Transcription Factor Egr-1 Regulates Glomerular Mesangial Cell Proliferation*

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Increase of glomerular mesangial cells (MCs) is a prominent histopathological finding in many types of glomerulonephritis. We have shown previously that expression of the zinc-finger transcription factor, early growth response gene-1 (egr-1), is closely correlated with the proliferation of cultured MCs. To elucidate whether Egr-1 is required for MC proliferation, we inhibited serum-induced Egr-1 expression by phosphothioate-modified antisense oligonucleotides (ODNs). Uptake of antisense ODNs into MCs was demonstrated, and five different egr-1 antisense ODNs were tested for their impact on serum-induced egr-1 mRNA and protein levels and on MC growth. The most potent egr-1 antisense ODN inhibited serum-induced egr-1 mRNA by 68%, protein induction by 58%, and MC replication as measured by [3H]thymidine uptake and cell counts by 78 and 46%, respectively. The effects of antisense ODNs on MC growth correlated closely with their ability to inhibit Egr-1 protein. ODNs acted in a dose-dependent manner, the minimal effective concentration being 1 μM. Control ODNs had no significant effects. In addition, antisense ODNs against egr-1 potently inhibited endothelin-1-induced Egr-1 expression and MC growth. Heparin, a known inhibitor of MC growth, suppressed serum-induced egr-1 mRNA expression by 39% and egr-1 mRNA expression by 44%. We conclude that Egr-1 is an essential part of the mitogenic signal transduction cascade in cultured MCs.

Mesangial hypercellularity is a prominent histological characteristic of many types of human glomerular kidney disease, such as IgA nephropathy, membranoproliferative glomerulonephritis, focal glomerulosclerosis, and lupus nephritis. Growth factors leading to a proliferative response of MCs1 may be derived from intrinsically glomerular cells or from infiltrating leukocytes or platelets. Inadequate control of MC proliferation may be an initial step leading to progressive glomerular alterations and end stage renal disease. Potentially detrimental effects of MC hypercellularity include mechanical obstruction of glomerular capillaries, increased production of proinflamma-

tory mediators, and disturbance of production and removal of extracellular matrix proteins promoting mesangial and glomerular sclerosis.

The immediate early gene, early growth response gene-1 (egr-1), encodes a 75–80-kDa transcriptional regulator that binds DNA through three zinc-finger domains (1–6). Egr-1, also known as zif 268, Krox 24, TIS 8, and NGFI-A, is rapidly and transiently induced in response to multiple mitogenic signals (7, 8). The induction of Egr-1 occurs mainly at the transcriptional level (9–11). This activation is mediated through serum response elements in the egr-1 promoter in fibroblasts and rat MCs (9, 12, 13). Egr-1 modulates specific gene expression (14–18).

So far, only a few specific biological functions can be ascribed to Egr-1. Egr-1 is involved in differentiation processes. It was shown to be essential and restrictive for differentiation of myeloblastic HL60 cells along the macrophage lineage (19). Egr-1 has been found to suppress v-sis-dependent transformation of NIH 3T3 cells (20). In addition, Egr-1 has recently also been shown to be involved in the regulation of proliferation of T lymphocytes (21) and astrocytes (22).

In previous studies, we observed a very close correlation between the stimulation of MC growth and induction of egr-1 mRNA expression by mitogens such as platelet-derived growth factor, serotonin, and arginine vasopressin. Angiotensin II, which was not mitogenic for cultured rat MCs, failed to induce egr-1 mRNA expression (7). Here, we report that different antisense oligonucleotides directed against egr-1 mRNA block mitogen-induced Egr-1 expression and inhibit proliferation of cultured MCs. This indicates that Egr-1 is an important component of the mitogenic signal transduction cascade in glomerular MCs.

