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Exogenous Attenuation of p21$^{Waf1/Cip1}$ Decreases Mesangial Cell Hypertrophy as a Result of Hyperglycemia and IGF-1

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Abstract. Renal mesangial cell hypertrophy is a characteristic of diabetic nephropathy as well as a response to renal stress or injury. Because hypertrophy is a result of increased protein content per cell without DNA replication, those proteins that control the cell cycle, such as the cyclin kinase inhibitor p21, represent fertile ground for studying the mechanism of this structural alteration. A key role for p21 in promoting mesangial cell (MC) hypertrophy has been established using p21 knockout mouse models. Furthermore, some of the biologic effects of IGF-1, including cell proliferation, have been shown to be positively influenced by p21. In an attempt to begin to translate these findings ultimately to the bedside, methods to attenuate p21 levels in wild-type kidney cells were examined. With the use of a phosphorothioated antisense oligodeoxynucleotide (ODN) to p21, which has previously been shown to decrease specifically and effectively p21 protein levels in a variety of cell types, it is shown that attenuation of p21 in MC leads to a dose-dependent reduction of hypertrophy in the milieu of hyperglycemic culture media. Furthermore, the hypertrophic effect of the IGF-1 on MC is also attenuated using the same antisense p21 ODN. There was no evidence of apoptosis or other toxicity in MC transfected with the concentrations of antisense p21 ODN used in these experiments. Because the use of antisense ODN in human disease is already established in other medical disciplines, the stage is now set for the use of antisense p21 ODN to attenuate renal cell hypertrophy *in vivo*, leading to a new strategy for treatment of diabetic nephropathy and other diseases characterized by MC hypertrophy.

Diabetes is the most prevalent single disease among the ESRD population, accounting for 30 to 40% of patients who receive renal replacement therapy. Rather than being a consequence of profuse glomerular cell proliferation, the increased kidney size seen in diabetic nephropathy is largely the result of glomerular cell hypertrophy (1). Other renal diseases, similar to those that are modeled by partial renal ablation, are also characterized by glomerular hypertrophy (2). Thus, mechanisms that control cell-cycle transit play a critical role in the pathogenesis of this disease and may in fact be targets for future therapies of diseases, such as diabetic nephropathy, characterized by glomerular cell hypertrophy.

Cell-cycle progression is a finely orchestrated sequence of events controlled by a number of highly conserved gene products, including the cyclins and their partners, the cyclin-dependent kinases (CDK) (3). The evolutionary significance of the importance of this system is highlighted by the appearance of prevalent terminal diseases, such as cancer and atherosclerosis, that occur with failure of this system. Cyclin/CDK activity is regulated at another level by the cyclin kinase "inhibitors" (CKI). Similar to the other members of the Cip/Kip family of CKI, p21 was originally described as a universal inhibitor of G1 CDK (4, 5). However, more recent work from a number of laboratories, including our own, has uncovered a variety of other functions of this pleiotropic protein, some that seem (at first glance) to be opposite to their originally described functions (reviewed in (6)). For example, under some conditions, p21 functions in an antiapoptotic (7, 8) as well as a pro-mitogenic (9–11) manner.

The relevance of p21 to diabetic renal disease lies in its ability to arrest cell-cycle transit while allowing protein synthesis to continue, resulting in cellular hypertrophy. Using p21 knockout (p21[−/−]) mice and tissues, several investigators have demonstrated an essential role of p21 in both the promotion of diabetic glomerular hypertrophy (12, 13) and the progression of chronic renal failure in an ablation model (14). However, the use of transgenic animals in the previously reported work on p21 and the kidney does not, of course, easily translate to the bedside. We have been studying the effects of p21 attenuation in vascular smooth muscle (VSM) and cancer cells using an antisense oligodeoxynucleotide (ODN) specific to p21. This 21-bp ODN was easily transfected into these cells and also had systemic effects on tumor allografts when injected subcutaneously into nude mice. Therefore, we asked whether reduction of p21 in mesangial cells (MC) using this approach may ultimately result in a salutary effect in those human renal diseases characterized by MC hypertrophy.

We now show that antisense attenuation of p21 protein

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levels in human MC results in decreased hypertrophy under conditions of hyperglycemia as well as IGF-1 stimulation, with no evidence of toxicity at the low concentrations used in this study, suggesting new avenues of research into methods of attenuating glomerular hypertrophy in diabetes and other common renal diseases.

Materials and Methods

Materials

Mouse monoclonal anti-recombinant full-length $p21^{Waf1/Cip1}$ antibody and HeLa cell nuclear extract were obtained from Upstate Biotechnology (Lake Placid, NY). Goat anti-mouse and anti-rabbit horseradish peroxidase–conjugated IgG was obtained from Bio-Rad (Richmond, CA). Rabbit anti-human polyclonal poly-ADP-ribose polymerase (PARP) antibody was purchased from Cell Signaling Technology (Beverly, MA). Hoechst 33258 and Sulfo-Rhodamine 101 were from Calbiochem (San Diego, CA). Lipofectamine 2000 was obtained from Invitrogen Life Technologies (Carlsbad, CA). [^{3}H]leucine, [^{3}H]thymidine, and ECL Western Blotting Detection Reagents were obtained from Amersham Biosciences (Buckinghamshire, UK). All other reagents, including mouse anti–α-actin monoclonal antibody, were from Sigma (St. Louis, MO).

Human MC Culture

Normal human MC line was obtained from Cambrex Bio Science (Walkersville, MD) and maintained according to the vendor's recommendations. The cells, used at passages 5 to 8 for the experiments, were subcultured and grown in Life Technologies RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) with normal glucose (NG; 5.5 mM) or high glucose (HG; 30 mM) supplemented with 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2-humidified incubator until approximately 75% confluence. The cells were growth-arrested, by placing them in serum-free NG or HG quiescent media for at least 12 h, and exposed to 5% FBS-containing media with NG or HG or to quiescent media with IGF-1 as indicated.

