Kidney International, Vol. 51 (1997), pp. 694–702

Expression of the transcriptional regulator Egr-1 in experimental glomerulonephritis: Requirement for mesangial cell proliferation

HARALD D. RUPPRECHT, GERHARD HOFER, EMILE DE HEER, R. BERND STERZEL, GERHARD FALLER, and HARALD O. SCHOECKLMANN

Medizinische Klinik IV and Pathologisches Institut der Universität Erlangen-Nürnberg, Erlangen, Germany, and Department of Pathology, University of Leiden, Leiden, The Netherlands

Expression of the transcriptional regulator Egr-1 in experimental glomerulonephritis: Requirement for mesangial cell proliferation. The early growth response gene-1 (Egr-1), a zinc finger transcriptional regulator, was induced in a rat model of mesangioproliferative glomerulonephritis (GN). Northern blot analysis revealed a maximal 14.9-fold increase in glomerular Egr-1 mRNA at day 6 of GN. By immunohistochemistry Egr-1 protein expression was demonstrated to be mainly confined to glomerular mesangial cells (MC). To test whether Egr-1 directly regulates MC proliferation, cultured MCs were stimulated with platelet-derived growth factor (PDGF) after preincubation with different Egr-1 antisense oligonucleotides (ASOs). PDGF-induced Egr-1 mRNA levels were inhibited by up to 75% and protein levels by up to 91%. In addition Egr-1-specific ASOs blocked PDGF-induced rise in ^{3}H-thymidine uptake by 83% and almost completely abrogated increase in MC number. We conclude that Egr-1 induction is of critical importance for PDGF-induced mitogenic signaling in MCs, and inhibition of Egr-1 *in vivo* may offer an approach to oppose glomerular MC proliferation in glomerular inflammatory disease.

A common reaction pattern of various glomerular kidney diseases—be the original insult of immunological, toxic or metabolic nature—is increased proliferation of intrinsic mesangial cells (MCs) and mesangial hypercellularity. Examples include IgA nephropathy, membranoproliferative glomerulonephritis, postinfectious endocapillary proliferative glomerulonephritis, lupus nephritis, diabetic nephropathy and focal segmental glomerulosclerosis. Persistent mesangial hypercellularity together with increased matrix deposition is considered an important factor in progressive glomerular sclerosis and deterioration in renal function. In various animal models of glomerular disease stimulation of MC proliferation resulted in increased deposition of extracellular matrix, and interventions inhibiting proliferation also blocked matrix expansion [1, 2]. In view of these findings, our work addresses two key questions: (1.) What are the molecular mechanisms regulating MC proliferation *in vitro* and *in vivo*? (2.) How can MC proliferation be inhibited or prevented and how can resolution of mesangial hypercellularity be achieved?

In previous studies, we could demonstrate a very close correlation between the proliferative response of cultured rat MCs and the induction of the early growth respone gene-1 (Egr-1) [3, 4]. Furthermore, inhibitors of MC growth, like atrial natriuretic peptide or dexamethasone, inhibited Egr-1 mRNA induction (H.D. Rupprecht, unpublished observations). Egr-1, also known as zif268, Krox 24, TIS 8 or NGFI-A, is a member of the family of immediate early genes. It is rapidly and transiently induced after a variety of mitogenic signals [3, 5], but also during fetal development in the mouse [6], following differentiation signals [7, 8], after ionizing radiation [9], or in response to stretch/relaxation [10]. Induction has been shown to occur mainly at the transcriptional level [4, 11, 12]. The gene encodes a 75 to 80 kDa nuclear phosphoprotein [13, 14], which has been shown to bind DNA at the consensus sequence GCGGGGGCG in a zinc-dependent fashion through three zinc-finger domains [14–16] and to activate transcription [14, 17–19].

The present study demonstrates that Egr-1 induction not only occurs after mitogenic stimulation of MCs in culture, but can also be observed with MC proliferation *in vivo* during the course of anti-Thy-1 nephritis, a rat model of mesangioproliferative glomerulonephritis. Using an antisense oligonucleotide strategy, we provide evidence that Egr-1 induction is not a mere epiphenomenon of MC proliferation, but is an essential part of the mitogenic signal transduction cascade elicited by PDGF in cultured MCs.

Methods

Experimental disease

Inbred male Sprague-Dawley rats (150 to 200 g) were obtained from Charles River Deutschland (Sulzfeld, Germany). Monoclonal antibody against Thy1.1 (ER4) is described elsewhere [20]. Anti-Thy-1 nephritis was induced by a single intravenous injection of 1 mg/kg body wt of ER4 into the tail vein. Groups of four animals were sacrificed on days 2, 6 and 12 after induction of nephritis and renal tissue was obtained for immunohistochemistry or RNA extraction. A group of four normal animals served as a control.

Immunohistochemistry

Five-micrometer cryostat kidney sections were air dried for 10 minutes, fixed in 3% paraformaldehyde for five minutes, incubated with 0.1% Triton X100 for 10 minutes, and subsequently in 5% fetal calf serum and 5% normal goat serum in phosphate

Received for publication July 23, 1996
and in revised form September 18, 1996
Accepted for publication September 19, 1996

buffered saline (PBS) for 20 minutes. For Egr-1 staining sections were incubated for 120 minutes with polyclonal anti-Egr-1 antibody R5232-2 (1:150), as described in detail elsewhere [13], followed by a 30 minute incubation with tetramethylrhodamin isothiocyanate (TRITC)-conjugated goat anti-rabbit antibody (1:200; Nordic Immunological Laboratories, Tilburg, Netherlands). The anti-Egr-1 antibody was provided by Dr. V.P. Sukhatme (Beth Israel Hospital, Boston, MA, USA). For double label immunofluorescence stainings, sections were either incubated with monoclonal antibody OX-7 (recognizing the Thy1.1 epitope on MCs; 1:200; PharMingen, San Diego, CA, USA) or ED-1 (recognizing monocytes/macrophages; 1:1500), descibed in detail elsewhere [21], for 60 minutes and fluorescein isothiocyanate (FITC)-conjugated goat F(ab')2 anti-mouse IgG1 antibody (1:100; Nordic) for 30 minutes, followed by incubation with anti-Egr-1 antibody R5232-2 (1:150) for 120 minutes and TRITC-conjugated goat anti-rabbit antibody (1:200; Nordic) for 30 minutes. All antibodies were diluted in PBS containing 1% bovine serum albumin and 0.5% Tween.