MATERIALS AND METHODS

Reagents—Phosphothioate-modified antisense and sense, scrambled, or mismatched control oligodeoxynucleotides (ODNs) were from MWG Biotech (Ebersberg, Germany) and were dissolved in H2O. ODNs were applied 16 h prior to stimulation of MCs. The sequences of the ODNs used and their positions relative to the translational AUG start site were as follows: AS1 (–118 to –99), 5'-GGCGGGTGCAGGGGGCAACAT-T3; S1, 5'-AGTGGCCCTCGACCCCGC-3; SCR1, 5'-AGGGTCGGC-TGCCGGAGGCA-3; AS2 (–107 to –93), 5'-TATCATGGCGGGTG-3; AS2M, 5'-TCAGATGGGTGCTGCC-3; S2, 5'-ATGCGCGCCCAAGGCGG-3; AS4 (485–499), 5'-GGCTGGCCTGCATGCT-3; S4, 5'-GAGCATGACCAACC-C3; SCR4, 5'-GTGCGTACCCCGTGC-3; ASS (–180 to –161), 5'-CTCGTGGACGGGCT-3. For visualization of cellular uptake of ODNs, AS2 was labeled with fluorescein at the 5' end. Endothelin-1 (ET-1) was from Bachem (Heidelberg, Germany), and heparin from bovine lung was from Sigma.

Mesangial Cell Isolation and Culture—Glomeruli from rat kidneys were isolated and glomerular outgrowth and subsequent subculturing of MCs were performed as described previously (7). MCs were used for experiments between passages 12 and 25.

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1 The abbreviations used are: MC, mesangial cell; Egr-1, early growth response-1; ODN, oligodeoxynucleotide; ET-1, endothelin-1; FCS, fetal calf serum.
Incubated with oligonucleotide AS2 (5’ modified oligonucleotides. For 16 h. The cellular uptake of oligonucleotides was examined by polyvinylpyrrolidone, 2% BSA, 2% Ficoll 400,000), 5’ ethanol-precipitated RNA was separated on a 1% agarose gel. Blots were incubated in phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk powder to block nonspecific binding, washed in phosphate-buffered saline containing

RNA Extraction and Northern Blot Analysis—MCs were grown in 10-cm dishes until subconfluency and growth arrested for 72 h in medium containing 0.4% FCS. After adequate stimulation with various ligands, total RNA was extracted by the method of Chomczynski and Sacchi (23). Ethanol-precipitated RNA was separated on a 1% agarose formaldehyde gel. Size fractionated RNA was transferred onto Hybond nylon membranes (Amersham Intl., Little Chalfont, United Kingdom). The Northern blot was prehybridized with 5 × Denhardt’s solution (2% polyvinylpyrrolidone, 2% BSA, 2% Ficoll 400,000), 5 × SSC (0.75 M NaCl, 0.075 M sodium citrate), 50% formamide, 50 mM Na3PO4, 0.1% SDS, 0.25 mg/ml salmon sperm DNA at 65 °C for 4 h. DNA hybridization probes were labeled with [α-32P]dCTP using a random primed labeling kit (Boehringer Mannheim). Egr-1 was visualized using the ECL system (Amersham Intl.).

Determination of [3H]Thymidine Uptake—MCs were seeded in 96-well plates at a density of 3 × 10^5 cells/well and growth arrested for 72 h in medium supplemented with 0.4% FCS. Stimulation of MCs was performed as for [3H]thymidine uptake. Cell number was determined 72 h after growth stimulation. Monolayers were washed twice in phosphate-buffered saline, and MCs were trypsinized and transferred into 10 ml of Isoton for counting in a cell counter (Coulter, Luton, United Kingdom). Statistical analyses were performed using Student’s t test for unpaired values.

