g for 10 min at 4°C). Five hundred
micrograms of total extracted protein was precleared with 10 μl of protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4°C. Cdk4 complexes were precipitated for 6 h at 4°C with 10 μl of protein A-Sepharose precoated with 10 μg of a mouse monoclonal antibody against cdk4 (Ab-1, clone DCS-35; Neomarkers, Fremont, CA). Precipitates were washed four times with immunoprecipitation buffer and twice with 50 mM Hepes, pH 7.5, containing 1 mM DTT. Next, the precipitates were resuspended in kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate) containing 1 μg/assay of the substrate protein glutathione S-transferase-pRb (Santa Cruz Biotechnology). ATP was added to a final concentration of 50 μM, in addition to 10 μCi of [γ-³²P]ATP (6000 Ci/mmol). The kinase assay was performed at 30°C for 30 min. The reaction was stopped by adding Laemmli sample buffer and boiling for 5 min. Finally, the samples were separated by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography.

Statistical Analyses
Statistical analyses were performed using t tests for unpaired values.

Results
MC Hyperplasia during Experimental Mesangioproliferative Glomerulonephritis Is Correlated with Increased Expression of Cyclin D1 Protein
In rat nephritis induced by injection of the ER4 anti-Thy1.1 antibody, maximal MC hyperplasia occurred on day 6 of the disease (data not shown). Immunohistochemical staining revealed an increase in cyclin D1-positive nuclei within the mesangium of nephritic rats on day 5 of the disease (Figure 1B), compared with healthy control animals (Figure 1A). Cy-
clin D1-positive nuclei in glomeruli from untreated control animals were predominantly podocytes, as identified by their localization outside the glomerular basement membrane. Glomerular cell numbers and percentages of cyclin D1-positive nuclei were determined. The percentage of cyclin D1-positive nuclei in glomeruli was increased from 15 ± 4.9% (control) to 26 ± 9.1% on day 2 and to 33 ± 5.5% on day 5 of the disease. Double staining with antibodies against the MC-specific surface marker Thy1.1 (green fluorescence) and cyclin D1 (red fluorescence) revealed colocalization of these proteins in MC nuclei, marked by yellow staining (Figure 1, C and D), on day 5. To further examine the relevance of these descriptive in vivo findings of enhanced cyclin D1 expression with MC hyperplasia, we began studies of MC growth control in cultured MC.

**TGF-β1 Reduces the PDGF-Mediated Increases in Cyclin D1 and p21Waf-1 Protein Abundance**

To examine the effects of the well characterized MC mitogen PDGF and the antimitogenic factor TGF-β1 on the regulation of cyclin D1 protein abundance, Western blot analyses were performed (Figure 2). Incubation of MC with PDGF (20 ng/ml) markedly upregulated the protein levels of cyclin D1 after 12 and 24 h. Coincubation of TGF-β1 (5 ng/ml) and PDGF reduced the PDGF-induced increases in cyclin D1 protein levels at 12 and 24 h, whereas TGF-β1 alone had no significant effect on cyclin D1 protein levels, compared with growth-arrested control MC. Next, we investigated whether PDGF and TGF-β1 affected the expression of the cyclin D1-associated proteins cdk4, p21Waf-1, and p27Kip-1 (Figure 2). The cdk inhibitor protein p21Waf-1 was markedly upregulated by stimulation with PDGF for 12 and 24 h, whereas TGF-β1 alone had no effect on p21Waf-1 protein levels. Coincubation with TGF-β1 slightly diminished the PDGF-induced increase in p21Waf-1 protein levels at 24 h. In contrast, protein levels of the CKI p27Kip-1 were reduced by PDGF at 12 and 24 h with or without coincubation with TGF-β1. Incubation with TGF-β1 alone for 12 or 24 h led to an increase in p27Kip-1 expression. Protein levels of cdk4 were not notably affected by PDGF or TGF-β1 at the time points examined (Figure 2).