ODN Transfections

Human $p21^{Waf1/Cip1}$ antisense and randomly scrambled sequence control ODN were synthesized by Oligos Etc. (Wilsonville, OR). The human p21 antisense ODN sequence was 5'-ATC CCC AGC CGG TTC TGA CAT-3', and the randomly scrambled control ODN sequence was 5'-TGG ATC CGA CAT GTC AGA-3'. The transfection procedure was performed using Lipofectamine 2000 following the manufacturer's recommended steps. Briefly, the normal human mesangial cells were grown to approximately 85 to 90% confluence and washed with sterile PBS twice. The ODN (50 nM or 100 nM) were mixed with Lipofectamine 2000 at the ratio of ODN (in μg):Lipofectamine 2000 (in μl) 1:2.5 in Opti-MEM media and were added to the cells for 6 h at 37°C. Serum-free media (without ODN) with NG or HG were added overnight, the media were changed in the next morning, and the cells were stimulated with 5% FBS-containing media with NG or HG or quiescent media with IGF-1 as indicated.

Western Blot Analysis

After incubation under appropriate conditions and different times, human MC were washed with cold PBS and lysed in lysis buffer at 4°C. The cell lysates were centrifuged (13,000 $\times$ *g*, 4°C, 10 min), the supernatants were resolved by SDS-PAGE, and the proteins were transferred to nitrocellulose membrane (Osmonics, Minnetonka, MN). Nonspecific binding sites were blocked in TBS-T buffer (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature. The membrane was then incubated with $p21^{Waf1/Cip1}$ or PARP primary antibody in TBS-T containing 2.5% nonfat dry milk overnight at 4°C and further incubated with a goat anti-mouse or goat anti-rabbit horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature. Bound antibodies were detected using ECL Western Blotting Detection Reagents after extensive washing of the membrane. Densitometry was analyzed using NIH Image J 1.29x software.

[^{3}H]Leucine and [^{3}H]Thymidine Incorporation

Human MC were plated in 24-well plates and incubated in serum-free media with NG or HG for 24 h. The cells were incubated under appropriate conditions for another 24 h and pulsed with 1 μCi [^{3}H]leucine or [^{3}H]thymidine in the last 12 h. In the case of leucine incorporation, the cells were counted and data are shown as incorporation of leucine per 10^6 cells. After washing in ice-cold PBS, the cells were precipitated with 15% TCA, solubilized in 1 N NaOH, and neutralized by 1 N HCl. The solutions were counted in ScintiVerse scintillation cocktail (Fisher Scientific, Pittsburgh, PA).

Measurement of Cell Protein and Cell Number

Human MC were treated with NG or HG media plus ODN as indicated. After 72 h, the cells were washed with PBS and trypsinized for counting the cell number in a hemacytometer chamber and lysed in lysis buffer at 4°C. The total protein content was determined by Lowry method. The ratio of cell protein to cell number was expressed as micrograms per 10^3 cells.

Flow Cytometry

Human MC were fixed in 95% ethanol after different treatment and frozen at −20°C until studied. On the day of flow cytometry, human MC were centrifuged and resuspended in cold PBS containing 2 μg/ml Hoechst 33258 and incubated on ice in the dark for 2 h, with the last 1 h adding Sulfo-Rhodamine 101 (20 μg/ml). Thirty thousand cells from each treatment were measured and the fluorescence signal of Hoechst 33258, and Sulfo-Rhodamine 101 was measured in a flow cytometer (13).

Measurement of Toxicity

For evaluating lactate dehydrogenase (LDH) release, human MC were plated in 24-well plates and treated as indicated. After being incubated under appropriate conditions for 48 h, the conditioned media were collected and LDH activity release into the media was determined by NADH oxidation using a Sigma Tox-7 *in vitro* toxicology kit as described previously (15).

Statistical Analyses

The results are expressed as means ± SD. Statistical analyses were performed with *t* test, and statistical significance was determined as $P < 0.05$.

Results

Antisense p21 ODN Attenuates p21 Levels in Human MC

In a variety of cell types, p21 is increased early after a mitogenic stimulus, likely reflecting its assembly factor role for G1 cyclin/CDK pairs (16, 17). We asked whether a similar effect occurs in MC, which are, in fact, modified VSM cells.

Human MC were grown to confluence in media containing 5% serum, then were serum-starved for 24 h and subsequently placed in 5% serum-containing media in the presence of a glucose concentration of 30 mM (simulating the hyperglycemic diabetic milieu) for various times. Western blotting of cell lysate with p21 antibody was performed using α-actin as a loading control and HeLa cell nuclear extract as a mobility standard for p21. Similar to what we have previously shown in VSM cells (18), p21 was initially increased after a mitogenic stimulus, starting at 4 h and persisting until at least 72 h, reaching statistical significance at 8 h (Figure 1). The higher molecular weight band, which we have observed in other human tissues (19), likely represents nonspecific binding as its intensity is not altered with time and it migrated higher than the protein of HeLa cell nuclear extract.

We and others have previously shown that our antisense p21 ODN reduces p21 protein levels in human (19) and rat (11, 16) VSM, as well as human breast cancer (20) cells. To determine whether p21 is similarly attenuated in human MC in a hyperglycemic environment by this ODN, we transfected human MC with several concentrations of the human antisense p21 or scrambled sequence control ODN and immunoblotted equal quantities of cell lysate harvested at various times after serum stimulation in the presence of HG. At 8, 24, and 48 h after serum stimulation, MC that were transfected with antisense p21 ODN demonstrated a dose-dependent attenuation of p21 protein level with no decrement in α-actin levels (Figure 2). There was no difference in p21:actin ratio between cells with and without Lipofectamine 2000. Cells that were incubated with scrambled sequence control ODN showed a slight, nonsignificant decrease in p21 protein level as compared with control and Lipofectamine 2000 only cells. This nonspecific effect of ODN has been observed to varying degrees in VSM (11, 16, 21) and other cells (20); however, specificity of this antisense ODN to p21 in VSM cells is shown by its lack of effect on α-actin in all studies, as well as the lack of significant attenuation of other non-p21 proteins in work from our (16) and other (11, 21) laboratories.

