In Vivo Identification of the Mitogen-Activated Protein Kinase Cascade as a Central Pathogenic Pathway in Experimental Mesangioproliferative Glomerulonephritis

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Abstract. Evidence was recently provided for the activation of extracellular signal-regulated kinase (ERK), the best characterized mitogen-activated protein kinase, as an intracellular convergence point for mitogenic stimuli in animal models of glomerulonephritis (GN). In this study, in vivo ERK activity was blocked, with a pharmacologic inhibitor (U0126) of the ERK-activating kinase, in rats with mesangioproliferative GN. After injection of the monoclonal anti-Thy1.1 antibody (OX-7), the rats were treated (days 3 to 6) with low (10 mg/kg body wt) or high (100 mg/kg body wt) doses of U0126 administered intraperitoneally twice daily. On day 6 after induction of the disease, whole cortical tissue and isolated glomeruli were examined by using kinase activity assays, Western blot analyses, and immunohistochemical assays. Treatment with U0126 significantly reduced glomerular stimulation of ERK in anti-Thy1 GN. In the high dose-treated group, this downregulation was accompanied by a reduction in the number of glomerular mitotic figures, back to healthy control levels, and significant decreases in the numbers of total (P < 0.05) and 5-bromo-2'-deoxyuridine-positive (P < 0.05) glomerular cells. Immunohistochemical double-staining of renal sections demonstrated that mesangial cells were the major glomerular targets of U0126 in anti-Thy1 GN. These observations point to ERK as a putative intracellular mediator of the proliferative response in GN and suggest that pharmacologic treatments that interfere with the activation of ERK may be of potential therapeutic interest.

Mitogen-activated protein (MAP) kinases are important mediators involved in the intracellular network of interacting proteins that transduce extracellular stimuli to intracellular responses (1). Extracellular signal-regulated kinases (ERK) were the first reported and are still the best described members of the group of MAP kinases. Two ERK isoforms have been described; ERK1 (or p44 MAP kinase) and ERK2 (or p42 MAP kinase) are serine/threonine kinases that regulate the expression of many genes via the phosphorylation of several transcription factors (1,2). The binding of extracellular stimuli to G protein-coupled receptors or protein tyrosine kinase receptors results in the formation of GTP-Ras, which induces the sequential activation of cytoplasmic protein kinases, leading to their phosphorylation and activation (1,2). MAP kinase/ERK kinase 1 (MEK1) and MEK2 are specific activators of ERK1 and ERK2. MEK are dual-specificity protein kinases that phosphorylate both threonine and tyrosine regulatory sites in ERK (3). Although an extensive body of data describes the pivotal role of this signaling pathway in the control of cellular proliferation in vitro (1,2), little is known regarding the roles of ERK1 and ERK2 in physiologic or pathophysiologic conditions or their activation in vivo. Other members of the rapidly growing group of MAP kinases include stress-activated protein kinases, p38 MAP kinases (α, β, γ, and δ), ERK3, and ERK5 (1,2,4,5). Compared with ERK1 and ERK2, however, the physiologic function of most of these kinases, even in vitro, is less well defined (1,2,4,5).

A major focus of research on the pathogenesis of proliferative glomerulonephritis (GN) has been to define extracellular growth-promoting factors (6). In contrast, little is known regarding the intracellular mediators that contribute to cellular growth in renal diseases. Such intracellular messengers represent likely targets for therapeutic pharmacologic interventions in proliferative glomerular diseases. We recently demonstrated for the first time, in rats with anti-glomerular basement membrane GN (7) or experimental mesangioproliferative GN (8), in vivo activation of ERK accompanying glomerular cell proliferation. Those data pointed to ERK as a regulator of cellular growth in proliferative GN. We performed the study described...
here to examine the effects of in vivo ERK inhibition on the progression of proliferative glomerular diseases. Therefore, we induced anti-Thy1.1 GN in rats. After the initial mesangiolysis after injection of the anti-Thy1.1 antibody, this form of GN is characterized by an overshooting proliferative phase that resembles human mesangio proliferative GN (9). We used the compound U0126 (10) to block ERK activation to block ERK activity in vivo and to assess its effect on the development of anti-Thy1.1 GN in rats. U0126 was demonstrated to be a potent and highly specific inhibitor of the ERK-activating kinases MEK1 and MEK2 (10).

Materials and Methods

Cell Culture

Primary rat mesangial cells (MC) were prepared and cultured as described previously (11). Before stimulation with platelet-derived growth factor-BB (PDGF-BB) (Sigma Chemical Co., St. Louis, MO), subconfluent cells were starved in serum-free medium for 24 h.

