The map kinase ERK regulates renal activity of cyclin-dependent kinase 2 in experimental glomerulonephritis

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Abstract

Background. In vitro, the extracellular signal-regulated kinase (ERK) is an intracellular convergence point of multiple stimuli, which affect the cell cycle. However, the role of ERK in cell cycle regulation in vivo is unknown.

Methods. To address this issue, ERK activity was blocked both in vitro in mesangial cells (MC) and in vivo in experimental glomerulonephritis (GN) by a pharmacological inhibitor (U0126) of the ERK-activating kinase.

Results. In stimulated MC, inhibition of ERK reduced cyclin-dependent kinase 2 (CDK2) phosphorylation, CDK2 activity and cyclin E/A expression, whereas downregulation of CDK inhibitor p27Kip1 expression was inhibited. In vivo, U0126 was given to rats in the acute phase of anti-Thy 1.1 GN. We previously showed that glomerular cell proliferation was reduced by 67% upon treatment with the inhibitor compared to nephritic controls. Now, we detected a significant increase in renal CDK2-activity/phosphorylation in the nephritic controls, that was significantly and dose-dependently reduced by ERK inhibition. CDK2 activation was accompanied by an increase in renal expression of cyclins E/A and the enhanced binding of these cyclins to CDK2 in the nephritic controls. These changes were blunted by U0126 treatment. Finally, we noted an increased expression and CDK2-binding of p27Kip1 protein in the nephritic controls which was decreased in U0126 treated rats.

Conclusions. Our observations provide the first evidence that ERK is an intracellular regulator of renal CDK2 activity in vivo in a glomerulonephritis model.

Keywords: cell cycle; cyclin A; cyclin E; MAP kinase; mesangial cell; p27Kip1

Introduction

Little is known about intracellular mediators contributing to cellular growth in renal diseases. We demonstrated activation of mitogen-activated protein (MAP) kinases in rats with anti-glomerular basement membrane (GBM)-GN [1] and experimental mesangioproliferative GN [2]. Blockade of MAP kinase activation in vivo during the mesangioproliferative phase in experimental GN blunted mesangial cell proliferation [3], thereby pointing to MAP kinases as regulators of cellular growth in proliferative GN.

MAP kinases are pivotal components in the network of proteins that transduce extracellular signals to intracellular proliferative responses [4,5]. Extracellular signal-regulated kinases (ERK) are the best described members of the group of MAP kinases. The two functionally redundant ERK isoforms are ERK1 (or p44 MAP kinase) and ERK2 (or p42 MAP kinase) [4,5]. MEK1 and MEK2 (MAP kinase/ERK kinase) are their specific activators. MEKs are dual specificity protein kinases that phosphorylate threonine and tyrosine regulatory sites in ERK [6]. The question of how stimulation of ERK culminates in cell-cycle progression is poorly understood and has never been investigated in proliferative renal disease.

Regulation of cell proliferation by extracellular signals occurs during the first gap (G1) phase of the cell division cycle via modulation of the activity of cyclin-dependent kinases (CDKs) (reviewed in [7,8]). Activation of CDK2 is a tightly regulated critical step to progress through the late G1 restriction point and to enter the S phase [8]. CDK2 activity is enhanced by an association with cyclin E in the late G1 phase and with
cyclin A in the G1/S phase transition. In contrast, binding to CDK inhibitors like p27KIP1 blunts CDK2 activity [9,10]. In addition to cyclin association, full CDK2 activity requires phosphorylation of Thr\textsuperscript{160} [11,12]. In mesangial cells, glucose activated ERK phosphorylates p27KIP1, thereby increasing its expression [13]. Blockade of MAP kinase activity abolished the reduction in p27\textsuperscript{KIP1} expression induced by EGF and PDGF [14,15], or inhibited PDGF-stimulated expression of cyclin D1 and CDK2 [15]. Other studies on the role of MAP kinases in regulating the expression and activation of CDKs and CDK inhibitors \textit{in vitro} were mainly performed in non-renal cell types [16–18].

We have previously shown that in experimental GN, treatment with the MEK inhibitor, U0126, blunted ERK activation and reduced mesangial cell proliferation to almost control levels [3]. However, in that study, we failed to identify the mechanisms responsible for the cessation of cell proliferation. We therefore now investigated the role of MAP kinase activation in regulating the expression/activation of CDK2, cyclins E/A and p27\textsuperscript{KIP1} in rat mesangial cells \textit{in vitro} and in experimental GN \textit{in vivo}.