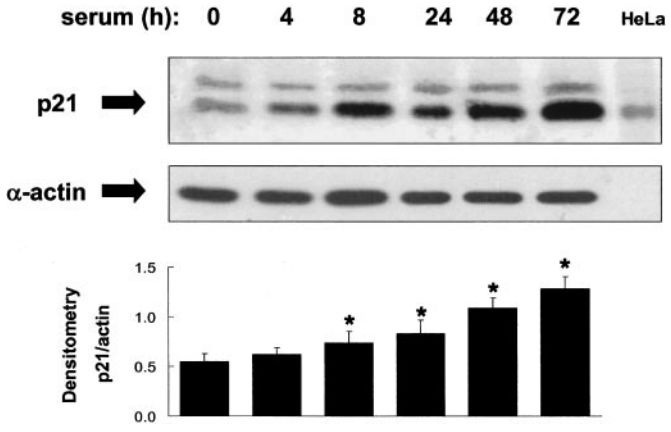

Figure 1. p21 protein in human mesangial cells (MC) is induced in high glucose (HG) media. Human MC were serum-starved for 24 h and then exposed to media containing 5% serum and HG (30 mM). At the times indicated after serum and glucose addition, the cells were lysed and equal protein quantities were immunoblotted with p21 or α-actin antibody. Densitometry of the bands was performed and displayed as p21/actin ratios. The experiment was performed three times and is presented as mean ± SD; $*P < 0.05$ compared with no serum.

Hyperglycemia Increases Protein Synthesis but Decreases DNA Synthesis in Human MC

Because of its established role as a G1 cyclin/CDK assembly protein, intact p21 is required for faithful cell-cycle progression in at least some cells; this phenomenon may explain the increase in p21 seen soon after mitogen stimulation (see Figure 1). Related to this finding, p21 has also been shown to be important in regulating whether an MC becomes hypertrophic or hyperplastic. To begin to investigate this phenomenon in the human MC used in this study, we examined the effects of normoglycemia and hyperglycemia on MC protein and DNA synthesis using radiolabeled leucine and thymidine, respectively. Compared with those incubated in normoglycemic medium, the human MC showed higher leucine incorporation but lower thymidine incorporation in hyperglycemic conditions (Figure 3). These data are consistent with the phenomenon of MC hypertrophy (22) in the hyperglycemic milieu, such as is seen in diabetic nephropathy.

Attenuation of p21 Inhibits MC Hypertrophy

Hypertrophy, which is the early and typical hallmark of diabetic nephropathy, is the result of a greater increase in cell protein than DNA and represents a phenomenon of increasing cell size. At a cellular level, this can be measured quantitatively by assessing the ratio of cell protein to cell number (22, 23), as hypertrophy is characterized by larger cells with a higher protein content than nonhypertrophic cells. In light of the increase in p21 seen in experimental diabetic nephropathy (13) and the lack of such hypertrophy seen in p21(−/−) animals (12), we next asked whether attenuation of p21 using antisense p21 ODN results in a decrease in MC hypertrophy; such a result would suggest future translational possibilities.

Human MC were transfected with either antisense p21 or random sequence control ODN at concentrations from 50 to 100 nM and stimulated with serum, and protein synthesis (as measured by [^{3}H]leucine incorporation per 10^6 cells; Figure 4A) or DNA synthesis (as measured by [^{3}H]thymidine incorporation; Figure 4B) were measured. The increased protein synthesis per cell of human MC seen in the HG media indicating hypertrophy (22) (see Figure 3) was attenuated (but not abolished) by antisense p21 ODN. DNA synthesis, although decreased in HG media, was also inhibited. The scrambled sequence control ODN did not show significant effects on either protein or DNA synthesis, although a slight decrement in both parameters was seen under these conditions as described above.

The ratio of protein to cell number is an accepted and widely used measure of cellular hypertrophy, as referenced above. Using this parameter, we found that the increased hypertrophy caused by placing the MC in HG media was partly but significantly reversed by antisense p21 ODN at 100 nM concentra-