Cell Counting

For cell counting, MC were seeded in 24-well culture plates (5 × 10^4 cells/well; well diameter, 12 mm) and cultured for 24 h. Under these conditions, a cell confluence of approximately 70% was reached. Cells were serum-starved for 24 h in serum-free medium before stimulation with PDGF (20 ng/ml), in the presence or absence of U0126. After 24 h, the cells were trypsinized and cell counting was performed by using the CASY-1 system (Scharfle, Reutlingen, Germany), which is based on the Coulter counter principle.

Induction of Anti-Thy1.1 GN and Experimental Design

All animal experiments were approved by the local review boards. Anti-Thy1.1 mesangial proliferative GN was induced in male Wistar rats (weighing 150 to 160 g; Charles River, Sulzfeld, Germany) by injection of 1 mg/kg monoclonal anti-Thy1.1 antibody (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, UK). After euthanasia, the rats were nephrectomized and a renal cortical section was obtained for light microscopy. Another cortical section was directly lysed in Triton X-100 buffer (see below), and the rest of the cortical tissue (approximately 80% of the whole cortex) was used to generate a preparation of glomeruli, via standard sieving methods (12), before lysis in Triton X-100 buffer.

Initially, eight healthy rats were studied for evaluation of the effect of a single dose of the MEK inhibitor U0126 on basal glomerular ERK activity. Subsequently, 30 rats were used to examine the effect of U0126 treatment on the progression of mesangio proliferative GN. Rats received twice-daily intraperitoneal injections of U0126 dissolved in DMSO (or vehicle alone), starting on day 3 after the induction of anti-Thy1.1 GN. Healthy control animals either were not treated or received U0126 according to the schedule described above. The animals were nephrectomized on day 6 and the last dose of U0126 1 h before euthanasia. In addition, the thymidine analogue 5-bromo-2’-deoxyuridine (BrdU) (20 mg/kg body wt; Sigma) was injected intraperitoneally 4 h before euthanasia on day 6. Twenty-four-hour urine collections were performed on day 5 after disease induction.

Renal Morphologic Assessments

Tissue for light microscopy was fixed in methyl Carnoy’s solution or formalin and embedded in paraffin (13). Four-micron sections were stained with the periodic acid-Schiff reagent and counterstained with hematoxylin. In periodic acid-Schiff-stained sections, the number of nuclei per glomerulus and the number of mitoses in 100 glomerular tufts were determined. In addition, cellular death in renal sections was assayed with the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end-labeling method (14).

Immunohistochemical Staining

Four-micrometer sections of methyl Carnoy’s solution-fixed tissue were processed by using the indirect immunoperoxidase technique, as described previously (13). Primary antibodies included BU-1 (a monoclonal antibody against BrdU) (Amersham, Braunschweig, Germany), IA4 (a monoclonal antibody against α-smooth muscle actin) (Dako, Glostrup, Denmark), JG-12 (a monoclonal endothelial cell-specific antiserum) (15), monoclonal anti-ED1 antibody (Camon, Wiesbaden, Germany), and the affinity-purified IgG fraction of a polyclonal rabbit anti-rat fibronectin antibody (Chemicon, Temecula, CA). Double-immunostaining was performed as described previously (16). Fibronectin staining was evaluated by using a point-counting method. For this procedure, a grid composed of 121 dots was superimposed on the glomeruli (range, 30 to 50; magnification, ×100) and the percentages of dots overlying stained areas were counted.

Western Blot Analyses

Whole cortical tissue or isolated glomeruli were homogenized in 2 ml of Triton X-100 lysis buffer [50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM ethylene glycol bis[β-aminoethoxy]ethyl]-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate] at 4°C. After incubation for 5 min, lysates were centrifuged at 4°C for 15 min at 10,000 × g. The soluble lysates were mixed 1:4 with 5% Laemmli buffer and were heated for 5 min at 95°C. Soluble lysates (80 µg) were loaded in each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 4 and 10% acrylamide for stacking and resolving gels, respectively. Proteins were transferred to nitrocellulose membranes (pore size, 0.45 µm; Schleicher and Schuell, Keene, NH) and probed with polyclonal antibodies against the carboxy-terminal peptide of p42 ERK (17), as well as polyclonal antibodies against the phosphorylated regulatory sequences of ERK (Calbiochem, Schwalbach, Germany). The primary antibodies (diluted 1:1,000) were detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG or horseradish peroxidase-conjugated Protein A and were observed with the Amersham ECL system (Amersham, Braunschweig, Germany), after extensive washing of the membranes. The intensities of the identified bands were quantified by densitometry, using the BioRad Gel Doc 1000 system and Multi-Analyst software (Bio-Rad, Munich, Germany).