RESULTS
Mitogen-activated Proliferation of Cultured MCs Correlates with Induction of Egr-1 Protein Expression—We recently reported a very close correlation between induction of egr-1 mRNA expression and MC growth by several growth-promoting, vasoactive peptides (7). Here we examined the effects of mitogenic 4% FCS and ET-1 on the expression of Egr-1 protein levels as detected by Western blot analysis. Egr-1 protein, barely detectable in control MCs, was found to be expressed in a dose-dependent manner following stimulation with FCS or ET-1 for 2 h (Fig. 1, upper panel). FCS- and ET-1-stimulated MC growth, as measured by [3H]thymidine uptake, exhibited a very similar dose dependence (Fig. 1, upper panel). To establish that Egr-1 induction is not a mere epiphenomenon but a necessary step in the mitogenic signal transduction cascade in MCs, we tried to prevent Egr-1 induction by the application of phosphothioate-modified antisense oligonucleotides directed against egr-1.

Uptake of Antisense Oligonucleotides by Cultured Rat MCs—To demonstrate cellular uptake of oligonucleotides by cultured MCs, 5’-fluorescein-labeled antisense oligonucleotides were applied to nonconfluent MCs for 16 h (Fig. 2). In Fig. 2B,
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Antisense Oligonucleotides against egr-1 Inhibit Serum-induced egr-1 mRNA and Protein Expression—MCs were incubated with antisense oligonucleotide AS1 (20 μM) or sense control oligonucleotide S1 (20 μM). Northern blot analysis was performed to test whether oligonucleotides directed against egr-1 mRNA effectively suppress egr-1 transcript levels 1 h after stimulation with 4% FCS (Fig. 3). We found that AS1 inhibited serum-induced increase in egr-1 mRNA levels by 68%, whereas the sense oligonucleotide S1 had only marginal effects. To demonstrate that oligonucleotides specifically inhibit egr-1 induction, Northern blots were also probed for the expression of c-fos, another immediate early gene that has been shown to exhibit induction characteristics similar to those of egr-1 (11, 24, 25). Neither AS1 nor S1 oligonucleotides influenced induction of c-fos mRNA expression significantly. Because efficacy of antisense oligonucleotides depends on elimination of the target protein, Western blot analysis was performed. Antisense oligonucleotide AS1 inhibited induction of Egr-1 protein by 58%, whereas application of sense and scrambled oligonucleotides resulted in only a slight reduction (Fig. 4A). Another antisense oligonucleotide, AS4, was less effective and inhibited serum-induced Egr-1 protein levels by 25% (Fig. 4B). Sense and scrambled control oligonucleotides were ineffective.

Antisense Oligonucleotides Directed against egr-1 Potently Inhibit MC Proliferation—To examine whether suppression of egr-1 mRNA and protein induction interfered with MC mitogenesis, we tested the effects of antisense oligonucleotides on serum-induced MC proliferation measured by [3H]thymidine uptake (Fig. 5A) and cell counts (Fig. 5B). [3H]Thymidine uptake was inhibited in a dose-dependent manner by antisense oligonucleotides AS1, AS2, and AS3 in a concentration range from 1 to 20 μM. The respective control oligonucleotides were without significant effects. Consistent with these results, antisense oligonucleotides AS1, AS2, and AS3 also significantly inhibited 4% FCS-induced increase in MC number (Fig. 5B). Again, control oligonucleotides were ineffective with the exception of inhibition of MC number by S3, which reached statistical significance. A summary of the effects of five different antisense oligonucleotides on 4% FCS-induced Egr-1 protein expression, [3H]thymidine uptake, and increase in MC number is given in Fig. 6. Antisense oligonucleotides that had the strongest effect on Egr-1 protein expression (upper panel) proved to be the most potent inhibitors of MC growth (78 ± 11% inhibition of [3H]thymidine uptake and 46 ± 14% inhibition of MC counts by AS1) (middle and lower panels). Likewise, egr-1 antisense oligonucleotide AS5, which did not affect serum-stimulated Egr-1 protein expression, failed to suppress MC growth.