**TGF-β1 Blocks the Nuclear Translocation of Cyclin D1 Induced by PDGF**

In addition, we investigated how PDGF and TGF-β1 regulate the subcellular localization of cyclin D1. Immunocytochemical analyses revealed that stimulation of growth-arrested MC with PDGF led to nuclear accumulation of cyclin D1 after 6 h. This phenomenon was markedly diminished by coincubation with TGF-β1 and PDGF for 6 h (Figure 3). The negative control, with irrelevant polyclonal rabbit IgG, revealed no detectable staining pattern (data not shown). These results were confirmed when the cyclin D1 protein abundance in nuclear extracts was analyzed by Western blotting (Figure 4, bottom row). Whereas TGF-β1 inhibited PDGF-induced accumulation of cyclin D1 protein in nuclear extracts, no notable alteration of total cellular cyclin D1 protein levels by TGF-β1 was observed at this time point in PDGF-stimulated MC (Figure 4, top row).
depicts ODN-induced changes in MC growth, as indicated by [3H]thymidine uptake and MC counts 24 and 72 h after stimulation with PDGF, respectively. Transfection with 0.1 mM levels of the antisense ODN AS2 reduced the PDGF-stimulated increase in cell counts to 35% (P, 0.01). The increase in cell number for MC treated with 0.1 mM levels of the corresponding sense control ODN S2 was 80% of the PDGF-stimulated control MC value. In addition, we examined whether reduction of cyclin D1 protein levels by transfection with antisense ODN against cyclin D1 led to induction of apoptosis. MC nuclei were observed by staining with Hoechst dye, and apoptotic MC were determined on the basis of nuclear morphologic features 24 h after transfection with 10 mM ODN. On average, 3% of the nuclei of untransfected control MC demonstrated an apoptotic phenotype. This percentage of apoptotic MC nuclei was not notably altered in MC transfected with either antisense ODN or sense control ODN (data not shown).

Transfection of Antisense ODN AS2 Against Cyclin D1 Inhibits Cyclin D1 Protein Expression Induced by FCS or PDGF

To confirm that the antimitogenic effects of antisense ODN against cyclin D1 are indeed attributable to reduced cyclin D1 protein levels, Western blot analyses of ODN-transfected MC were performed. The antisense ODN AS2 markedly decreased cyclin D1 protein levels induced by 2% FCS (Figure 8). Densitometric analyses revealed reductions of cyclin D1 protein levels to 42 and 27% by 0.05 and 0.1 mM concentrations of antisense ODN AS2, respectively. Application of 0.1 mM sense control ODN S2 reduced cyclin D1 protein levels to 68% of control levels. To assess nonspecific effects of ODN on overall MC protein synthesis, we also performed Western blot analyses for the G1 phase protein cdk2. Neither antisense nor sense ODN altered cdk2 protein expression levels in these experiments (Figure 8). PDGF-induced cyclin D1 protein ex-peptides ODN-induced changes in MC growth, as indicated by [3H]thymidine uptake and MC counts 24 and 72 h after stimulation with PDGF, respectively. Transfection with 0.1 mM levels of the antisense ODN AS2 reduced the PDGF-stimulated increase in cell counts to 35% (P, 0.01). The increase in cell number for MC treated with 0.1 mM levels of the corresponding sense control ODN S2 was 80% of the PDGF-stimulated control MC value. In addition, we examined whether reduction of cyclin D1 protein levels by transfection with antisense ODN against cyclin D1 led to induction of apoptosis. MC nuclei were observed by staining with Hoechst dye, and apoptotic MC were determined on the basis of nuclear morphologic features 24 h after transfection with 10 mM ODN. On average, 3% of the nuclei of untransfected control MC demonstrated an apoptotic phenotype. This percentage of apoptotic MC nuclei was not notably altered in MC transfected with either antisense ODN or sense control ODN (data not shown).

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pression was reduced by the antisense ODN AS2 (Figure 9). Densitometric analyses revealed reductions of cyclin D1 protein levels to 45 and 10% by 0.1 and 1 μM antisense ODN AS2, respectively. Control ODN S2 had no significant effect on cyclin D1 protein levels (Figure 9).

**Antisense ODN Against Cyclin D1 Inhibit Cdk4-Associated Kinase Activity**

Mitogenic stimulation of serum-starved MC with 2% FCS led to a maximal 1.9-fold increase in cdk4-associated kinase activity after 4 h, as assessed by pRb-kinase assays (Figure 10) and densitometric analyses. Transfection with 0.1 μM antisense ODN AS2 prevented the FCS-induced increase in cdk4-associated kinase activity, resulting in a relative activity that was only 37% of the sense ODN control value.