ERK Activity Assays

Soluble lysates (400 µg; prepared as described above) were incubated for 90 min with 2 µl of polyclonal antibody recognizing p42 ERK (17). Immunocomplexes were adsorbed to Protein A-Sepharose (Pharmacia, Freiburg, Germany), washed twice with lysis buffer and twice with kinase buffer (10 mM MgCl2, 20 mM Hepes, pH 7.4, containing 200 µM sodium orthovanadate), and resuspended in 60 µl of kinase buffer containing 50 µM ATP. The final reaction buffer also contained 2 µg of glutathione-S-transferase-Ellk (Santa Cruz Biotechnology, Santa Cruz, CA). The reaction was initiated by incubation at 30°C for 45 min. Then, 20 µl of 4× Laemmli buffer was added to terminate the reaction, and samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then analyzed by Western blot analysis, as described above, with a polyclonal...
anti-phospho-Elk1 antibody (New England Biolabs, Beverly, MA) that recognized only the phosphorylated forms of Elk1.

**Statistical Analyses**

Values are expressed as means ± SEM. Statistical analyses were performed with the Bonferroni t test. $P < 0.05$ was considered statistically significant.

**Results**

**MEK Inhibition Blocks the Mitotic Activity of Cultured MC**

To establish an effect of ERK signaling cascade inhibition on the proliferation of MC, we first examined the effect of the MEK inhibitor U0126 on PDGF-BB-induced mitotic activity of cultured rat MC. As demonstrated by anti-phospho-ERK Western blot analysis, which identified only active ERK forms phosphorylated within the regulatory sequence, U0126 demonstrated dose-dependent inhibition of PDGF-induced ERK activation in MC (Figure 1A). As expected, incubation of MC with PDGF stimulated cellular proliferation. U0126 dose-dependently led to a complete inhibition of this proliferative response to PDGF (Figure 1B). In this experimental setting, U0126 did not induce apoptosis or reduce the cellular viability of cultured MC in the presence of PDGF, as measured with nucleosome assays and cellular lactate dehydrogenase release, respectively (data not shown).

**U0126 Inhibits Glomerular ERK Activity In Vivo**

Next, we evaluated whether an intraperitoneal injection of U0126 would inhibit renal glomerular ERK activity in vivo. Healthy male Wistar rats received a single intraperitoneal injection of U0126, 1 h before nephrectomy and isolation of the glomeruli. As demonstrated by anti-phospho-ERK Western blot analysis (Figure 2A, upper), U0126 dose-dependently blocked the basal phosphorylation of ERK. Equal loading of glomerular proteins was confirmed by reprobing of immunoblots with a polyclonal antiserum that detected total ERK2 (Figure 2A, middle). The results of the anti-phospho-ERK Western blot analysis were confirmed with an immunocomplex ERK activity assay using a glutathione-S-transferase fusion protein of the transcription factor Elk1 as the substrate in the kinase reaction. The extent of Elk1 phosphorylation was determined by anti-phospho-Elk1 Western blot analysis (Figure 2A, lower). Collectively, these results established that U0126 could inhibit glomerular ERK activity in vivo.

On the basis of these findings, rats with mesangiolysis GN were treated with a low (10 mg/kg body wt, twice daily) or high (100 mg/kg body wt, twice daily) dose of U0126 or received vehicle (DMSO) alone. It is known that the rapid mesangiolysis that occurs within 24 h after injection of the anti-Thy1.1 antibody is critical for the overshooting glomerular proliferation. Therefore, treatment with U0126 was initiated 48 h after induction of the disease, i.e., at a time point when the nephritis was well established. Rats were euthanized on day 6, the time point with maximal glomerular ERK activation and glomerular proliferation (8). We evaluated the in vivo activity of ERK both in the renal cortex and in isolated glomeruli. As described previously (8), we observed an increase in ERK activity in crude cortical lysates on day 6 of anti-Thy1.1 GN (Figure 2B), with an even more pronounced increase in isolated glomeruli (Figure 2C), indicating that the glomeruli were the major source of the increased cortical ERK activity in anti-Thy1.1 GN. Renal phosphorylation of ERK was reduced, in a dose-dependent manner, in rats treated with U0126 (Figure 2B and C, middle). Densitometric analysis of the ERK activity assays in isolated glomeruli of rats with anti-Thy1 GN demonstrated statistically significant effects of the MEK inhibitor U0126 on ERK activity at a dose of 100 mg/kg body wt, administered twice daily (Figure 2D). These analyses also suggested that U0126 reduced basal ERK activity in healthy rats, although this finding was not statistically significant (Figure 2D).
ERK Inhibition Blunts Glomerular Proliferation in Anti-Thy1.1 GN