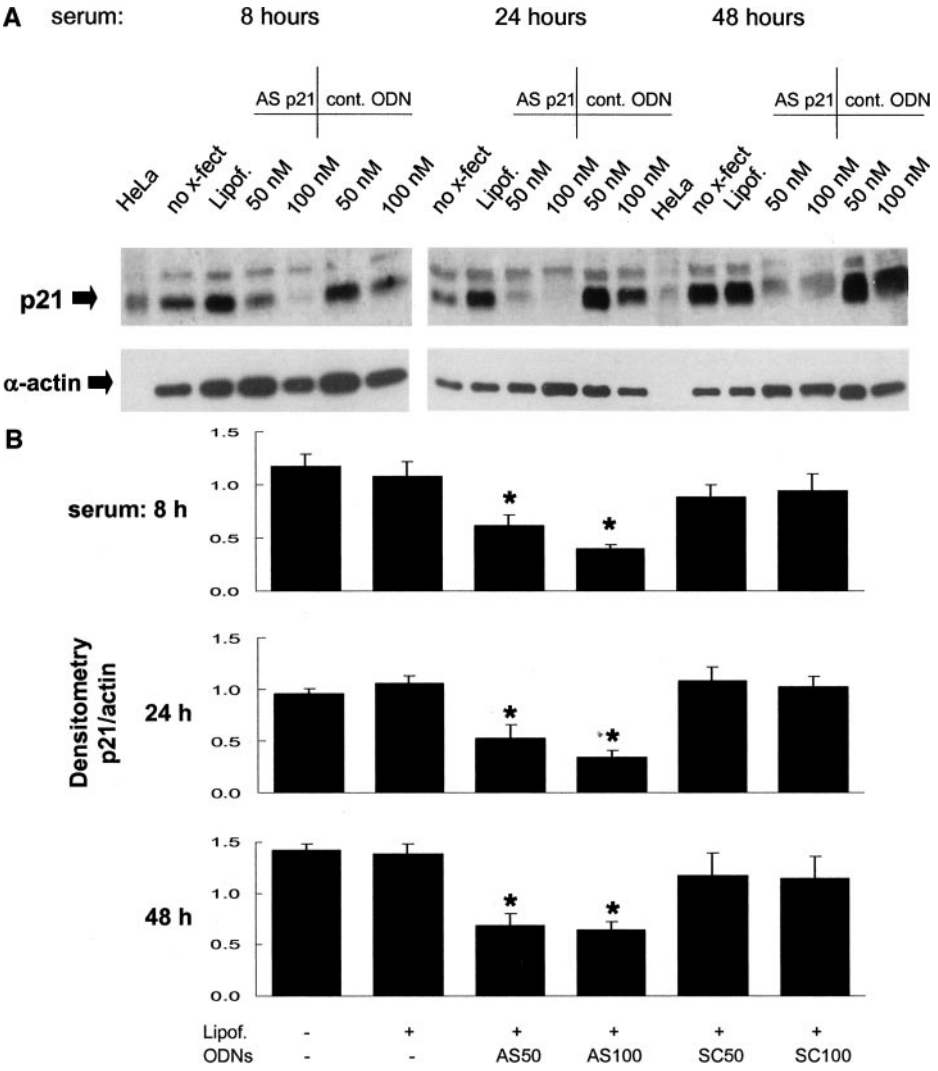

Figure 2. Antisense p21 ODN attenuates p21 levels in human MC in a hyperglycemic environment. Human MC were transfected with antisense p21 or scrambled sequence control oligodeoxynucleotides (ODN) as described in the Materials and Methods section, serum-starved, and then exposed for the times indicated to media containing 5% serum with HG (30 mM). (A) The cells were lysed and subjected to immunoblotting with p21 or α-actin antibodies. (B) Densitometry of the bands was performed and displayed as p21/actin ratios. The experiment was performed three times and is presented as mean $\pm$ SD; $^*P < 0.05$ compared with Lipofectamine 2000 only cells.

tion, as measured both by protein per cell and by FACS analysis (Figure 5); transfection of the human MC with this ODN concentration resulted in substantial decrease in p21 protein level at times from 8 to 24 h after transfection (see Figure 2). Thus, hypertrophy of human MC seen in the hyperglycemic milieu was dramatically attenuated *in vitro* using antisense p21 techniques.

IGF-1–Mediated MC Hypertrophic Response Is Attenuated by Antisense p21 ODN

IGF-1 plays important roles in a variety of cellular functions, including survival, proliferation, and terminal differentiation (reviewed in (24, 25)); recent data have implicated IGF-1 in the pathogenesis of both MC hypertrophy (26) and proliferation (27). In patients with type 1 diabetes, elevated levels of IGF-1 are seen in the urine and correlate with renal hypertrophy (28). Although the signaling pathways that regulate early responses of IGF-1 are well established, recent data have implicated p21 in the proliferative (10) and antiapoptotic (29) properties of IGF-1. In MCF-7 human breast cancer cells, a construct encoding antisense p21 decreased cell-cycle progression as a result of IGF-1, consistent with our work in VSM cells with PDGF-BB (16); and IGF-1–induced p21 expression inhibited ultraviolet-induced cell death (29), consistent with our work in human breast cancer cells (20).

In light of the critical effects of p21 with respect to IGF-1 function, we asked whether p21 mediates the hypertrophic effect of IGF-1 in human MC. In PDGF-BB–stimulated rat VSM cells, p21 is increased early (18), likely reflecting its assembly factor function (16). To confirm that a similar effect occurs after stimulation with the MC growth factor IGF-1, serum-starved MC were stimulated with IGF-1 in normal glu-

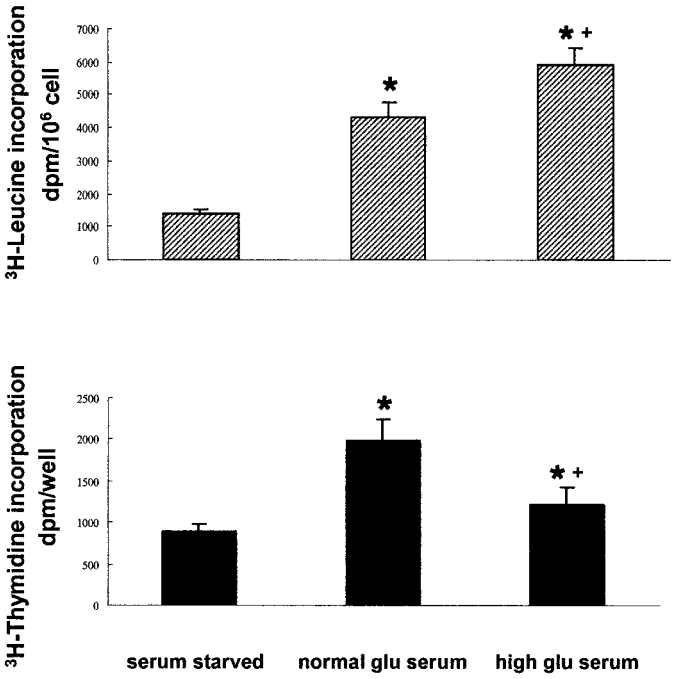

Figure 3. Hyperglycemia increases protein synthesis but decreases DNA synthesis in human MC in serum-containing HG media. Human MC were serum-starved for 24 h and subsequently stimulated with 5% serum containing either normal glucose (NG; 5.5 mM) or HG (30 mM) for 24 h. Leucine (dpm/10^6 cell) or thymidine (dpm/well) incorporation into cellular protein or DNA, respectively, was determined as described in the Materials and Methods section. $*P < 0.05$ compared with serum-starved; $+P < 0.05$ compared with NG serum. The experiment was performed four times and is presented as mean ± SD.