\section*{Materials and methods}

\subsection*{Cell culture}

Primary rat mesangial cells (MC) were prepared and cultured, as described [19]. Prior to stimulation with 17\% FCS (Biochrom, Berlin) for 0, 8, 16, 24 or 30 h, with or without 10μM MEK inhibitor U0126 [20], subconfluent cells were starved in 0.2\% FCS-supplemented medium for 72 h. Treatment with U0126, under these conditions, completely blunts the phosphorylation of MAP kinases ERK1/2 as well as mesangial cell proliferation [3].

\subsection*{Experimental model and experimental design}

All animal experiments were approved by the local review boards.

The renal tissue taken for analyses was identical to that described in our previous study [3]. Briefly, anti-Thy 1.1 mesangial proliferative GN was induced in male Wistar rats by injection of monoclonal anti-Thy 1.1 antibody. At sacrifice, 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 [3] while the rest (about 80\% of the whole cortex) was used to generate a preparation of glomeruli using standard sieving methods [21] prior to lysis.

Twenty-four rats were employed to examine the effect of MAP kinase inhibition on cell cycle regulatory proteins in mesangio proliferative GN. Twice a day, nephritic rats were starved in 0.2\% FCS-supplemented medium for 72 h. Treatment with U0126, under these conditions, completely blunts the phosphorylation of MAP kinases ERK1/2 as well as mesangial cell proliferation [3].

\subsection*{Immunohistochemical stainings}

Four micrometer tissue sections were processed by an indirect immunoperoxidase technique, as described [22]. Primary antibodies included a polyclonal rabbit anti-cyclin A antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) and a mouse monoclonal antibody against p27\textsuperscript{KIP1} (BD Biosciences, San Jose, CA). Negative controls consisted of substitution of the primary antibody with equivalent concentrations of normal rabbit or mouse IgG. An additional negative control for the specificity of the cyclin A immunohistology included overnight preincubation of the specific antibody with an excess of the appropriate blocking peptide (Santa Cruz Biotechnology), in which case no staining was observed. For quantification, positively stained nuclei from at least 100 glomerular cross sections of each animal were counted in the day 6 tissues. All slides were evaluated by an observer, who was unaware of the origin of the slides.

\subsection*{Western blot analysis}

Western blot analyses were performed, as described previously [3]. Nitrocelluloses were probed with polyclonal rabbit antibodies directed against the C-terminal part of mouse cyclin A (C-19), of rat cyclin E (M-20) and of human CDK2 (M2; all antibodies from Santa Cruz Biotechnology). For the detection of phospho-CDK2 (Thr\textsuperscript{160}) the membrane was probed with an affinity purified polyclonal rabbit antibody directed against a synthetic phospho-peptide corresponding to residues surrounding Thr\textsuperscript{160} of human CDK2 (Cell Signaling, New England Biolabs GmbH, Frankfurt/Main, Germany). Expression of p27\textsuperscript{KIP1} was detected by an affinity purified polyclonal rabbit antibody directed against the C-terminal part of the human protein (Delta Biolabs, Campbell, CA). To test the specificity of...
bands were subjected to densitometry (see above). An additional control was overnight preincubation of the polyclonal anti-p27KIP1 antibody with an excess of the appropriate blocking peptide (Delta Biolabs) prior to incubation with the membrane. The primary antibodies were detected using horseradish peroxidase (HRP)-conjugated protein A (BioRad Laboratories, Hercules, CA) and visualized by the Amersham ECL system (Amersham, Barunschweig, Germany).

For studying a potential binding of cyclin A, cyclin E and p27KIP1 to CDK2, protein lysates underwent an immunoprecipitation with antibodies directed against CDK2 or cyclin E at 4°C overnight. Protein A sepharose (Pharmacia Biotech Europe, Freiburg, Germany) was added for 1 h at 4°C. Samples were centrifuged, the pellet washed threefold with Western blot lysis buffer (see above) and resuspended in Laemmli buffer. Following SDS-PAGE and blotting, the membranes were probed with antibodies against cyclin A, cyclin E and p27KIP1 (after immunoprecipitation of CDK2) or against cyclin E (after immunoprecipitation of cyclin E).

For quantification, Western blot bands were subjected to densitometry, using the BioRad Gel Doc 1000 system and Multi-Analyt software (Bio-Rad, Munich, Germany).