**Antisense ODN Against Cyclin D1 Inhibit p21Waf-1 Protein Expression Induced by PDGF**

Finally, we investigated whether the expression of the E2F-dependent gene product p21Waf-1 (31) was affected by the reduction in cyclin D1 protein levels. As depicted in Figure 11, the abundance of p21Waf-1 protein was markedly decreased by MC transfection with antisense ODN against cyclin D1. Densitometric analyses revealed that 0.1 and 1 μM concentrations of antisense ODN AS2 diminished PDGF-stimulated p21Waf-1 protein levels to 29 and 19%, respectively. Similar results were obtained when MC were transfected with antisense ODN and stimulated with 2% FCS (data not shown). Sense control ODN had no significant effects on PDGF-related p21Waf-1 increases.

**Discussion**

In this study, we examined the expression of the G1 phase regulatory protein cyclin D1 during the development of MC hyperplasia in a rat model of glomerulonephritis and its involvement in the cell cycle regulation of cultured rat MC. A central feature of many types of glomerulonephritis is MC hyperplasia. The anti-Thy1.1 model of mesangial proliferative glomerulonephritis is characterized by intense MC proliferation and has been used to study the in vivo expression of cell cycle regulators in glomerular cells. Shankland et al. (32) demonstrated that the onset of MC replication in this model is associated with significant and transient increases in cyclin A and cdk2 protein levels. Using immunohistochemical analyses, we observed that increased nuclear abundance of cyclin D1 protein in MC precedes the peak of MC hypercellularity in anti-Thy1.1-induced glomerulonephritis, suggesting the involvement of cyclin D1 in the development of mesangio proliferative diseases. Similar to our results, Yamada (39) reported increases in cyclin D1 mRNA levels in the anti-Thy1.1 model, which peaked on day 5 of the disease, whereas cyclins D2 and D3 showed no significant changes. We also detected constitutive expression of cyclin D1 protein in glomerular podocytes in normal and nephritic glomeruli. This finding was unexpected, because it has been shown that podocytes lose the ability to divide after they have developed their differentiated phenotype (40). A recent study investigated developmental cyclin D1 expression in the glomerular podocyte lineage (41). In podocytes, cyclin D1 was expressed in the mesenchymal state and downregulated during the S-shaped and comma-shaped body stages. This expression was again markedly increased at the capillary loop and in maturing stages (41). Clearly, the biologic relevance of cyclin D1 expression in podocytes is presently unclear and requires further elucidation.

To further examine the relevance of the in vivo findings of enhanced cyclin D1 expression in MC hyperplasia, we studied the effects of soluble regulators of MC growth on cyclin D1 expression and cellular localization. TGF-β1 causes growth arrest in G1 phase in several cell types and is a potent inhibitor of MC proliferation. Previously, we showed that TGF-β1 almost completely prevented the PDGF-driven transition of MC from G0/G1 phase into S phase, when TGF-β1 was coincubated with PDGF within the first 6 h (11). TGF-β1-induced growth arrest attributable to reductions in cyclin D1 mRNA and protein levels was demonstrated in rat intestinal epithelial cells (42). However, in other cell types (e.g., mouse keratinocytes [(43)] or human breast adenocarcinoma cells [(10)]), TGF-β1 caused growth inhibition without altering cyclin D1 protein levels. In rat MC, entry into S phase occurred 14 h after PDGF stimulation. Previously, we noted that coinubation with TGF-β1 did not affect the PDGF-induced abundance of cyclin D1 protein up to 10 h after mitogenic stimulation (11). The data presented here demonstrate that PDGF caused marked increases in cyclin D1 protein expression even at later time points, i.e., 24 h after mitogenic stimulation (after completion
of G1 phase) (11). Moreover, PDGF markedly increased the translocation of cyclin D1 to the nucleus after 6 h, i.e., during the progression of MC through G1 phase (11). At late time points, TGF-β1 diminished PDGF-induced cyclin D1 protein expression, suggesting that downregulation of cyclin D1 protein abundance by TGF-β1 is a late mechanism of its growth-inhibiting action. In addition, TGF-β1 inhibited the PDGF-induced nuclear localization of cyclin D1 during G1 phase, which may reflect an early mechanism of the growth-inhibiting action of TGF-β1 in MC. Although the molecular mechanisms of TGF-β1-mediated effects on the subcellular localization of cyclin D1 are presently unknown, TGF-β1 could possibly inhibit the association of cyclin D1 with proteins that contain recognizable NLS or otherwise mediate nuclear importing. Alternatively, TGF-β1 could affect posttranslational modifications of cyclin D1 or associated proteins important for retention of cyclin D1 in the nucleus. In this context, TGF-β1 was recently found to facilitate increased nuclear exporting of cyclin D1.
Expression of p21Waf-1 was determined by Western blot analyses. Protein extracts were fractionated by SDS-polyacrylamide gel electrophoresis. Protein expression was evaluated and protein was extracted 24 h after the addition of PDGF. Co1, Co2, and Co3 MC were cotransfected with 0.1 or 1 μM antisense ODN against cyclin D1. Quiescent MC were preincubated with 0.1 or 1 μM ODN (sense control S2 or antisense AS2) for 16 h and growth-stimulated with PDGF (20 ng/ml). MC were collected and protein was extracted 24 h after the addition of PDGF. Co1, quiescent MC; Co2, MC stimulated with PDGF without transfection of ODN. Fifteen micrograms of protein lysates per lane was size-fractionated by SDS-polyacrylamide gel electrophoresis. Protein expression of p21Waf-1 was determined by Western blot analyses.