Because FCS contains a mixture of MC mitogens, we tested the effect of antisense oligonucleotides on Egr-1 protein expression and MC growth induced by the individual mitogen, ET-1 (Fig. 7). Antisense oligonucleotides AS1, AS2, and AS4 (10 μM) markedly inhibited ET-1-mediated induction of Egr-1 protein and MC growth by ET-1. Again, AS1 proved to be the
most potent antisense oligonucleotide and almost completely inhibited stimulation of MC \[^{[3]H}\]thymidine incorporation and cell counts by ET-1. Control oligonucleotides were without significant effects (data not shown).

**Inhibitors of MC Proliferation Inhibit Egr-1 Induction**—Having shown that Egr-1 induction is essential for serum- and ET-1-induced MC mitogenesis, we investigated whether heparin (26), an agent with reported antiproliferative effects on MCs, would inhibit egr-1 expression. Heparin led to a 39% suppression of 4% FCS-induced \[^{[3]H}\]thymidine uptake by MCs at a concentration of 50 units/ml and concomitantly diminished egr-1 mRNA expression by 44% (Fig. 8).

**DISCUSSION**

Induction of the transcriptional regulator Egr-1 has been demonstrated in diverse biological processes, including development (24), cell differentiation (1, 19, 27), depolarization (1), stretch/relaxation (28), ionizing radiation (29), and growth (1, 7, 21, 22). Our own previous studies revealed a very close correlation between the induction of proliferation of cultured glomerular mesangial cells and the induction of egr-1 mRNA by a variety of mitogens. Second messenger systems involved in the proliferative response were also critical for up-regulation of Egr-1 (7, 10). Using antisense oligonucleotides directed against egr-1, we demonstrated that induction of Egr-1 is required for the growth of cultured MCs. Because unmodified oligonucleotides have a short half-life (30), we used phosphorothioated oligonucleotides to increase resistance to exonucleolytic cleavage. Using fluorescein-labeled antisense oligonucleotides, we demonstrated that cultured MCs incorporate oligonucleotides from the culture medium, probably through endocytosis, as suggested by predominant granular cytoplasmic staining. However, oligonucleotides are likely to be released from granular compartments because we observed nuclear staining as well.

Because no useful guidelines exist for the selection of the best target sites, we tested five different egr-1 antisense oligonucleotides directed to different regions in the egr-1 mRNA. Several reports suggested that either 5'-untranslated sequences or the AUG start for translation are the most effective target sites (31, 32), whereas in other studies, internal sites were found to be more effective (33). Lima et al. (34) suggest that single-stranded loop structures are promising target sites. Laptev et al. (35), however, identified effective target sites in double-stranded regions of the predicted secondary structure for the mRNA. We found that antisense oligonucleotides located at or slightly upstream of the AUG translational start site (AS1, AS2, and AS3) were more effective than those located at greater distances upstream (AS5) or downstream (AS4) of the AUG codon. The most effective antisense oligonucleotide (AS1) inhibited serum-induced egr-1 mRNA by 68% and protein by 58%. This extent of inhibition is in agreement with reports using antisense oligonucleotides against Tis 8 and collagen IAI (22, 35). One important aspect of the use of antisense oligonucleotides is specificity, because nonspecific binding of oligonucleotides to small molecules and proteins has been described (36). We think that the effects of egr-1 antisense oligonucleotides on MC growth are specific for several reasons: 1) inhibition of egr-1 was shown on mRNA and protein levels; 2) egr-1 mRNA induction but not the induction of another immediate early gene, c-fos, was inhibited; 3) antisense oligonucleotides acted in a dose-dependent manner; 4) sense, scrambled,
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and mismatched oligonucleotides served as controls for nonspecific effects and had no significant impact on egr-1 mRNA and protein levels or on MC growth; and 5) various egr-1 antisense oligonucleotides were tested and a clear correlation between their ability to inhibit Egr-1 protein and their impact on serum- and ET-1-induced MC growth was apparent. Because several different oligonucleotides were effective, it is highly unlikely that target genes other than egr-1 were responsible for the observed effects.