The renal morphologic features in the proliferative phase of anti-Thy1.1 GN are characterized by pronounced glomerular hypercellularity (Figure 3, A and B). In the group receiving high-dose U0126, these histologic changes were potently blunted in most glomeruli (Figure 3C). In accordance with the glomerular histologic features demonstrated in Figure 3, we detected an increase in the number of glomerular cells (assessed as the number of glomerular nuclei) in rats with anti-Thy1 GN, compared with control animals (Figure 4A). Treatment with U0126 induced a significant reduction of glomerular...
cells in rats with anti-Thy1 GN. The changes in glomerular cell proliferation were further examined with assessments of the number of glomerular mitotic figures and BrdU-positive nuclei. In rats with anti-Thy1 GN that were treated with the MEK inhibitor at 100 mg/kg body wt, we observed a significant reduction in mitoses (Figure 4B). This effect of U0126 was also demonstrated by quantification of BrdU-positive nuclei (Figure 4C). In addition to the action of U0126 in proliferative GN, we observed significant reductions in the numbers of glomerular mitotic figures and BrdU-positive cells in healthy control animals (Figure 4, B and C). However, this effect did not induce a significant reduction in the number of glomerular cells in healthy rats treated with U0126 (Figure 4A). We previously described a massive induction of apoptosis within hours and a slight increase in glomerular apoptosis up to 10 d after the induction of anti-Thy1 GN (8). In the study presented here, treatment with U0126 did not affect the level of glomerular apoptosis (Figure 4D). Considering the link between MC proliferation and MC matrix expansion, we quantified glomerular staining for fibronectin by grid-counting. The data support this link (Figure 4E), but the reduction in glomerular fibronectin staining in rats treated with U0126 was not statistically significant. Anti-Thy1.1 GN is not a model of severe proteinuria. However, compared with control rats, a moderate increase in albuminuria in rats with GN and an even smaller increase in healthy rats receiving U0126 were observed (Figure 4F). In nephritic rats, U0126 induced a dose-dependent, statistically nonsignificant reduction in albuminuria (Figure 4F).

To identify the type of proliferating glomerular cells targeted by inhibition of the MEK-ERK module in anti-Thy1.1 GN, immunohistochemical double-staining was performed. Staining with BrdU was combined with cell type-specific immunohistochemical marker staining. The number of proliferating MC was significantly reduced in rats treated with high-dose U0126 (100 mg/kg body wt, twice daily), compared with untreated rats with anti-Thy1.1 GN (Figure 5A). Compared with proliferating MC, the total number of proliferating glomerular monocytes/macrophages was much lower (Figure 5B). Furthermore, the number of double-positive (BrdU- and ED1-positive) cells in rats with mesangioproliferative GN was not affected by U0126 (Figure 5B). Double-staining for proliferating glomerular endothelial cells demonstrated no effect of treatment with the MEK inhibitor in nephritic animals (data not shown).

Discussion

In this study, we demonstrate for the first time that pharmacologic compounds can inhibit renal ERK activity in vivo and, more importantly, that such interventions can prevent overshooting cellular proliferation in mesangioproliferative GN. These data provide strong evidence for ERK being a putative regulator of the proliferative response to renal immune injury in vivo.