Antisense oligonucleotides

Phosphothioate-modified antisense and sense, scrambled or mismatched control oligodeoxynucleotides (ODNs) were from MWG Biotech (Ebersberg, Germany) and were dissolved in H_2O. ODNs were applied 16 hours prior to stimulation of MCs. The sequences of the ODNs used and the positions relative to the translational AUG start site are as follows:

AS1: 5'-GCGGGGTGCAGGGGCACACT-3' (−118 to −99),
S1: 5'-AGTGTGCCCCTGCACCCCGC-3',
SCR1: 5'-AGGCTGGCTGCCGGGAGCGA-3',
AS2: 5'-TTACATGCGGGGTGC-3' (−107 to −93),
AS2M: 5'-TTAGATGCGGGGTCC-3',
AS3: 5'-CGGCCTTGGCCGCTGCCAT-3' (1 to 19),
S3: 5'-ATGGCAGCGGCCAAGGCGG-3',
AS4: 5'-GGGTTGGTCATGCTC-3' (485 to 499).

Mesangial cell isolation and culture

Glomeruli from rat kidneys were isolated and glomerular outgrowth and subsequent subculturing of MCs were performed as previously described [3]. MCs were kept in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated (50°C, 30 min) fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamin, 5 μg/ml insulin in a 95% air-5% CO_2 humidified atmosphere at 37°C. MCs were used for experiments between passages 5 and 20.

RNA extraction and Northern blot analysis

MCs were grown in 10 cm dishes until subconfluency and growth arrested for 72 hours in medium containing 0.4% FCS. After adequate stimulation cells were washed twice in phosphate buffered saline (PBS) and total RNA was extracted by the method of Chomczynski and Sacchi [22]. For the extraction of glomerular RNA renal cortex was dissected from kidneys from experimental animals, sacrificed at various time points after disease induction, and glomeruli were isolated by the sieving technique as previously described [3]. RNA was size-fractionated on a 1% agarose formaldehyde gel and transferred onto Hybond nylon membranes (Amersham, Little Chalfont). The Northern blot was baked at 80°C for two hours, prehybridized with 5× Denhardt's solution,

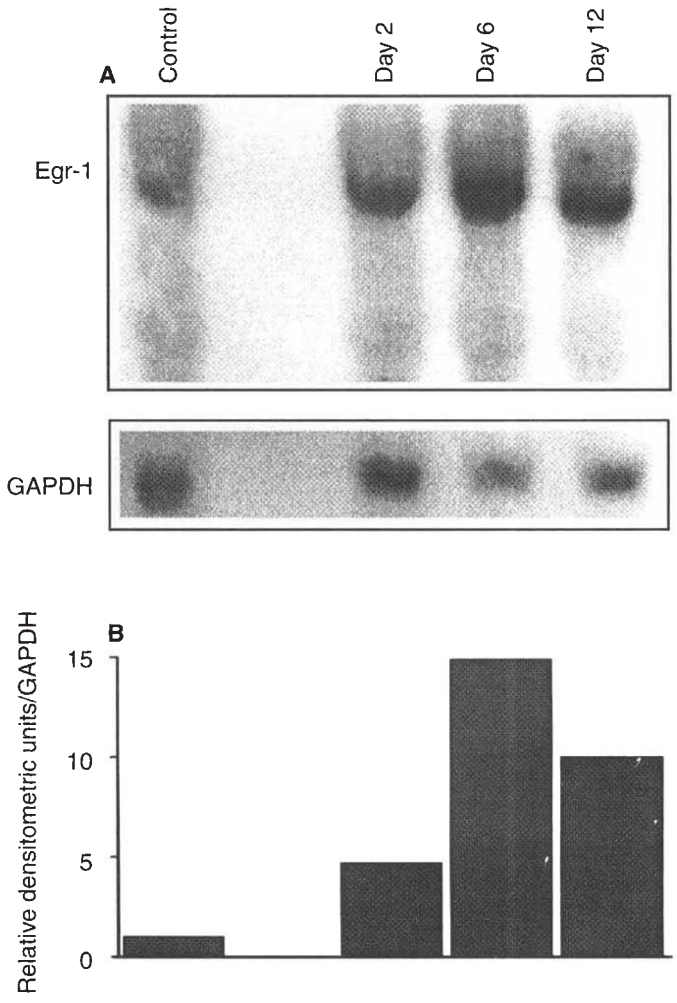


Fig. 1. *Egr-1 mRNA expression during the course of anti-Thy-1 nephritis.* Total RNA was extracted from glomeruli isolated by the differential sieving technique from control rats or from animals rendered nephritic by a single injection of anti-Thy1.1 antibodies at day 2, 6 or 12. RNA obtained from 4 animals per time point was pooled and 15 μg RNA were size-fractionated on an agarose gel and blotted. **A.** Northern blot was probed for Egr-1 or GAPDH to control for equal loading. **B.** Expression levels for Egr-1 were quantified by densitometric analysis and are expressed as relative densitometric units corrected for expression of GAPDH. Egr-1 expression under control conditions was defined as one densitometric unit.

5 × SSC, 50% formamide, 50 mM Na_3PO_4, 0.1% SDS, 0.25 mg/ml salmon sperm DNA at 39°C for four hours. DNA hybridization probes were labeled with [α-^{32}P]dCTP using a random primed labeling kit (Boehringer Mannheim, Mannheim, Germany). The blots were hybridized in prehybridization solution containing 2 × 10^6 cpm/ml of probe at 39°C for 20 hours. The blot was washed twice for 15 minutes with 2 × SSC containing 0.1% SDS and then 30 minutes with 0.1 × SSC containing 0.1% SDS. Blots were exposed to Kodak XAR-2 films with intensifying screens at −80°C.

