An oligonucleotide decoy for transcription factor E2F inhibits mesangial cell proliferation in vitro

NARUYA TOMITA,1 MASATSUGU HORIUCHI,1 SAWAKO TOMITA,1 GARY H. GIBBONS,1 JOHN Y. S. KIM,1 DANA BARAN,2 AND VICTOR J. DZAU1

1Department of Medicine, Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts 02115; and 2Division of Nephrology, McGill University, Montreal, Canada H3A 2B4

Tomita, Naruya, Masatsugu Horiuchi, Sawako Tomita, Gary H. Gibbons, John Y. S. Kim, Dana Baran, and Victor J. Dzau. An oligonucleotide decoy for transcription factor E2F inhibits mesangial cell proliferation in vitro. Am. J. Physiol. 275 (Renal Physiol. 44): F278–F284, 1998.—The transcription factor E2F controls expression of several genes involved in cell proliferation including c-myc, c-myc, proliferating cell nuclear antigen (PCNA), and cdk2 kinase. Having established that both PCNA and cdk2 kinase are induced in rat mesangial cells (MC) by serum stimulation, we attempted to inhibit MC proliferation in vitro by transfecting these cells with cationic liposomes containing a synthetic double-stranded oligodeoxynucleotide (ODN) with high affinity for E2F. Using a gel mobility shift assay, we detected increased specific binding of E2F in MC following serum stimulation. This binding was completely inhibited by preincubation of MC nuclear extracts with the double-stranded ODN with high affinity for E2F but not by preincubation with a missense ODN containing two point mutations. MC were also transfected with a luciferase reporter gene construct containing three E2F binding sites. Luciferase activity was enhanced by serum stimulation of MC, and this effect was specifically abolished by cotransfection of MC with E2F decoy ODN. Furthermore, RT-PCR analysis revealed that serum-induced upregulation of PCNA and cdk2 kinase gene expression was inhibited by E2F decoy ODN transfection but not by transfection of missense ODN. These changes in gene expression were paralleled by a reduction in PCNA and cdk2 kinase protein expression in E2F decoy ODN transfected cells. MC number increased following serum stimulation. This effect was blunted by transfection with E2F decoy ODN but not by transfection of missense ODN. These data suggest that the transcription factor E2F plays a crucial role in the regulation of MC proliferation and that this factor can be successfully targeted to inhibit MC cell cycle progression.

MATERIALS AND METHODS

Design of synthetic ODN. The E2F decoy ODN is a double-stranded phosphorothioate 14-mer that exhibits a high sequence-specific binding affinity to the transcription factor E2F (15). A control missense ODN was also synthesized containing a two-base substitution that has been shown to abolish E2F binding in VSMC (23). The E2F consensus sequence is shown in boldface, and two mutations are shown in italics, as follows: E2F decoy ODN, 5′ CTAGATTTCCC-GCG 3′ and 3′ TAAAGGGCGCTAG 5′; missense ODN, 5′ CTAGATTTCCGAGCG 3′ and 3′ TAAAGCAGGCCTAG 5′.
Synthetic ODNs were dissolved in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) purified over a NAP-10 column (Pharmacia Biotech, Uppsala, Sweden) and quantitated by spectrophotometry. Each pair of single-stranded ODN was annealed for 2 h, during which the temperature was reduced from 80 to 25°C (35).

Cell culture. MC were cultured from renal cortical fragments of 4- to 6-wk-old Sprague-Dawley rats according to published methods and were characterized as described previously (13). MC were used between passages 9 and 15 and were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 15% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a 5% CO2 incubator.