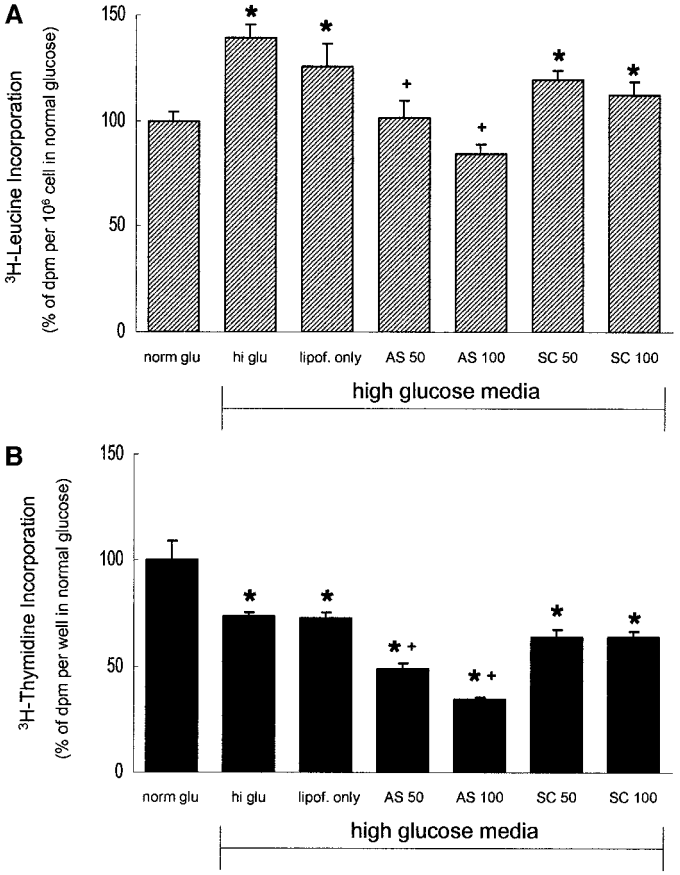

Figure 4. Antisense p21 ODN decreases protein synthesis greater than DNA synthesis in human MC. Human MC were transfected with antisense p21 (AS) or scrambled sequence control (SC) ODN, serum-starved, and subsequently stimulated with 5% serum containing either NG (5.5 mM) or HG (30 mM) as in Figure 3. [^{3}H]Leucine (A) or [^{3}H]thymidine (B) incorporation was assessed as described in the Materials and Methods section. This experiment was performed simultaneously with the experiment depicted in Figure 3; the data for NG and HG is recapitulated. $*P < 0.05$ compared with NG; $+P < 0.05$ compared with Lipofectamine 2000 only in HG media. The experiment was performed four times and is presented as mean ± SD.

cose media at concentrations of 10 and 100 nM for times ranging from 1 to 24 h, and the cell lysates were immunoblotted with p21 or α-actin antibodies. Both IGF-1 concentrations investigated increased p21 levels with a maximum at 4 h (Figure 6A). To begin to ascertain whether IGF-1 causes hypertrophy in MC through p21 induction, we first assessed whether our antisense p21 ODN has the ability to attenuate levels of this protein after IGF-1 stimulation. Human MC were transfected with antisense p21 or control ODN, serum-starved, and placed in normal (5.5 mM) glucose media with or without IGF-1 at 10 or 100 nM. At 8 or 24 h after IGF-1 stimulation, the cells were lysed and lysates were immunoblotted with p21 or α-actin antibodies. At both 8 and 24 h after IGF-1 incubation, antisense p21 ODN caused attenuation of IGF-1–induced p21 levels with no effect on α-actin levels (Figure 6, B and C).

To determine whether p21 is required for the hypertrophic effect of IGF-1 on MC, we measured radiolabeled leucine incorporation by these cells in response to IGF-1 in a normoglycemic environment, after transfection with either antisense p21 or random sequence control ODN. Because we showed above, using several experimental techniques, that changes in leucine incorporation per cell correlate with hypertrophy in this system (see Figures 4A and 5 and accompanying discussion), we used this measurement in the following experiments.

IGF-1 at 10 and 100 nM caused a dose-dependent increase in leucine incorporation per cell, consistent with the known hypertrophic effect of IGF-1 on MC (26) (Figure 7). After transfection with 50 or 100 nM of antisense p21 ODN at IGF-1 concentrations of 10 or 100 nM (conditions at which p21 was attenuated; see Figure 6), there was a significant decrease in leucine incorporation; no significant decrease was seen after transfection with control ODN (Figure 7). Furthermore, we previously showed that in these cells, [^{3}H]leucine incorporation per cell parallels protein per cell (compare Figure 4A with 5A), an expected finding because the amino acid leucine is found in the vast majority of cellular proteins. These data indicate that antisense p21 ODN attenuate the MC hypertrophy seen with two different concentrations of IGF-1. Thus, p21 is a mediator of MC hypertrophy in the hyperglycemic milieu and when stimulated by IGF-1; furthermore, antisense p21 ODN

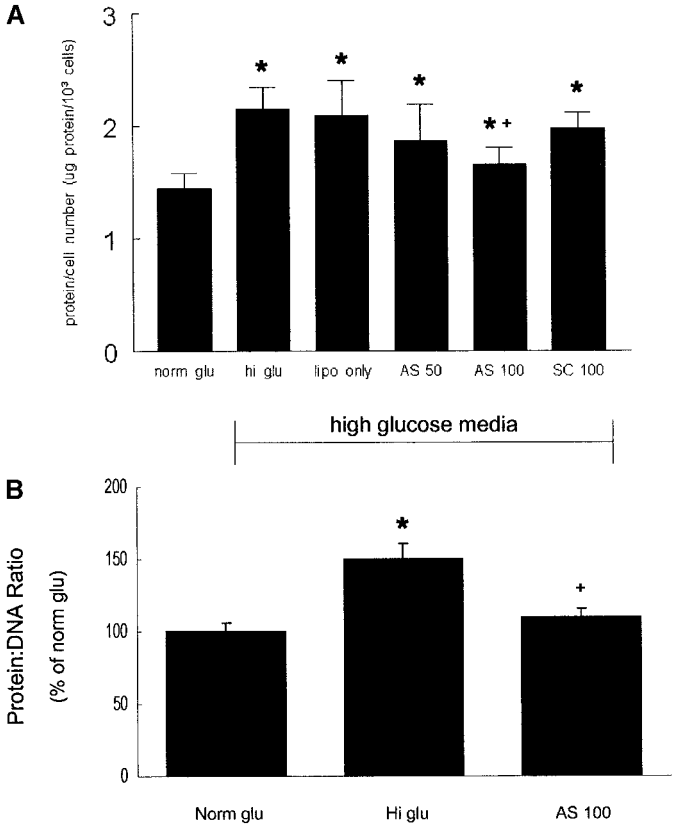

Figure 5. Antisense p21 ODN decreases the human MC hypertrophy in HG media. Human MC were transfected with AS or scrambled SC ODN, serum-starved, and subsequently stimulated with 5% serum containing either NG (5.5 mM) or HG (30 mM) as in Figures 3 and 4. (A) The measurement of cell protein and cell number was made after 72 h as described in the Materials and Methods section and is shown as a protein/cell number ratio. $*P < 0.05$ compared with NG; $+P < 0.05$ compared with Lipofectamine 2000 only in HG media. (b) FACS analysis of representative populations of cells in A were analyzed by FACS analysis and shown as ratio of Sulfo-Rhodamine 101 to Hoechst 33258. $*P < 0.05$ compared with NG; $+P < 0.05$ compared with HG. These experiments were performed three times and are presented as mean ± SD.