Protein extraction and Western blot analysis

MCs were grown in 3.5 cm dishes until subconfluency, growth arrested for 72 hours in medium containing 0.4% FCS and

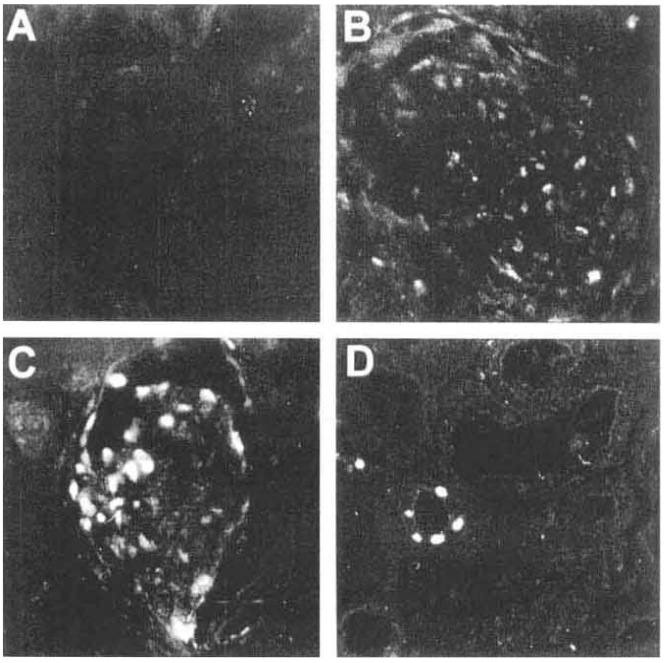


Fig. 2. *Immunofluorescence of cryosections showing Egr-1 expression during anti-Thy-1 nephritis.* (**A**) Representative glomerulus of control animal showing no or very weak nuclear Egr-1 staining. (**B**) Representative glomerulus six days after disease induction showing clear nuclear staining of multiple glomerular cells in mesangial location and (**C**) non-representative glomerulus showing very bright nuclear Egr-1 staining in isolated glomerular cells locating to the mesangium. (**D**) Isolated tubular cells six days after disease induction showing nuclear Egr-1 staining (magnification ×400). Paraformaldehyde-fixed frozen sections were incubated with a polyclonal anti-Egr-1 antibody for 120 minutes, followed by a 30 minute incubation with a TRITC-conjugated goat anti-rabbit antibody.

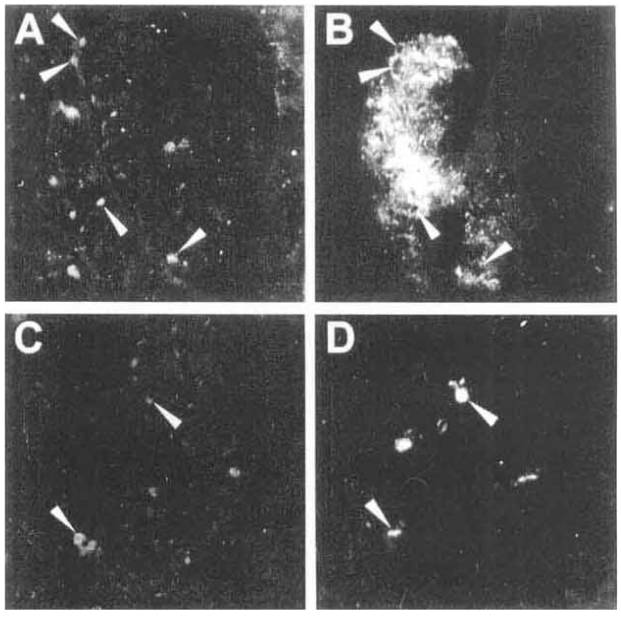


Fig. 3. *Double label immunofluorescence for Egr-1 and Thy1.1 or Egr-1 and monocytes of frozen sections six days after induction of anti-Thy-1 nephritis.* (**A**) glomerular expression of Egr-1 (polyclonal rabbit anti-Egr-1 antibody/TRITC-conjugated goat anti-rabbit antibody) and (**B**) the mesangial cell epitope Thy1.1 (mouse monoclonal antibody OX-7/FITC-conjugated goat F(ab')2 anti-mouse IgG1 antibody) showing many Egr-1+/OX-7+ cells (some marked by arrowheads). (**C**) Glomerular expression of Egr-1 and (D) the monocyte/macrophage marker ED-1 (mouse monoclonal antibody ED-1/FITC-conjugated goat F(ab')2 anti-mouse IgG1 antibody) showing two Egr-1+/ED-1+ cells (marked by arrowheads), whereas most Egr-1-expressing cells were of non-macrophage origin (Egr-1+/ED-1−). A and B or C and D each show an identical area of the same section viewed through different filters for visualization of TRITC- or FITC-conjugated antibodies.

harvested after adequate stimulation in 100 μl RIPA solution (1% Triton X-100, 1% sodiumdeoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.2, 10 mM EDTA, pH 7.2, 1 mM PMSF, leupeptin 2 μg/ml). Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules). Protein samples containing 20 μg total protein were denatured by boiling for five minutes and separated on a 7.5% denaturing SDS-PAGE gel. After electrophoresis, the gels were electroblotted onto NC membranes and the transfer was controlled by Ponceau-S staining. Blots were incubated in PBS containing 0.1% Tween-20 and 5% nonfat dry milk powder to block unspecific binding, washed in PBS containing 0.1% Tween-20 and incubated with the primary anti-Egr-1 antibody R5232-2 (1:3000), described in detail elsewhere [13]. Egr-1 was visualized with a secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody using the ECL system (Amersham).

Determination of ^{3}H-thymidine uptake

MCs were subcultured in 96-well plates in medium supplemented with 10% FCS until subconfluency and growth-arrested for 72 hours in medium supplemented with 0.4% FCS. Quiescent MCs were then exposed to fresh medium containing 0.4% FCS with and without ODNs for an additional 16 hours before stimulation with agonists. Cells were pulsed with 1 μCi/ml [^{3}H-methyl]-thymidine (specific activity 5 mCi/mmol; ICN, Hyland, TX, USA) from 0 to 24 hours after addition of agonists. The cells were washed twice with PBS, lysed with distilled water and harvested onto filters by an automated cell harvester (Bibby Dunn, Watertown, MA, USA). Incorporated counts were measured by a liquid scintillation counter (Beckmann, Fullerton, CA, USA).