Figure 10. Reduction of cdk4-associated kinase activity by antisense ODN against cyclin D1. Growth-arrested MC were transfected for 3 h with 0.1 μM antisense ODN AS2 or 0.1 μM sense control ODN S2 in the presence of transfection reagent. Control MC were incubated with transfection reagent alone. Whole cellular protein lysates were obtained from quiescent MC (Co1) or from transfected or untransfected (Co2 [MC stimulated with 2% FCS without transfection of ODN] and Co3) MC after stimulation with 2% FCS for 4 h. Cdk4-associated kinase activity was assessed by retinoblastoma tumor suppressor protein (pRb) kinase assays. Antibodies used for immunoprecipitation were directed against cdk4; as a negative control, an isotype-matched irrelevant antibody was also used for immunoprecipitation (Co3). Phosphorylated pRb substrate protein was assessed by autoradiography (top row), and precipitated cdk4 protein was observed by Western blot analyses (bottom row).

Figure 11. Inhibition of PDGF-induced p21Waf-1 protein expression by antisense ODN against cyclin D1. Quiescent MC were preincubated with 0.1 or 1 μM ODN (sense control S2 or antisense AS2) for 16 h and growth-stimulated with PDGF (20 ng/ml). MC were collected and protein was extracted 24 h after the addition of PDGF. Co1, quiescent MC; Co2, MC stimulated with PDGF without transfection of ODN. Fifteen micrograms of protein lysates per lane was size-fractionated by SDS-polyacrylamide gel electrophoresis. Protein expression of p21Waf-1 was determined by Western blot analyses.

Smad7 (44). In renal epithelial normal rat kidney 49F cells, intracellular localization of cyclin D1–cdk4 complexes was demonstrated to be involved in the regulation of their activity (19). Calmodulin seems to be essential for the nuclear accumulation of cyclin D1 and cdk4 (19), as well as for the nuclear translocation of p21Waf-1 (45). Also, p21Waf-1 was demonstrated to be important for the translocation of cdk4 and cyclin D1 to the nucleus (21). At present, little is known regarding the effects of soluble growth factors on the nuclear translocation of cyclin-cdk complexes. It remains to be determined whether the inhibitory effect of TGF-β1 on the nuclear translocation of cyclin D1 is critical for the antimitogenic action of TGF-β1 in MC.