has the capability of attenuating MC hypertrophy under conditions of hyperglycemia or increased IGF-1 levels seen in the diabetic state.

No Evidence of Apoptosis or Other Toxicity at Low Concentration of Antisense p21 ODN Transfection

At higher concentrations, antisense p21 ODN can cause apoptosis by disruption of the normal cell cycle checkpoints (6, 20). Because the observed decrease in thymidine and leucine incorporation after antisense ODN transfection could be the result of a toxic or an apoptotic effect of the ODN, we performed several assays to rule out this possibility.

To evaluate apoptosis of the MC at the ODN concentrations used, we examined cleavage of PARP. This protein is cleaved by caspases and results in appearance of 89- and 24-kD fragments, the latter of which binds irreversibly to broken ends of DNA, which ensures irreversibility of apoptosis. MC were transfected with antisense p21 and random sequence ODN and immunoblotted with PARP antibody. Whereas marked PARP cleavage, as evidenced by appearance of the 89-kD cleavage product, was seen in the Jurkat whole-cell lysate control, there was no such cleavage seen under any of the experimental conditions (Figure 8, top).

LDH is contained in living cells such that the appearance of this enzyme in the media is an indication that cells have died and released this protein. LDH appearance in the conditioned media from the above experiment was assayed and found to be unchanged in cells with and without ODN transfection (Figure 8, bottom). Thus, ODN transfection at 50 to 100 nM into human MC results in no evidence of apoptosis or other toxicity.

Discussion

Glomerular cell hypertrophy is a consequence of the diabetic milieu and is an indicator of renal damage that ultimately leads to kidney failure. In addition, IGF-1, which is increased in the urine of patients with diabetes, has been shown to induce both glomerular cell proliferation (30) and hypertrophy (12, 13), consistent with its putative role in the pathogenesis of diabetic nephropathy. Because hypertrophy is characterized by increased protein synthesis in the absence of a corresponding increase in DNA replication, those proteins that control DNA synthesis and other aspects of cell cycle progression are critical in regulating this process.

The CKI of the Cip/Kip family lie downstream of the tumor suppressor p53 and, among their other functions, dictate whether cells whose DNA is damaged are repaired or targeted for apoptosis. p21 has pleiotropic functions in a variety of cells, including VSM cells, and has the capacity to inhibit as well as stimulate cell-cycle progression (reviewed in (3, 6)) and, critical to its function in the p53 pathway, has the ability to prevent apoptosis (20, 31). The antisense p21 ODN used in this study has been shown to inhibit assembly of cyclinD and cdk4 (16, 21) and to decreased Rb phosphorylation (11); the latter event lies downstream of cdk2 and cdk4 activation and thus can be used as a readout of both. At the low concentrations used in this study, although we saw assembly factor effects on DNA synthesis consistent with previous reports in VSM (16) and other (32, 33) cells, we saw no evidence of apoptosis at these concentrations (Figure 8), suggesting that the actions of the antisense p21 ODN are concentration dependent.

In VSM cells, p21 plays an assembly factor function (16) that is evidenced by its early increase in quantity after mitogen stimulation in VSM cells (18) as well as MC (34). In MC, which are modified smooth muscle cells, p21 has been shown to limit glomerular cell proliferation and hypertrophy, as can be seen in p21 knockout mouse studies (12, 30). This function of p21 in renal MC suggests that this CKI may be a suitable target for therapy in the several kidney diseases characterized by glomerular hypertrophy; however, before the present report, no available studies examined the feasibility of using exogenous means to attenuate this protein in human MC.