Determination of cell number

MCs were seeded in 96-well plates at a density of 3×10^3 cells/well and growth arrested for 72 hours in medium supplemented with 0.4% FCS. Stimulation of MCs was performed as for ^{3}H-thymidine uptake. Cell number was determined 72 hours after growth stimulation. Monolayers were washed twice in PBS, cells were trypsinized and transferred into 10 ml of Isoton for counting in a coulter counter (Coulter, Luton).

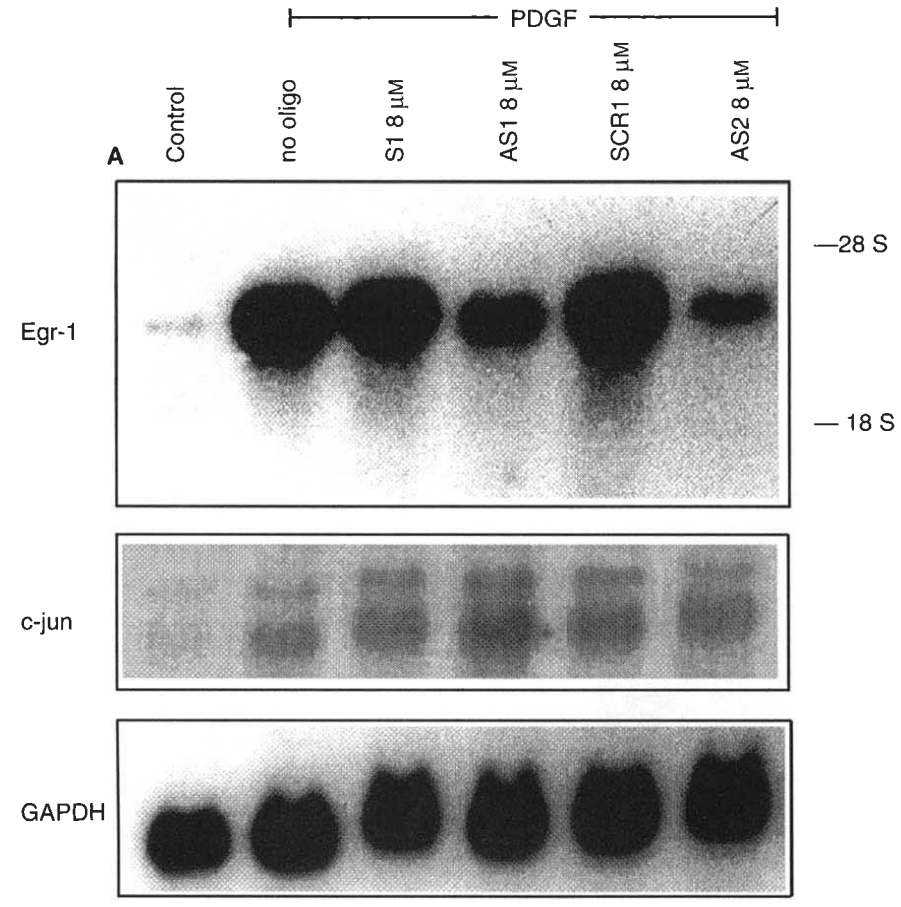
Statistical analysis

Statistical analysis was performed using the Student's *t*-test for unpaired samples.

Results

Glomerular expression of Egr-1 mRNA in experimental mesangioproliferative glomerulonephritis

We recently reported a close correlation between the induction of Egr-1 mRNA expression and MC growth by a number of



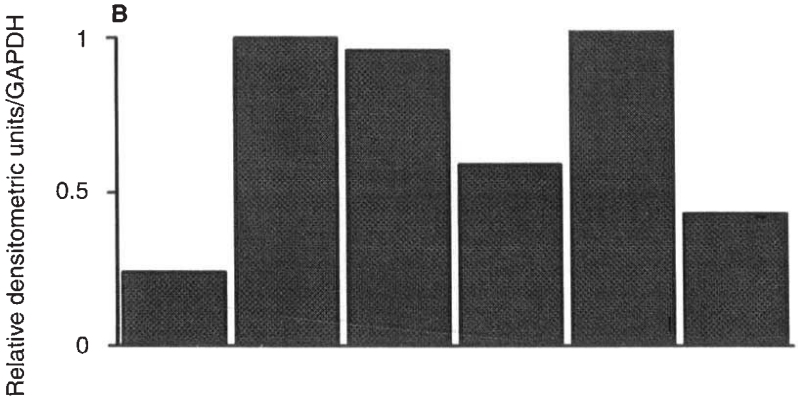


Fig. 4. *Inhibition of PDGF-induced Egr-1 mRNA expression in cultured rat mesangial cells by antisense oligonucleotides against Egr-1.* Growth arrested MCs were preincubated for 16 hours with 8 µM Egr-1 antisense oligonucleotides (AS1, AS2) or sense (S1) or scrambled (SCR1) control oligonucleotides before stimulation with PDGF 20 ng/ml for one hour. Total RNA was harvested and size-fractionated on an agarose gel. **A.** Northern blot was probed for Egr-1 (upper panel), c-*jun* (middle panel) or GAPDH (lower panel). **B.** Relative densitometric units corrected for GAPDH expression are given (Egr-1 expression after PDGF-stimulation without preincubation with oligonucleotides = 1).

mitogenic, vasoactive peptides [3]. To test whether Egr-1 expression also occurred *in vivo*, we determined Egr-1 transcript levels during the course of anti-Thy-1 nephritis, an experimental model of mesangioproliferative glomerulonephritis elicited by a single injection of monoclonal antibody ER 4. This model is characterized by initial mesangiolysis and acute inflammation followed by a phase of predominant MC proliferation peaking at days 3 to 6 after disease induction [20, 23]. Disease progression was monitored by measurement of 24-hour urinary protein excretion (mean ± SD; $N = 4$; controls, 8.7 ± 2.1 mg/day; Thy-1 at day 2, 129.5 ± 164.6 mg/day; at day 6, 277.4 ± 76.5 mg/day; at day 12, 109.4 ± 30.1 mg/day), which was comparable to that reported in the literature [20]. Total RNA was harvested from whole kidneys or from isolated glomeruli and Northern blot was probed for Egr-1. No significant change in Egr-1 mRNA expression between control animals and animals six days after disease induction was observed when RNA was isolated from total kidneys (data not shown). However, if RNA was extracted from isolated glomeruli,