Because cyclin D1 is a key regulator of early G1 phase progression, we examined whether reduction of cyclin D1 protein abundance by transfection of antisense ODN is sufficient to inhibit cdk4 kinase activity and MC proliferation. Several studies successfully used transfection of antisense ODN to examine the roles of MC growth regulators and to block MC replication. In cultured MC, proliferation could be inhibited by using antisense ODN against the early growth response gene EGR1 (34) or other cell cycle-associated proteins, such as proliferating cell nuclear antigen or Ki-67 (35). The results presented here demonstrated that transfection of antisense ODN against cyclin D1 very effectively reduced MC proliferation. Antisense ODN led to reductions in cyclin D1 protein levels and profoundly inhibited both FCS- and PDGF-induced DNA synthesis and replication of rat MC. Furthermore, we demonstrated that transfection with antisense ODN against cyclin D1 reduced the FCS-induced increase in cdk4-associated kinase activity. Because the arrest of cell cycle progression in G1 phase can lead to the induction of apoptosis under certain conditions (46), we investigated whether the growth arrest mediated by antisense ODN against cyclin D1 might cause apoptosis in MC. We observed no increase in the percentages of apoptotic MC in antisense ODN-transfected MC, compared with sense ODN-transfected or untransfected MC. However, overexpression of cyclin D1 has been reported to lead to increases in apoptosis for fibroblasts and epithelial cells (47,48).

One of the cyclin D1/cdk4-associated proteins is p21Waf-1, which can act as an assembly factor or as an inhibitor of cdk–cyclin complexes (29,30). Shankland et al. (32) reported de novo expression of p21Waf-1 in anti-Thy-1.1-induced glomerulonephritis that coincided with the reduction in MC hyperplasia, suggesting that p21Waf-1 may be an important factor in the resolution of MC proliferation. In investigating how soluble growth regulators and antisense ODN against cyclin D1 affect p21Waf-1 expression in cultured MC, we observed that PDGF caused marked increases in p21Waf-1 protein levels in MC. Furthermore, we transfected MC with a reporter plasmid containing the chloramphenicol acetyltransferase gene under the control of the p21Waf-1 promoter. Our preliminary data indicated that p21Waf-1 gene expression in MC was transcriptionally induced by PDGF (data not shown). In contrast, protein levels of the CKI p27Kip1 were significantly suppressed by incubation with PDGF for 12 h. Induction of p21Waf-1 protein by PDGF was also observed in p53-deficient and normal mouse fibroblasts, supporting the interpretation of p53-independent upregulation of p21Waf-1 by PDGF (28). In other cell types, TGF-β1 was observed to enhance p21Waf-1 expression by p53-independent mechanisms (49,50). However, our experiments showed that in cultured rat MC, TGF-β1 led to a slight reduction in the p21Waf-1 expression induced by PDGF, suggesting that the patterns of TGF-β1-mediated effects on cell cycle regulatory proteins can differ considerably among vari-
ous cell types. The expression of p21\textsuperscript{Waf-1} was also reduced by transfection with antisense ODN against cyclin D1. Hiyama et al. (51) recently demonstrated that ectopic expression of cyclin D1 in human glioma and rodent fibroblast cell lines induced p21\textsuperscript{Waf-1} gene expression and that coexpression of the transcription factors E2F-1 and DP-1 could specifically transactivate the p21\textsuperscript{Waf-1} promoter. Furthermore, an active E2F binding site was identified within the p21 Waf-1 promotor (31). Therefore, downregulation of p21\textsuperscript{Waf-1} by antisense ODN against cyclin D1 might be attributable to an E2F-dependent mechanism. Conceivably, other cell cycle regulatory proteins that are involved in the activation of G\textsubscript{1} phase cdk (e.g., cyclin E/ckd2) might also promote p21\textsuperscript{Waf-1} gene expression by enhancing the release of E2F.

The results of in vitro studies with rat MC demonstrated that mitogenic PDGF strongly induced protein expression and nuclear translocation of cyclin D1 in cultured MC. Both effects were inhibited by antimitogenic TGF-\beta1. Reduction of cyclin D1 protein levels by transfection with antisense ODN against cyclin D1 resulted in marked inhibition of MC proliferation, without changes in the percentage of apoptotic cells. Furthermore, cdk4-associated kinase activity and expression of the E2F-dependent protein p21\textsuperscript{Waf-1} were negatively affected by antisense ODN against cyclin D1. Antisense ODN against cyclin D1 have also been shown to diminish the endothelin-1-induced increase in DNA synthesis in rat MC (25). Therefore, strategies aiming to reduce cyclin D1 expression, e.g., by transfer of antisense ODN or with specific cyclin antagonists, might represent effective methods to inhibit MC proliferation in vivo.

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