Transfection of ODN into cells. Cationic liposomes (Life Technologies) were used to transfect double-stranded decoy ODN into MC. Liposome-ODN complexes were formed by adding 10 µg of lipid in 300 µl of OPTI-MEM (Life Technologies) to 3.3 µg of ODN in 300 µl of OPTI-MEM. The solution was mixed gently and incubated at room temperature for 45 min to allow liposomes to form. Ten milligrams of lipid in a volume suitable for transfection were used per 25-cm2 flask for a final concentration of 2 µM. We confirmed that this quantity of DNA and cationic liposomes had no toxic effect on cell viability. MC were grown either in 25-cm2 flasks or in 24-well plates. Cells were rendered quiescent by placing them for 48 h prior to transfection in a defined serum-free (DSF) medium as previously reported (19, 26). Just before transfection, cells were washed with DSF medium and then incubated for 6 h at 37°C with freshly prepared liposome-ODN complexes. The medium was then changed to fresh medium supplemented with 15% FBS or DSF medium.

Nuclear protein extraction. Nuclear extract was prepared from glomerular MC as previously described for VSMC (23). In brief, cultured cells were first washed with cold phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4) and then homogenized with a Potter-Elvehjem homogenizer in 4 vol of ice-cold homogenization buffer [10 mM HEPES, pH 7.5, 0.5 M sucrose, 0.5 mM spermidine, 0.15 mM spermin, 5 mM EDTA, 0.25 mM EGTA, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After centrifugation at 12,000 g for 30 min at 4°C, the pellet was lysed in a Douce homogenizer in 1 vol of ice-cold homogenization buffer containing 0.1% Nonidet P-40 (NP-40), and centrifuged at 12,000 g for 30 min at 4°C. The nuclear pellet was washed twice with ice-cold homogenization buffer containing 0.35 M sucrose. After washing, nuclei were preextracted with 1 vol of ice-cold homogenization buffer containing 0.05 M NaCl and 10% glycerol for 15 min at 4°C. The nuclei were then extracted in the same buffer containing 0.3 M NaCl and 10% glycerol for 1 h at 4°C. The concentration of DNA was adjusted to 1 mg/ml. After pelleting the extracted nuclei at 12,000 g for 30 min at 4°C, the supernatant fraction was brought to 45% (NH4)2SO4 and stirred for 30 min at 4°C. The precipitated proteins were collected at 17,000 g for 30 min, then resuspended in homogenization buffer containing 0.35 M sucrose.

Gel mobility shift assay. Double-stranded 2′F ODN probe was 32P-labeled with a 3′-end labeling kit (Clontech, Palo Alto, CA) as described previously (23). After end labeling, 32P-labeled ODN probe was purified by Nick column (Pharmacia Biotech). Ten microliters of a mixture of 32P-labeled ODN probe (0.5–1 ng, 20,000 cpm) and 1 µg of polydeoxynosinic-polydeoxycylic acid (Sigma Chemical, St. Louis, MO) were incubated with 10 µg of nuclear extracts from MC for 30 min at room temperature prior to loading onto a 5% polyacrylamide gel. The gels were subjected to electrophoresis, dried, and the labeled DNA was visualized by autoradiography (16, 17). For the competition assay, unlabeled competitor double-stranded ODN for 2′F or missense ODN for 2′F or missense was preincubated with parallel samples 10 min prior to the addition of the labeled probe.

Cotransfection of luciferase plasmid and 2′F decoy ODN. Using the cationic liposome method, we cotransfected an E2F reporter plasmid and decoy ODN into MC. An E2F-luciferase reporter plasmid driven by three tandemly repeated E2F binding sites was kindly donated by Dr. Wilhelm Krek (Dana Farber Cancer Institute, Boston, MA) (22). Preparation of liposomes was performed as described above. In this case, 2 µg of plasmid was incubated with 10 mg of lipids. Cells were collected 48 h after transfection, and cell extracts were prepared using the reporter lysis buffer in the Luciferase Assay System (Promega, Madison, WI). The protein content was determined using BSA as a standard. The luciferase values were measured by the Luciferase Assay System (Promega). The luciferase values were normalized by the protein content.