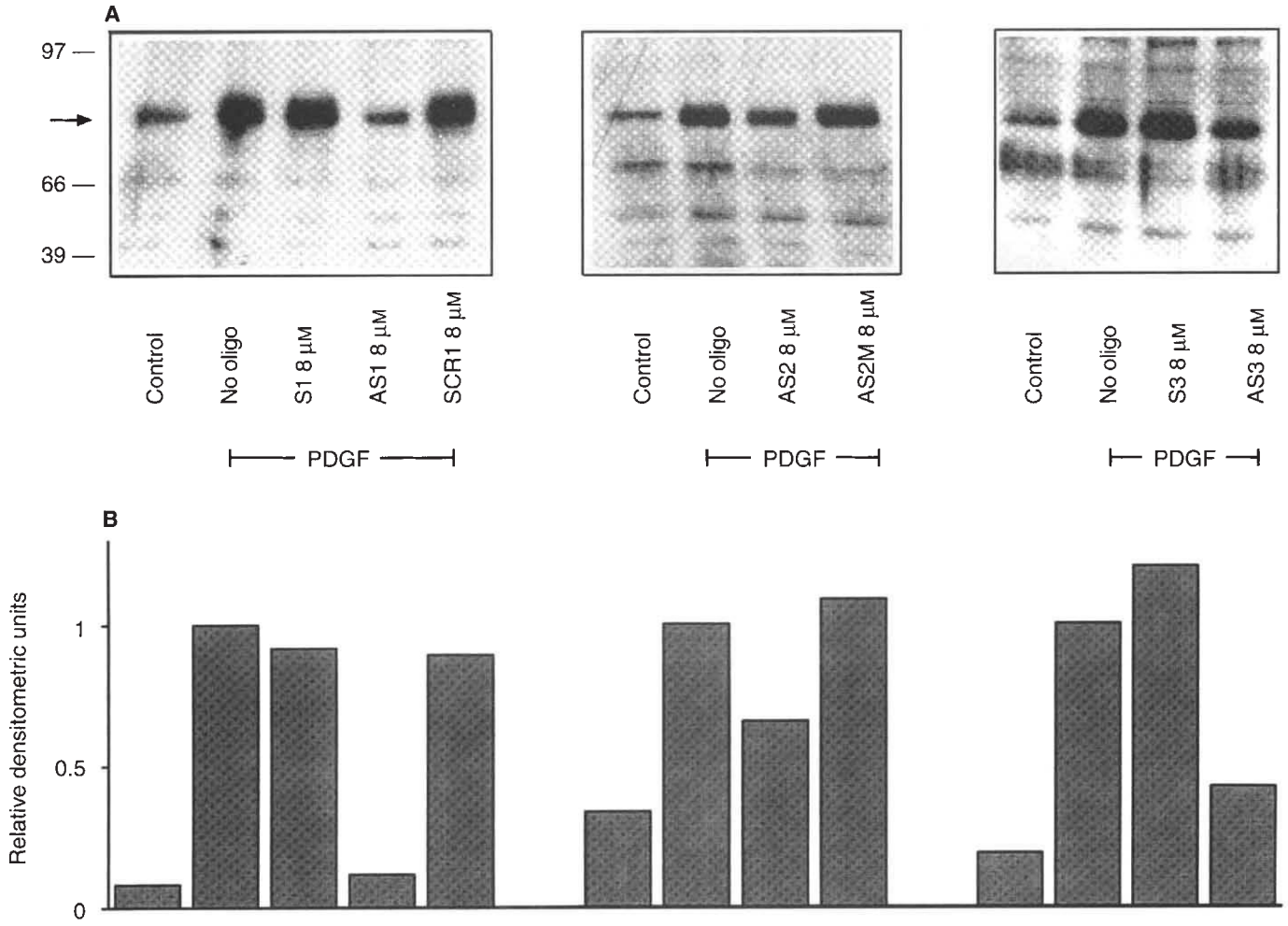


Fig. 5. *Inhibition of PDGF-induced Egr-1 protein expression in cultured rat mesangial cells by antisense oligonucleotides against Egr-1.* Growth arrested MCs were preincubated for 16 hours with 8 µM Egr-1 antisense oligonucleotides (AS) or sense (S), scrambled (SCR) or mismatched (ASM) control oligonucleotides before stimulation with PDGF 20 ng/ml for two hours. **A.** Twenty milligrams of protein were size fractionated on a SDS-PAGE gel and Egr-1 was visualized on Western blots using the ECL system (Amersham). **B.** Relative densitometric units are given (Egr-1 expression after PDGF-stimulation without addition of oligonucleotides = 1). A representative of three experiments is shown for antisense oligonucleotides AS 1 and AS2. The Western blot with antisense oligonucleotide AS3 was only performed once.

a maximal 14.9-fold increase in Egr-1 mRNA expression was observed on day 6 after induction of anti-Thy-1 nephritis (Fig. 1). This demonstrates that Egr-1 mRNA is up-regulated in an experimental model of mesangioproliferative nephritis and is of predominant glomerular origin.

Egr-1 protein expression in anti-Thy-1 nephritis is predominantly up-regulated in glomerular mesangial cells

To identify the cell type expressing Egr-1, immunohistochemistry was performed on paraformaldehyde-fixed cryostat kidney sections. Control animals showed virtually no or only very weak nuclear staining within the glomeruli, and no staining was observed in tubular cells (Fig. 2A). Six days after induction of anti-Thy-1 nephritis, a marked increase in nuclear staining of multiple cells was observed within the glomeruli (Fig. 2B). The time course of Egr-1 protein expression paralleled that of Egr-1 mRNA induction. Only a marginal increase in nuclear staining was observed on day 2, and the maximum staining was detected on day 6 after disease induction and decreased thereafter. On day six of nephritis, but not on days 2 or 12, some scattered tubular cells were found to express Egr-1 protein (data not shown). Double label immunofluorescence staining demonstrated that most Egr-1 expressing cells were also Thy1.1-positive, identifying them as mesangial cells (Egr-1+/OX-7+) (Fig. 3 A, B). To test whether in addition to MCs infiltrating monocytes expressed Egr-1 we performed stainings with the monocyte/macrophage marker ED-1. We found 0.77 ± 0.67 ED-1+ cells per glomerular cross section (GCS) in control animals, 7.86 ± 3.36 cells/GCS on day 2, 4.29 ± 2.68 cells/GCS on day 6, and 1.45 ± 1.37 ED-1+ cells/GCS on day 12 (mean ± SD; $N = 30$). Double label immunofluorescence staining for Egr-1 and ED-1 revealed the presence of few Egr-1+/ED-1+ cells. Most Egr-1 positive cells, however, did not stain for the monocyte/macrophage marker ED-1 (Fig. 3 C, D). These results identify the majority of Egr-1 expressing glomerular cells as MCs. However, few monocyte/macrophages also contribute to glomerular Egr-1 expression in anti-Thy-1 nephritis.