Most of our current knowledge regarding the physiologic relevance of MAP kinases is based on in vitro studies with cultured cells. The MAP kinase ERK plays a pivotal role in the regulation of important cellular functions such as proliferation, differentiation, and apoptosis (1). In cultured cell lines, mitogenic stimulation by various extracellular agonists is correlated with ERK activation (1). More importantly, dominant-negative interfering mutants of Ras or Raf-1 (components upstream of MEK in the ERK signaling cascade) have been demonstrated to inhibit growth factor-induced cell proliferation (18,19), whereas constitutively activated Raf-1 induces cell proliferation (18). Furthermore, dominant-negative or constitutively active mutants of MEK inhibit or accelerate NIH3T3 cell proliferation, respectively (20,21), and constitutively active MEK has been demonstrated to induce cellular transformation (22). Finally, catalytically inactive mutants of ERK and its antisense cDNA inhibit proliferation (23). These in vitro data indicate an essential role for the ERK cascade in the control of cellular proliferation. Recently, we were able to describe the in vivo activation of ERK in renal proliferative inflammatory glomerular diseases in rats, namely anti-glomerular basement membrane GN (7) and experimental mesangioproliferative GN (8). During the course of anti-Thy1.1 GN, we demonstrated that maximal activation of ERK was detectable in phases of the disease characterized by augmented glomerular cell prolifera-
tion (8). Although our data suggested that ERK contributed to glomerular proliferation in vivo, no interventional studies were available that examined whether inhibition of ERK activation could prevent cellular proliferation in inflammatory diseases, especially with respect to the kidney. Because we demonstrated the induction of MEK protein expression in anti-Thy1.1 GN (8), a pharmacologic inhibitor of MEK (U0126) seemed to be an attractive tool to prevent activation of the MEK-ERK module. In this study, U0126, when administered intraperitoneally twice daily, dose-dependently prevented glomerular ERK activation in anti-Thy1.1 GN. Furthermore, treatment with the MEK inhibitor reduced glomerular cell proliferation (the characteristic feature of experimental mesangioproliferative GN) and seemed to decrease pathologic proteinuria in this disease model. In accordance with our findings, another group recently demonstrated in vivo that inhibition of the ERK signaling cascade could reduce the growth of colon cancer in mice (24).

As observed previously (8), the kinetics of ERK activation...
during the course of anti-Thy1.1 GN suggested that resident glomerular cells, rather than infiltrating cells, are the sites of altered intracellular MAP kinase signaling. The study presented here supports that conclusion, because our data demonstrated that MC, rather than infiltrating monocytes/macrophages, were the main glomerular targets of MEK-ERK inhibition in mesangioproliferative GN. Cellular proliferation of intrinsic glomerular cells is thought to be essential for the progression of proliferative GN to end-stage renal disease (16). The proliferative response to injury in GN may be augmented by a convergence of multiple cytokines at ERK, inducing its activation. We previously demonstrated that PDGF is a major extracellular factor in the progression of anti-Thy1.1 GN (25). Because we demonstrated here that inhibition of ERK in cultured MC potently decreased PDGF-induced proliferation, it is tempting to speculate that the demonstrated effect of ERK inhibition on glomerular proliferation in mesangioproliferative GN might be attributable, in part, to inhibition of PDGF-induced intracellular signaling events in vivo.

The use of the MEK inhibitor U0126 allowed us to establish that inhibition of intracellular ERK activity substantially decreased glomerular cell proliferation in experimental mesangioproliferative GN, thus providing a novel strategy for the treatment of proliferative responses to immune injury. Such a mechanism-based therapy might be a potential approach not only in mesangioproliferative GN but also in other renal or extrarenal immune diseases associated with increased cellular proliferation. However, it is essential to establish the pathophysiologic relevance of the MEK-ERK module in each form of immune injury, to identify the diseases that are most likely to benefit from treatment with a MEK inhibitor. Such studies, other than this study, have not been performed. In healthy control rats, inhibition of basal glomerular proliferation, in association with mild albuminuria, was observed. These changes were not correlated with detectable pathologic features in renal histologic assessments. No side effects of treatment with U0126 were observed in rats with anti-Thy1 GN during the course of this study. However, on the basis of knowledge regarding the ERK signaling cascade, the question of whether other proliferating cells, e.g., hematopoietic cells, may be affected by long-term systemic suppression of the MEK-ERK signaling module must be explored.

In conclusion, this study provides new insights into the pathogenesis of glomerular proliferation in response to immune injury, with compelling support for the pivotal role of the ERK signaling cascade in mesangioproliferative GN. Furthermore, our data point to a new therapeutic strategy for the treatment of proliferative GN, i.e., inhibition of MEK as part of a mechanism-based therapeutic concept.

Acknowledgments

We gratefully acknowledge the assistance of Jennifer Zimmer, Gabriele Dietzel, Gertrud Minartz, Kerstin Schenk, and Andrea Cosler. This work was supported by Grants Bo 1288/13-1 (to Dr. Bokemeyer) and SFB 542/C7 (to Drs. Floege and Ostendorf) from the Deutsche Forschungsgemeinschaft and in part by a BONFOR research grant from the Faculty of Medicine, University of Bonn. We thank A. Christine Tababka, Jia-Sheng Yan, and Dr. Christopher A. Teleha for the synthesis of U0126.

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