RT-PCR. RNA was extracted from glomerular MC treated with E2F or missense ODN using RNAzol (Tel-Test, Friendswood, TX) on day 2 posttransfection. Levels of PCNA, cdk2 kinase, and β-actin mRNA were measured by RT-PCR as previously described from glomerular MC as previously described for VSMC (23). The PCNA 5′ primer was 5′-ACTCTGGCTCTCGAGGG 3′; the PCNA 3′ primer was 5′-TCTCATTAGCCTAGG 3′ (32). The cdk2 kinase 5′ primer was 5′-CGGTTCCATGGAACCTTC 3′; the cdk2 kinase 3′ primer was 5′-ATGCGCAAAGCTAGGGC 3′ (27). The β-actin 5′ primer was 5′-TTGTAACCAACTGGGACGATAGG 3′; the β-actin 3′ primer was 5′-GATCTTGATCTCTGAGGTGCT 3′ (32). The luciferase values were normalized by the protein content.
(Amersham). The antibody reaction was detected using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham). Quantitative analysis of Western blot was performed by densitometry with a CS-9300 PC image analyzer (Shimazu, Kyoto, Japan). Sample signals (numerical values) were given as arbitrary units, and the ratio of the densitometric values compared with the values in nontransfected cells was calculated for each to enable statistical comparison. For densitometric analysis, we first determined the linear range of exposing time and then exposed the immunoblotting film within this time range.

Cell number measurement. After transfection, MC were seeded in 24-well tissue culture plates. After the cells became adherent, they were rendered quiescent by incubating them for 24 h in DSF medium. The medium was then switched to fresh RPMI supplemented with 10% or 15% FBS. After 3 days, cell proliferation was measured using the colorimetric WST assay (Wako, Osaka, Japan) (18).

Statistical analysis. The results are expressed as means ± SE. Statistical analysis was performed by ANOVA followed by
RESULTS

We first verified that double-stranded ODN tagged with FITC at their 3' end could be introduced efficiently into MC nuclei using the cationic liposome gene transfer method. Six, 12, and 24 h after transfection, MC were fixed with methanol and observed by fluorescence microscopy. In preliminary studies, we established that a DNA-liposome ratio of 1:3 resulted in optimal uptake and nuclear localization of ODN. As shown in Fig. 1, the FITC signal was observed both in cytoplasm and nuclei at each time point. Twenty-four hours after transfection using cationic liposomes, we detected FITC-labeled ODN in the nuclei of approximately 60% of the cells. No FITC signal was seen in untransfected cells. In the absence of cationic liposomes, FITC-labeled ODN were not taken up significantly by MC (data not shown).

We documented by gel mobility shift assay that E2F binding activity was enhanced in serum-stimulated but not in unstimulated MC (Fig. 2A). This E2F binding was abolished by preincubation of nuclear extracts with excess amount of the unlabeled E2F ODN but not with excess unlabeled missense ODN (Fig. 2B).

We then utilized the E2F-luciferase reporter gene to assess the effect of the E2F decoy on mitogen-stimulated transcriptional activation. As shown in Fig. 3, serum treatment of MC stimulated luciferase gene expression that was driven by three repeated E2F binding sites. Increased luciferase activity was not seen in transfected MC that were maintained in serum-free medium, nor was it seen when a control luciferase reporter plasmid lacking E2F binding sites was transfected (data not shown). Transfection of E2F decoy ODN (2 mM) resulted in significant inhibition of serum-induced luciferase activity. In contrast, transfection of the missense ODN failed to influence serum-stimulated E2F transcriptional activity. In these experiments, luciferase light units were adjusted by the protein content. Transfection of the E2F transcription factor decoy had no effect on reporter gene expression driven by a viral promoter.

We also examined the effect of E2F decoy ODN on endogenouse levels of cdk2 kinase and PCNA mRNA, which are under the control of transcription factor E2F. After serum stimulation for 2 days, MC mRNA levels of cdk2 kinase and PCNA were clearly increased (Fig. 4). In MC transfected with E2F decoy ODN, significant reductions in cdk2 kinase and PCNA mRNA levels were observed. In contrast, transfection with the missense ODN had no significant effect on cdk2 kinase and PCNA mRNA expression. In control amplifications using the β-actin primers, no significant differences among cells were detected.