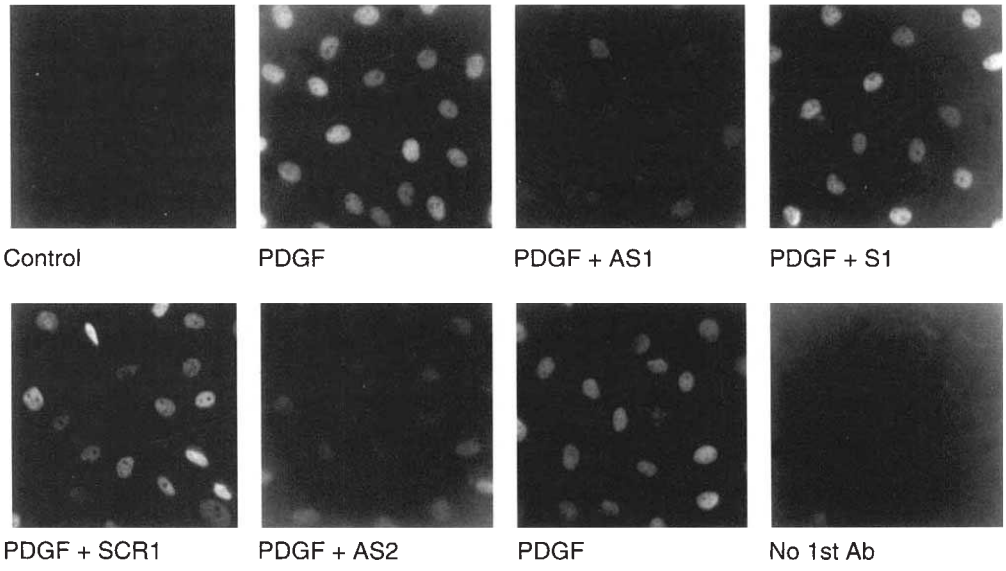


Fig. 6. *Inhibition of PDGF-induced nuclear Egr-1 protein expression by antisense oiligonucleotides against Egr-1.* Immunofluorescence of cultured MCs seeded on chamber slides showing Egr-1 expression after 16 hours preincubation with Egr-1 antisense (AS) or sense (S) or scrambled (SCR) control oligonucleotides (8 μM) and stimulation with PDGF 20 ng/ml for two hours. A representative of three independent experiments is shown.

Antisense oligonucleotides directed against Egr-1 inhibit mRNA and protein expression in cultured MCs

To elucidate the function of Egr-1 in the mitogenic signal transduction cascade in cultured MCs, we attempted to eliminate Egr-1 by the application of antisense oligonucleotides (ODN) directed against Egr-1. Preincubation of quiescent MCs with antisense ODN AS1 or AS2 for 16 hours prior to PDGF stimulation for one hour inhibited Egr-1 mRNA levels by 54% and 75%, respectively (Fig. 4). Sense or scrambled control ODNs were without significant effects. The induction of another immediate early gene, c-*jun*, was not affected. Since the efficacy of antisense oligonucleotides depends on elimination of the target protein, Western blot anlaysis was performed. AS1 inhibited PDGF-induced Egr-1 protein levels by 91% ± 9% (N = 3; Fig. 5). Two other antisense ODNs, AS2 and AS3, inhibited Egr-1 protein induction by 55% ± 8% (N = 3) and 74% (N = 1), respectively. Again sense (S1, S3), scrambled (SCR1), as well as a mismatched control ODN (AS2M) had no detectable effect on Egr-1 protein induction (Fig. 5). We also tested the effects of Egr-1 antisense ODNs on PDGF-induced Egr-1 protein levels by immunocytochemistry to demonstrate that indeed nuclear accumulation of Egr-1 was prevented. A clear decrease in PDGF-induced nuclear staining for Egr-1 was observed when MCs were preincubated with antisense ODNs (AS1, AS2; Fig. 6).

Antisense oligonucleotides against Egr-1 potently inhibit mesangial cell proliferation

To examine whether suppression of Egr-1 mRNA and protein induction interfered with MC mitogenesis, we tested the effect of Egr-1 antisense oligonucleotides on PDGF-induced MC proliferation measured by ^{3}H-thymidine uptake (Fig. 7A) and cell counts (Fig. 7B). ^{3}H-thymidine uptake was dose-dependently inhibited by antisense oligonucleotides AS1, AS2 and AS3 in a concentration range from 1 to 8 μM. The respective control oligonucleotides (8 μM) were without significant effects. Consistent with these results, antisense oligonucleotides also significantly inhibited PDGF-induced increase in MC number. Again, control oligonucleotides were ineffective. A summary of the effects of the different antisense oligonucleotides on PDGF-induced Egr-1 protein expression and ^{3}H-thymidine uptake is given in Table 1. Antisense oligonucleotides maximally inhibiting Egr-1 protein expression proved to be the most effective inhibitors of MC growth. Likewise, Egr-1 antisense oligonucleotides that were less effective inhibitiors of PDGF-induced Egr-1 protein expression suppressed MC growth to a lower extent.