During the course of our studies, nonspecific effects of c-myc and c-myb antisense oligonucleotides containing stretches of four consecutive guanine nucleotides on the proliferation of smooth muscle cells were reported (37). The oligonucleotides AS1 and AS2, used in our study, contained stretches of four G residues. Based on two findings, we do not believe that the antiproliferative effects of the applied antisense oligonucleotides were predominantly due to non-antisense mechanisms. First, antisense oligonucleotides not containing stretches of four consecutive G residues (AS3 and AS4) were effective in inhibiting egr-1 mRNA and Egr-1 protein as well as MC growth. Second, we used a mismatched control oligonucleotide (AS2M), in which the stretch of four G residues was left in place and the overall nucleotide composition was unchanged. AS2M did not inhibit Egr-1 protein expression (data not shown) and had no significant effects on FCS-induced [3H]thymidine uptake or cell number. Because AS1 and AS2 were slightly stronger inhibitors of MC growth than the other oligonucleotides, we cannot fully exclude the possibility of some additional nonspecific effects. Despite these limitations, our data indicate that Egr-1 is an important regulatory component of the mitogenic signal transduction cascade in MCs and that antisense oligonucleotides directed against egr-1 can be useful tools to inhibit mitogen-elicted MC proliferation in cell culture and in experimental glomerulonephritis.

In agreement with the involvement of Egr-1 in proliferative cell responses, demonstrated by our data, are recent studies showing that endothelin-3-induced mitogenesis of astrocytes was inhibited by egr-1 antisense oligonucleotides (22) and that overexpression of Egr-1 in LLC-PK1 renal cells led to up-regulated transcription of the Gαq proto-oncogene (15), which has been implicated in cell growth and oncogenic transformation (38). Additionally, Egr-1 was recently shown to delay interleukin-1-inducible tumor growth arrest of melanoma cells (39). Finally Egr-1 contributes to the regulation of the thymidine kinase promoter at the G1-G0 transition of the cell cycle (40).

In addition to these studies, our data obtained in glomerular mesangial cells provide further evidence for an important role of Egr-1 in the regulation of cellular growth events. They emphasize the need to identify additional physiologically relevant target genes of this transcriptional regulator in order to elucidate signal transduction events downstream of Egr-1 induction.