PCNA and cdk2 kinase were detected in cell extracts by Western blotting (Fig. 5, A and B). In E2F decoy
ODN transfected cells, PCNA protein content was reduced by 38% and cdk2 kinase by 46% compared with missense decoy transfected cells.

Finally, we examined the effect of E2F decoy ODN on MC proliferation 72 h after serum stimulation. Control MC number increased significantly in response to 10% serum and 15% serum stimulation. Cell proliferation was blunted by transfection of E2F decoy ODN (P < 0.01), whereas the missense ODN alone did not affect cell number (Fig. 6).

**DISCUSSION**

In this study, we used a transcription factor decoy to inhibit MC proliferation. This novel strategy is based on previous observations indicating that double-stranded ODN decoys can block the binding of nuclear transcription factors to specific promoter regions of target genes (3, 4). We were able to determine the specific conditions under which cationic liposomes could be used to successfully transfect a decoy ODN for the transcription factor E2F into MC without causing cellular toxicity. Our data show that the cationic liposome delivery method results in both cytoplasmic and nuclear uptake of FITC-labeled ODN (Fig. 1).

The transcription factor E2F plays a key role in the expression of the cell cycle regulatory genes including c-myc, c-myb, cdk2 and cdc2 kinase, and PCNA (9, 29, 33, 34). Little or no E2F binding activity is detectable in quiescent cells, but the level of E2F mRNA has been reported to increase following serum stimulation in some cell lines (28). Our data demonstrate that E2F protein levels in nuclear MC extracts were markedly increased by serum stimulation for 6 h (Fig. 2 A). The specificity of the radiolabeled E2F probe was confirmed by adding a 100-fold excess of unlabeled double-stranded E2F ODN to the reaction compared with similar quantities of missense ODN that do not bind E2F (Fig. 2 B). As shown in Fig. 3, transfection of MC with an E2F-luciferase reporter gene resulted in enhancement of luciferase activity following stimulation of the cells with serum. Luciferase activity was reduced significantly when MC were cotransfected with E2F decoy ODN but not with missense ODN transfection (3rd bar). Values are means ± SE; n = 6. N.S., not significant.

**Fig. 5. Changes in PCNA and cdk2 kinase protein expression following decoy transfection.** Cell lysates were prepared from MC 3 days after transfection with E2F decoy ODN or missense ODN. Fifty micrograms of cell lysate was used in each reaction. Lanes 1–4 show the results of immunoblotting with the cell lysates from MC grown in DSF medium (lane 1), MC stimulated with 15% FBS (lane 2), MC stimulated with serum after missense ODN transfection (lane 3), and MC stimulated with serum after E2F decoy ODN transfection (lane 4). In E2F decoy ODN transfected cells, PCNA protein and cdk2 kinase content were reduced. Representative result from 4 separate experiments is shown in A, and quantitative analysis by densitometer is shown in B. Values are group means ± SE; n = 4. P < 0.01 vs. the missense ODN.

**Fig. 6. Effects of decoy ODN on MC proliferation.** After 10% serum (A) or 15% serum (B) stimulation, MC cell proliferation was induced (2nd bar). This proliferation was considerably attenuated by E2F decoy ODN transfection (4th bar) (P < 0.01), but not by missense ODN transfection (3rd bar). Values are means ± SE; n = 6. N.S., not significant.
Maneuvers that result in decreased nuclear E2F binding and decreased gene transcriptional activation should result in the inhibition of cell cycle regulatory gene expression. Indeed, transfection of MC with E2F decoy ODN but not missense decoy ODN resulted in decreased mRNA levels for PCNA and cdk2 kinase in serum-stimulated cells (Fig. 4). These changes were paralleled by a reduction in detectable PCNA and cdk2 kinase protein in MC extracts (Fig. 5). E2F decoy ODN transfection also resulted in a significant reduction in MC proliferation following stimulation with 10% FBS (Fig. 6). Presumably, further inhibition was not achieved because the addition of serum to the cell cultures activated alternative pathways of proliferation not regulated by E2F. Thus blockade of E2F-mediated gene transcription resulted in attenuated MC proliferation consistent with the hypothesis that E2F decoy ODN can inhibit cell cycle progression by preventing transcriptional activation of critical cell cycle regulatory genes.