Discussion

Glomerular mesangial cell hypercellularity is considered one of the critical early pathogenetic events leading to progressive glomerular scarring, loss of renal function and end-stage renal disease. Here we demonstrate a marked up-regulation of the transcriptional regulator Egr-1 in an *in vivo* model of mesangioproliferative glomerulonephritis. The well established and highly reproducible anti-Thy-1 model, elicited by a single injection of monoclonal antibody ER4, was chosen for this purpose. The model is characterized by a pronounced mesangial proliferative response after an initial mesangiolytic phase. MC proliferation, as determined by staining for the δDNA polymerase accessory protein PCNA (proliferating cell nuclear antigen), peaks at around days 3 to 6 after induction of the model [24–27]. Examining glomerular total RNA, we found a maximum 15-fold increase in Egr-1 mRNA expression on day 6 after disease induction, demonstrating that *in vivo* Egr-1 mRNA expression in anti-Thy-1 nephritis is largely restricted to the glomerular compartment, and that peak expression correlates with the time point of maximal MC proliferation reported [2, 25, 26]. These data were confirmed by immunohistochemistry, which identified the majority of Egr-1 protein expressing cells as mesangial cells. A few cells exhibited double staining for ED-1 and Egr-1, indicating that in

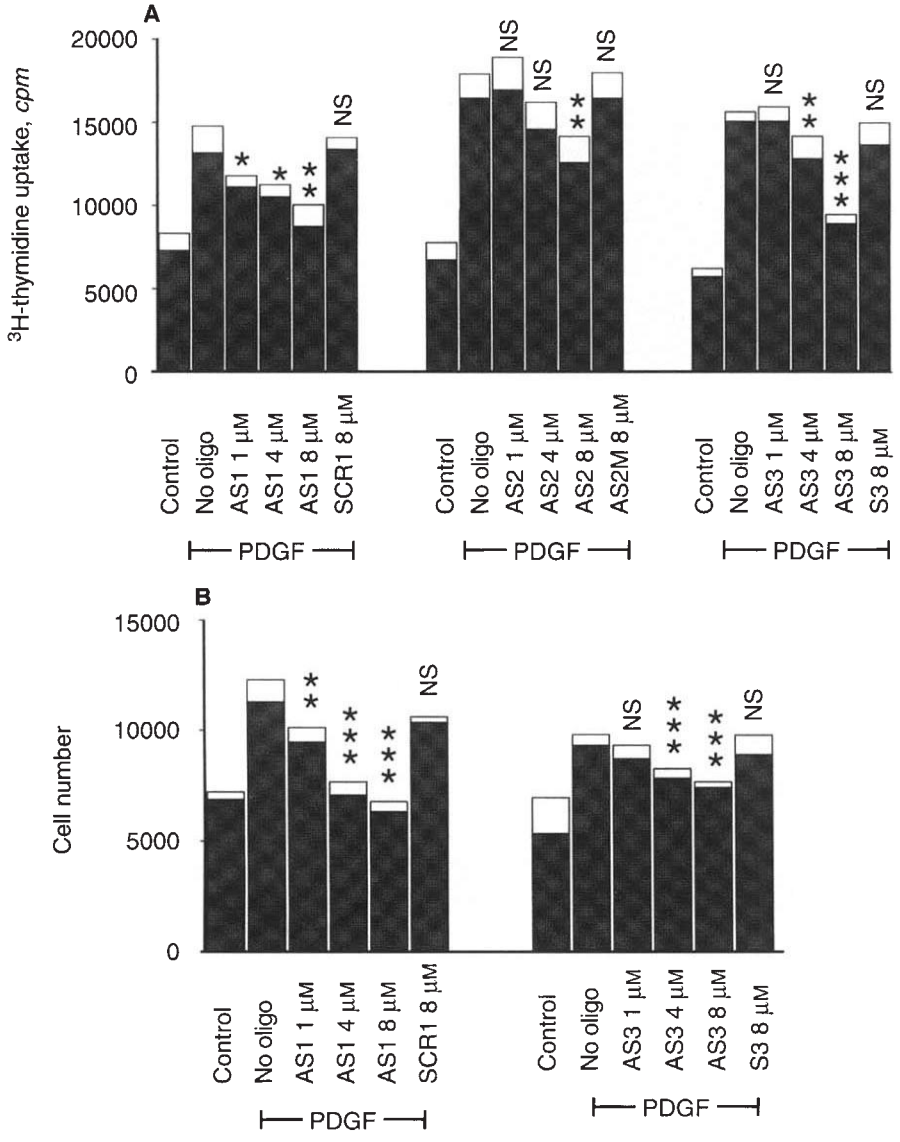


Fig. 7. *Dose-dependent inhibition of MC proliferation by antisense oligonucleotides against Egr-1.* MCs were seeded in 96-well plates, serum deprived for 72 hours, preincubated for 16 hours with the indicated concentrations of antisense (AS) or control sense (S), scrambled (SCR) or mutated (ASM) oligonucleotides and then stimulated with PDGF 20 ng/ml. MC proliferation was assessed by (**A**) ^{3}H-thymidine incorporation measured from 0 to 24 hours after PDGF-stimulation and (**B**) obtaining cell counts 72 hours after the addition of PDGF. Values given are means ± SD of five samples. Significances were calculated comparing PDGF-stimulated cells after preincubation with oligonucleotides to PDGF-stimulated cells without the addition of oligonucleotides. N.S. is nonsignificant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1. Effects of various antisense oligonucleotides directed against Egr-1 on PDGF-stimulated Egr-1 protein expression and MC growth

% Inhibition	AS1	AS2	AS3	AS4
Egr-1 protein (Western blot)	91% ± 9% (N = 3)	55% ± 8% (N = 3)	74% (N = 1)	24% (N = 1)
^{3}H-thymidine uptake	83% ± 40% (N = 3)	44% ± 9% (N = 3)	75% ± 32% (N = 5)	33% (N = 1)

Quiescent MCs were incubated for 16 hours with the indicated antisense oligonucleotides (8 µM) before stimulation with PDGF (20 ng/ml). Total cellular protein for Western blotting was extracted 2 hours after PDGF-stimulation. Egr-1 expression was quantitated by densitometry and the percent inhibition relative to PDGF-stimulated controls without addition of oligonucleotides is given. ^{3}H-thymidine incorporation was measured in the time interval from 0 to 24 hours after PDGF-stimulation (N = number of independent experiments each consisting of 5 samples).

addition to MCs, infiltrated macrophages can contribute to glomerular Egr-1 expression. Expression of Egr-1 by activated macrophages in culture has previously been shown by us and others [8, 28]. Since the number of macrophages per glomerular cross section peaked on day 2 after induction of nephritis, declining rapidly thereafter, whereas Egr-1 mRNA expression was only marginal at day 2 and peaked at day 6, it is unlikely that macrophages are a major source of glomerular Egr-1. As expected by the biological function of Egr-1 as a DNA-binding transcriptional regulator, staining was clearly nuclear.