In this study, we show that an antisense ODN to p21, which

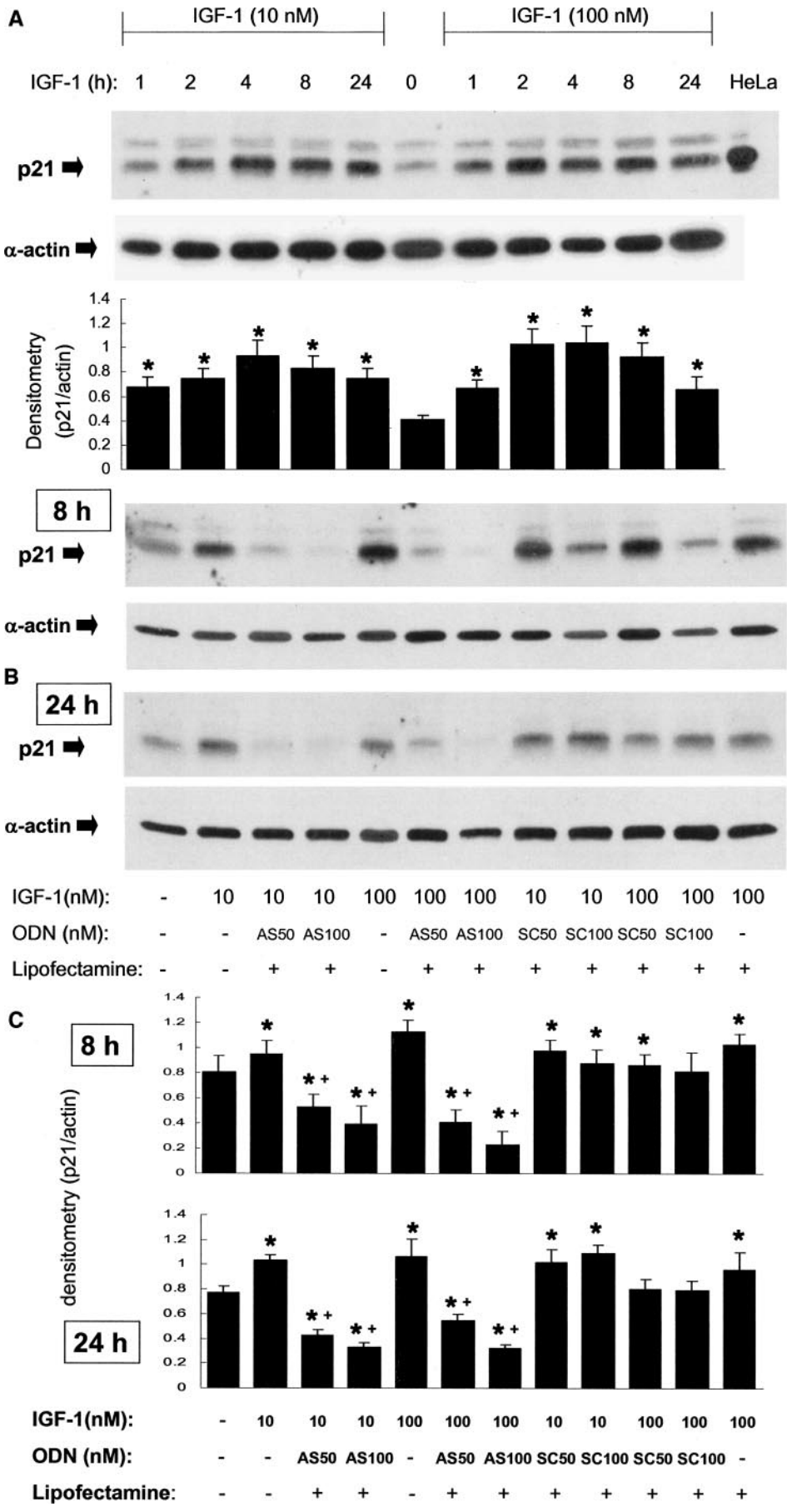

Figure 6. Antisense ODN attenuates IGF-1–mediated p21 induction in human MC in HG media. (A) Human MC were serum-starved for 24 h, then incubated in media with NG (5.5 mM) in the presence of IGF-1 for the times and at the concentrations indicated. The cells were lysed, and equal amounts of protein were electrophoresed and immunoblotted with p21 or α-actin antibodies. Densitometry was performed and expressed as p21/actin ratios. *$P < 0.05$ compared with IGF-1 (0 h). (B) Human MC were transfected with antisense (AS) or scrambled sequence control (SC) ODN at the concentrations indicated under conditions of NG (5.5 mM). After serum starvation, IGF-1 was added at the indicated concentrations and times. The cells were lysed, and equal amounts of protein were electrophoresed and immunoblotted with p21 or α-actin antibodies. (C) Densitometry of B was performed and expressed as p21/actin ratios. *$P < 0.05$ compared with control; +$P < 0.05$ compared with either IGF-1 10 nM or 100 nM with Lipofectamine 2000 only. This experiment was performed three times and is presented as mean ± SD.

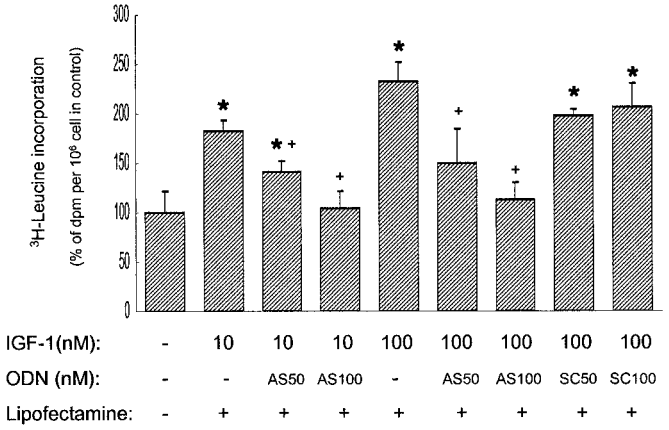

Figure 7. AS ODN attenuates IGF-1–mediated MC hypertrophy. Human MC were transfected with AS or scrambled SC ODN at the concentrations indicated under conditions of NG (5.5 mM). After serum starvation, IGF-1 was added at the concentrations indicated, and [^{3}H]leucine (dpm/10^6 cell) incorporation was assessed as described in the Materials and Methods section. $*P < 0.05$ compared with control; $+P < 0.05$ compared with either IGF-1 10 nM or 100 nM with Lipofectamine 2000 only. This experiment was performed three times and is presented as mean ± SD.

we have previously demonstrated to attenuate specifically and significantly p21 protein levels in several cell types, has the capacity to decrease specifically and effectively (although not completely) p21 levels in human MC in culture. We further show that this antisense p21 ODN decreases indicators of MC hypertrophy in response to HG media and after stimulation of the cells with IGF-1. These data are the first such demonstration of the use of antisense p21 in renal disease and suggests that exogenous means of attenuating p21 may ultimately prove useful in the therapy of glomerular hypertrophic diseases. The lack of complete inhibition of p21 by these antisense ODN may in fact be an advantage, because complete inhibition of p21 (as with p21[−/−] models) may result in disruption of other critical functions of this protein, such as its possible contribution to tumor suppression downstream of p53 (35).

Another mechanism that leads to a progressive loss of renal function relates to the ability of MC and other cells to secrete matrix proteins, such as collagen and fibronectin. In the case of renal disease, these proteins can take up residence extracellularly and lead to decreased glomerular filtration. Our finding that transfection of a homologous antisense p21 ODN into rat VSM cells causes decreased fibronectin and laminin secretion into conditioned media in these cells that are closely related to MC (36) suggests that a similar mechanism may be at play in MC; this property of the ODN would markedly increase its utility in renal disease and is currently under active investigation in our laboratory.