On the basis of our in vitro observations, the predicted effect of E2F inhibition in vivo would be to prevent cell entry into S phase in models where a stimulus is applied to induce cell proliferation. We have indeed shown that local delivery of E2F decoy ODN in the balloon-injured rat carotid attenuates VSMC proliferation in the vessel wall (23). This does not preclude other potential roles for E2F in other cell types. For example, E2F-1 knockout mice have been generated that exhibit a defect in thymocyte maturation (11). These mice develop normally and have similar numbers of cells in S phase in both spleen and gut epithelium. However, they display visible enlargement of the thymus and lymph nodes attributed by the authors to the defects in normal T cell apoptosis. These observations suggest that other members of the E2F family (E2F-2 to E2F-5), and indeed other transactivators of cell cycle regulatory genes, may substitute for E2F-1 in certain tissues. The study also draws attention to the importance of E2F-1 in promoting apoptosis in thymocytes. These results of widespread E2F gene disruption are somewhat unexpected, but they emphasize that there are important differences between total gene knockout and site-directed in vivo inhibition of E2F in a selected target tissue or organ.

MC proliferation is the hallmark of many human glomerular diseases. Many in vitro studies have identified growth factors and cytokines such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and interleukin-1 as MC mitogens (12). These factors can be locally produced in the glomerulus by MC and endothelial cells and/or by infiltrating hematopoietic cells. Growth factor and cytokine stimulation of MC results not only in proliferation but also in a wide variety of biological responses, including alterations in matrix protein synthesis and degradation, as well as the generation of many effector molecules potentially involved in glomerular pathological processes (21). Although the factors that regulate MC proliferation in vivo are still ill-defined, studies in the anti-Thy 1 rat model of glomerulonephritis emphasize the link between PDGF and bFGF upregulation and MC proliferation (12), as well as the association between transforming growth factor-β upregulation and the development of glomerular sclerosis (12).

Since the induction of cell cycle progression appears to be critical for the activation of MC both in vitro and in vivo, therapeutic strategies designed to arrest cell cycle progression may prove to be effective in treating proliferative forms of glomerulonephritis. Our strategy using E2F decoy ODN is particularly attractive because inhibition of a single transcription factor results in quiescence of several cell cycle regulatory genes that are essential for orderly cell cycle progression (14, 20). We have recently reported successful gene introduction into the rat kidney, especially into glomeruli using the hemagglutinating virus of Japan (HVJ) liposome technique (30), and we and others have initiated studies designed to analyze the effects of antiproliferative ODN and other gene sequences on glomerular biology and pathophysiology in vivo. In the anti-Thy 1 rat model, we have preliminary data to show that MC proliferation and sclerosis can be attenuated with intrarenal infusions of E2F decoy ODN.

We would like to thank T. Luu, of McGill University, for technical assistance.

This study was presented in abstract form as an oral communication, at the Annual Meeting of the American Society of Nephrology, in San Diego, 1995.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-46831, HL-35252, HL-35610, HL-48638, HL-07709, and HL-58616 and by grants from Ciba-Geigy, Bristol Meyers Squibb, and Smith Kline Beecham Foundation. V. J. Dzau is the recipient of National Institutes of Health MERIT Award HL-35610. D. Baran is a Research Scholar of the Fonds de la Recherche en Sante du Quebec. J. Y. S. Kim is a Howard Hughes Medical Student Research Fellow.

Address for reprint requests: V. J. Dzau, Dept. of Medicine, Brigham and Women’s Hospital, Harvard Medical School, 75 Francis St., Boston, MA 02215.

Received 6 January 1997; accepted in final form 4 May 1998.

REFERENCES