In a next objective we tried to elucidate whether the observed expression of the transcriptional activator Egr-1 bears functional relevance for MC growth induction. Egr-1 antisense oligonucleotides were used to block Egr-1 expression in cultured rat MCs. PDGF was chosen as the mitogenic stimulus, since it is one of the most potent MC mitogens *in vitro* and since it had been demonstrated by antibody infusion that PDGF is one of the major cytokines involved in MC proliferation in the ATS (anti-thymocyte serum) model of glomerulonephritis [29]. Furthermore, our own previous studies showed that cultured MCs express high levels of Egr-1 mRNA after PDGF stimulation [3]. Here we

demonstrate that phosphothioate-modified antisense oligonucleotides directed against Egr-1 prevent or diminish PDGF-induced increase in Egr-1 mRNA and protein levels. The effect was dose-dependent in a concentration range from 1 to 8 μM. AS oligonucleotides also potently inhibited MC growth as measured by ^{3}H-thymidine uptake and cell counts in a dose-dependent fashion.

We included several controls to provide conclusive evidence for the specificity of the observed effects: (1.) Inhibition of Egr-1 was shown on mRNA and protein level. (2.) Egr-1, but not another immediate early gene, c-*jun*, was inhibited by Egr-1 AS oligonucleotides. (3.) Inhibition at the protein level was also shown by immunocytochemistry, proving that indeed nuclear staining for Egr-1 was abolished. We felt this was necessary since the existence of a 54 kDa cytoplasmic form of Egr-1, which cannot function as a transcriptional regulator, has been reported in PC12 cells [30]. (4.) Sense, scrambled and mismatched oligonucleotides served as controls for nonspecific effects and had no significant impact on Egr-1 mRNA, protein levels or MC growth. (5.) A number of different Egr-1 antisense oligonucleotides was tested. We observed a clear correlation between the ability of the various oligonucleotides to inhibit Egr-1 protein expression and their ability to inhibit PDGF-induced growth. Since several different oligonucleotides were effective it is unlikely that target genes other than Egr-1 were responsible for the observed effect.

Some of the oligonucleotides used in our study contain a stretch of four consecutive guanine residues. During the course of this study Burgess et al reported that AS oligonucleotides directed against c-*myc* and c-*myb* containing such stretches of four guanine residues might inhibit proliferation of smooth muscle cells due to a non-antisense mechanism [31]. Several reasons argue against major nonspecific effects of antisense ODNs used in our study: (1.) We demonstrate that oligonucleotides not carrying four consecutive guanine residues effectively inhibit Egr-1 protein and MC growth. (2.) When we introduced two base pair mismatches into an AS oligonucleotide containing four guanine residues, leaving the stretch of four guanine residues in place, this mismatched oligonucleotide was no longer capable of modulating Egr-1 protein levels or MC proliferation. (3.) We found a consistently close correlation between the abiltiy of the oligonucleotides to inhibit Egr-1 protein and their ability to suppress MC growth.

Our *in vitro* studies in cultured MCs demonstrate that induction of Egr-1 is a necessary step in the PDGF-induced mitogenic signal transduction cascade. Antisense oligonucleotides to Egr-1 could offer the possibility to interfere with MC proliferation, one of the major histopathological abnormalities in the early course of many forms of glomerulonephritis. Our observations add to a list of few reported biological functions of Egr-1. Egr-1 is essential for and restricts differentiation of HL60 cells along the macrophage lineage [8]. In addition to its role in macrophage differentiation, Egr-1 was implicated, albeit only indirectly, in the differentiation process of megakaryocytes [32]. More in line with our observations, some studies have implicated Egr-1 in growth control. Sells et al demonstrated that Egr-1 impedes IL-1-inducible tumor growth arrest in melanoma cells and that a dominant negative mutant WT-1/Egr-1 chimera accelerated growth arrest [33]. Overexpression of Egr-1 in LLC-PK$_1$ cells, a renal epithelial cell line, accelerated growth rate possibly via induction of the guanine nucleotide binding protein Gαi2 [18]. Further evidence for a possible role of Egr-1 in proliferative processes came from studies on the ras/MAP kinase cascade, known to be critical for mitogenic signaling. McMahon et al showed that expression of activated p^{21}ras results in Egr-1 up-regulation and overexpression of a dominant negative p^{21}ras mutant inhibits Egr-1 induction [34]. Hipskind et al showed that MAP kinase leads to phosphorylation of Elk-1, a DNA binding protein which assembles with the serum response factor (SRF) over the serum response elements (SREs) in the promotor region of the c-*fos* and also the Egr-1 gene. Phosphorylation of Elk-1 was necessary for transactivating activity of this transcription factor complex [35]. Interestingly, it was demonstrated in various cell types, and we have shown in MCs that the SREs in the Egr-1 promotor region are critical for transcriptional induction of Egr-1 [11, 36, 37]. Therefore, these studies establish a possible link between mitogen receptor generated second messenger systems, the activation of the MAP kinase cascade, and the transcriptional activation of the immediate early gene Egr-1.

To our knowledge, our study is one of the first that describes a function of Egr-1 in *in vitro* studies and provides evidence for clear up-regulation in the corresponding cell type in an *in vivo* model. Further studies should now address the question of whether inhibition of Egr-1 in the *in vivo* model of glomerulonephritis by gene transfer techniques will be helpful in limiting the proliferative response of MCs. Furthermore, downstream target genes of the transcriptional activator Egr-1 need to be identified to obtain more insight into the early nuclear signal transduction events involved in MC mitogenesis.

Acknowledgments

This study was supported by a research grant to H.D. Rupprecht from the Deutsche Forschungsgemeinschaft, Bonn (Klinische Forschergruppe: Molekulare Regulationssysteme in glomerulären Zellen der Niere).

Reprint requests to Dr. Harald D. Rupprecht, Medizinische Klinik IV der Universität Erlangen-Nürnberg, Nephrologische Forschungslabors, Loschgestraße 8, 91054 Erlangen, Germany.

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