Some clinicians may harbor concerns about attenuation of such a key protein as p21, which lies in the all important tumor

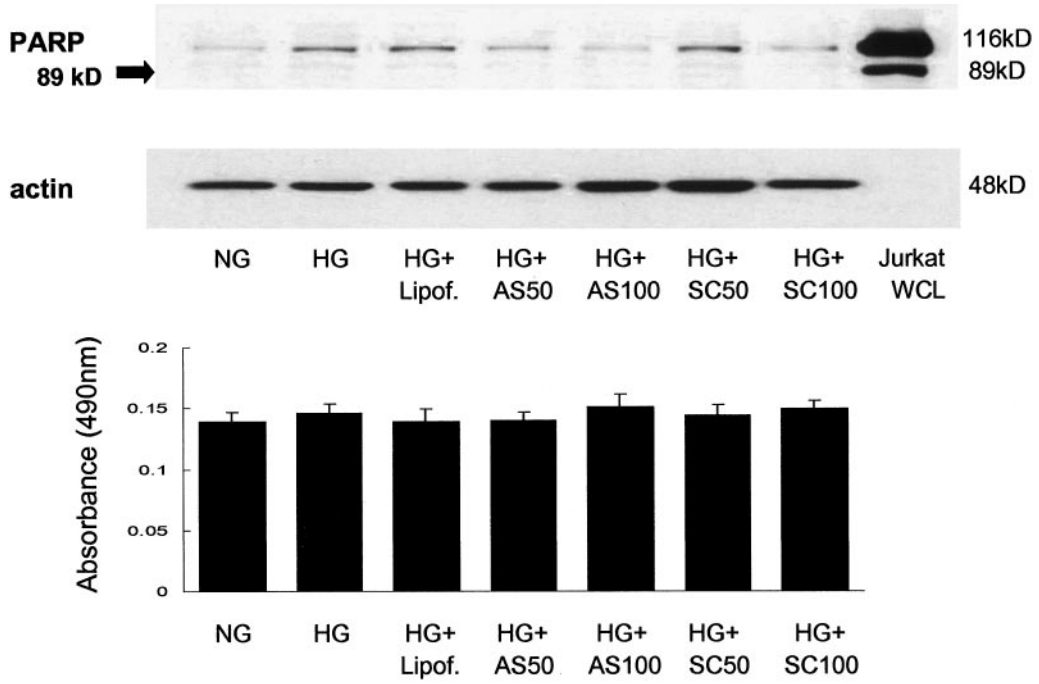

Figure 8. Lack of an apoptotic or toxic effect of AS ODN at the concentrations used. Human MC were transfected with AS or scrambled SC ODN at the concentrations indicated under conditions of NG (5.5 mM) or HG (30 mM). After serum starvation, the cells were incubated with serum for 48 h and the cell lysate was immunoblotted with PARP and α-actin antibodies (top). The poly-ADP-ribose polymerase (PARP) degradation product at 89 kD (representing apoptosis) can be seen in the positive control lane containing Jurkat whole-cell lysate but not in any of the experimental conditions. The conditioned medium from the above experiments was analyzed for lactate dehydrogenase (LDH) release as described in the Materials and Methods section (bottom). The LDH experiments were performed in triplicate and are presented as mean ± SD; the PARP experiment was performed twice with identical results.

suppressor pathway. These issues may be allayed by the following evidence. First, as discussed above, attenuation of p21 protein using our antisense ODN is only transient and not complete, thus repair mechanisms which are in response to ongoing or subsequent DNA damage will remain intact. Furthermore, although p21 lies in such a key location of the tumor suppressive pathway, there are clearly redundant mechanisms by which p53 is able to assist in DNA repair. This is evidenced by the fact that p21(−/−) mice do not have increased rates of malignancy when followed for 7 mo (37), although a subsequent study showed that these mice, when followed longer, showed an increase in spontaneous tumor development at an average age of 16 mo (38). In any case, the function of p21 in malignancy has not been fully established, although it is clear that absence of this protein is not identical to p53 absence, and p21 attenuation may actually be beneficial in malignancy (6). Finally, it is not at all unusual to find a pharmaceutical agent that inhibits a key signaling pathway yet has a specific (rather than globally toxic) effect on the patient; for example, the hepatic hydroxymethyl glutaryl CoA reductase inhibitors (the statins) decrease farnesylation of many signaling proteins including Ras yet lead to minimal patient toxicity.

Although elevated quantities of IGF-1 are seen in the urine of individuals with type 1 diabetes (28), whether increased intrarenal or circulating levels of IGF-1 are pathogenic for the detrimental changes of the diabetic state on the kidneys is an open question (39). However, it is abundantly clear that IGF-1 is important in tissue culture models of MC hypertrophy, which have been correlated with human disease. Recent data from several investigators have demonstrated a positive role of p21 in IGF-1–mediated breast cancer cell proliferation, such that abolition of p21 using antisense techniques resulted in decreased cell proliferation (10). This positive role of p21, consistent with our work in VSM (9) and breast cancer cells (20), suggested that p21 may be mediating the mesangial hypertrophic effect seen under diabetic (*i.e.*, hyperglycemic) conditions. In this report, we confirm that hypothesis, lending further ammunition to the possibility of using antisense p21 ODN as a therapeutic intervention.

Antisense ODN therapy is in fact a well-accepted and effective therapy in medicine; however, most of the existing pharmaceutical studies exploiting this technology are used by cancer researchers and oncologists. Although beyond the scope of this discussion, many published reviews address the specificity and efficacy of this therapeutic technique (40–42). The toxicity, stability, and tissue distribution of small ODN has been well studied; some of the early pharmacokinetic analyses of organ distribution in monkeys and rats showed very high concentrations (>5 μg/g tissue) and persistence (up to 3 d) after a single injection of a radiolabeled ODN (43) in several organs, including kidneys. Supporting its possible future translational utility, we have found that our antisense p21, when injected subcutaneously, results in decreased angiogenesis and attenuated tumor growth in a mouse allograft breast cancer model (44). Thus, the stage is now set for an animal study of our antisense p21 ODN in a rodent diabetic or other hypertrophic kidney disease model, which may lead to the development of new pharmaceutical agents based on this paradigm.

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