REFERENCES
1. Sukhatme, V. P., Cao, X. M., Chang, L. C., Tsai Morris, C. H., Stamenkovich, D., Ferreira, P. C., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., Le Beau, M. M., and Adamson, D. E. (1988) Cell 53, 37–43
2. Christy, B., and Nathans, D. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 8757–8761
3. Pavlath, K. G., and Pabo, C. O. (1991) Science 252, 809–817
4. Lemaire, P., Vanasse, C., Schmitt, J., Stunnenberg, H., Frank, R., and Charnay, P. (1990) Mol. Cell. Biol. 10, 3456–3467
5. Cao, X. M., Koski, R. A., Gashler, A., McKiernan, M., Morris, C. F., Gaffney, R., Hay, R. V., and Sukhatme, V. P. (1990) Mol. Cell. Biol. 10, 1931–1939
6. Gashler, A., and Sukhatme, V. P. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 191–224
7. Rupprecht, H. D., Dann, P., Sukhatme, V. P., Sterzel, R. B., and Coleman, D. L. (1992) Am. J. Pathol. 140, F263–F266
8. Sukhatme, V. P., Kartha, S., Toback, F. G., Taub, R., Hoover, R. G., and Tsai Morris, C. H. (1987) Oncogene Res. 1, 343–355
9. Rupprecht, H. D., Sukhatme, V. P., Rupprecht, A. P., Sterzel, R. B., and Coleman, D. L. (1994) J. Cell Physiol. 159, 311–323
10. Rupprecht, H. D., Sukhatme, V. P., Lacy, J., Sterzel, R. B., and Coleman, D. L. (1994) Am. J. Pathol. 145, F351–F360
11. Lau, L. F., and Nathans, D. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 1182–1186
12. Christy, B., and Nathans, D. (1989) Mol. Cell. Biol. 9, 4889–4895
13. Qureshi, S. A., Cao, X., Sukhatme, V. P., and Foster, D. A. (1991) J. Biol. Chem. 266, 10892–10896
14. Gupta, M. P., Gupta, M., Zalk, R., and Sukhatme, V. P. (1991) J. Biol. Chem. 266, 12515–12516
15. Kinane, T. B., Finder, J. D., Kawashima, A., Brown, D., Abbate, S., Shang, C., Fredericks, W. J., Rauscher, F. J., III, Sukhatme, V. P., and Eronlian, L. (1994) J. Biol. Chem. 269, 72503–72509
16. Molnar, G., Crozat, A., and Pardee, A. B. (1994) Mol. Cell. Biol. 14, 5242–5248
17. Mutem, A., Camp, S., and Taylor, P. V. (1995) J. Biol. Chem. 270, 1866–1872
18. Ackerman, S. L., Minden, A. G., Williams, G. T., Bobonis, C., and Young, C. Y. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 7523–7527
19. Nguyen, H. Q., Hoffman Liebemann, B., and Liebemann, D. A. (1993) Cell 72, 197–209
20. Huang, R. P., Darland, T., Okamura, D., Mercella, D., and Adamson, E. D. (1994) Oncogene 9, 1367–1377
21. Perez-Castillo, A., Pipoén, C., García, I., and Alemán, S. (1993) J. Biol. Chem. 268, 14945–14950
22. Hu, R. M., and Levin, E. R. (1994) J. Clin. Invest. 93, 1820–1827
23. Chmeczyński, P., and Saeche, N. (1987) Anal. Biochem. 162, 156–159
24. McMahon, A. P., Champion, J. E., McMahon, J. A., and Sukhatme, V. P. (1990) Development 108, 281–287
25. Greenberg, M. E., and Ziff, E. B. (1984) Nature 311, 433–438
26. Castells, F. J., Jr., Hoover, R. L., Harper, P. A., and Kornowski, M. J. (1985) J. Am. Pathol. 120, 427–435
27. Cheng, T., Wang, Y., and Dai, W. (1994) J. Biol. Chem. 269, 30848–30853
28. Akai, Y., Homma, T., Burns, K. D., Yasuda, T., Badr, K. F., and Harris, R. C. (1994) Am. J. Pathol. 144, C482–C489
29. Hallahan, D. E., Sukhatme, V. P., Sherman, M. L., Virudachalam, S., Kufe, D., and Weichselbaum, R. R. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2156–2160
30. Fisher, T. L., Terhorst, T., Cao, X., and Wagner, R. W. (1993) Nucleic Acids Res. 21, 3857–3865
31. Chiang, M.-Y., Chan, H., Zounes, M. A., Freier, S. M., Lima, W. F., and Martin, F. H., and Weichselbaum, R. R. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 4051–4055
32. Gupta, S. K., Gallego, C., and Johnson, G. L. (1992) Mol. Cell. Biol. 3, 123–128
33. Sells, S. F., Muthukumar, S., Sukhatme, V. P., Crist, S. A., and Bangnakar, V. M. (1995) Mol. Cell. Biol. 15, 682–692
34. Pardee, A. B. (1989) Science 246, 603–608

Fig. 8. Effect of heparin on egr-1 mRNA expression and MC growth. Quiescent MCs were preincubated for 12 h with heparin (50 units/ml) prior to stimulation with FCS (4%). A, [3H]thymidine uptake was determined between 0 and 24 h after serum stimulation. B, total RNA was obtained 1 h after serum stimulation, and Northern blot was probed for egr-1 (upper panel) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (middle panel). egr-1 mRNA expression was quantitated by densitometry. Relative densitometric units (expression after FCS stimulation alone = 1) corrected for gdh expression are given. **, p < 0.01.