**CDK2 kinase activity assay**

The kinase activity of CDK2 was determined in cell lysates and lysates of renal cortical tissue by first immunoprecipitating CDK2 (antibody M2, Santa Cruz Biotechnology) and then using Histone H1 (Roche Diagnostics GmbH, Mannheim, Germany) in an *in vitro* phosphorylation assay as substrate. Briefly, immunoprecipitated CDK2 was resolved in 17 μl kinase buffer (50 mM N-hydroxyethylpiperezine-N’-2-ethanesulfonic acid (HEPES); 1 mM dithiothreitol (DTT); 10 mM MgCl2; 2.5 mM ethyleneglycol-bis-(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA); 10 mM β-glycerophosphate; 5 mM MnCl2, pH 7.5; 0.5 mM NaF; 0.4 mM sodium orthovanadate) and 1 μl Histone H1 (1 μg/μl), 1 μl ATP (10 mM) and 1 μl γ-32P-ATP (10 mCi/ml, Amersham Pharmacia Biotech, Freiburg, Germany) were added to the reaction mix. The samples were incubated for 45 min at 30°C and 10 μl of 5x Laemmli buffer was added to terminate the reaction. Subsequently, samples were heated for 5 min at 95°C and subjected to SDS-PAGE. Finally, the gel was dried and exposed to autoradiographic film (Amersham Pharmacia Biotech). For quantification, 32P-Histone H1 bands were subjected to densitometry (see above).

**Statistics**

All values are expressed as means ± SD. Statistical significance (defined as *p* < 0.05) was evaluated using ANOVA and Bonferroni *t*-tests.

**Results**

We previously described the effects of MEK/ERK pathway inhibition on the proliferation of mesangial cells in those rats used for the present study [3]. Briefly, on day 6 after induction of anti-Thy 1.1 GN, treatment with high dose U0126 reduced the number of glomerular mitoses significantly by 67%, compared to nephritic controls, to the level of healthy controls. We thereby detected a significant reduction of mesangial cell proliferation by approximately 50% [3]. Based on these findings, we now investigated the role of MAP kinase activation in regulating cell cycle proteins important for the progression through the late G1 restriction point, in rat mesangial cells *in vitro* and in experimental GN *in vivo*.

**Effect of MEK inhibition by U0126 on CDK2, cyclins A and E and p27KIP1 in rat mesangial cells**

MEK blockade inhibits CDK2 phosphorylation and activation *in vitro*. As demonstrated by anti-phospho-CDK2 Western blot analysis, CDK2 phosphorylation at Thr160 was effectively induced in FCS-stimulated mesangial cells at 24 and 30 h following stimulation (Figure 1A, upper panel). Inhibition of the activation of MAP kinases ERK1/2 by the specific MEK inhibitor, U0126, however, reduced the FCS-induced CDK2 phosphorylation significantly at every time point investigated (Figure 1A, upper panel). The expression of total CDK2 in these cells did not change significantly by any treatment in the course of this experiment (Figure 1A, lower panel).

The results of the anti-phospho-CDK2 Western blot analysis were confirmed with an immunocomplex CDK2 kinase activity assay using Histone H1 as a substrate (Figure 1B and C). CDK2 activity rapidly increased upon FCS stimulation, whereas blockade of the activation of ERK1/2 by U0126 reduced the CDK2 kinase activity significantly at every time point following stimulation of the cells (Figure 1B and C).

MEK blockade inhibits cyclin A and E expression and stabilizes p27KIP1 *in vitro*. Western blot analysis showed that unstimulated MC expressed little or no cyclin A and cyclin E (Figure 2, upper and middle panel, respectively), whereas CDK2 inhibitor p27KIP1 was strongly expressed (Figure 2, lower panel). This expression pattern turned around upon stimulation of the mesangial cells with FCS: cyclin A expression was induced after 16 h of FCS stimulation and continuously increased thereafter. Cyclin E expression was first noticed 24 h following stimulation (Figure 1A, upper panel). The expression of total CDK2 in these cells did not change significantly by any treatment in the course of this experiment (Figure 1A, lower panel).
in p27KIP1 expression at 16, 24 and 30 h of stimulation (Figure 2).

CDK inhibitor p27KIP1 and not cyclins A and E, bind to CDK2 in stimulated MC following blockade of MEK. In contrast to cyclin A, a strong p27KIP1 signal in CDK2 immunoprecipitates from unstimulated MC was detected by Western blot analysis (Figure 3A). In FCS-stimulated cells, binding of cyclin A to CDK2 was detected at 16 h of stimulation, with maximum binding at 24 h. A strong p27KIP1 signal was still detected in the CDK2 immunoprecipitates at 8 h of FCS stimulation and then decreased significantly upon longer stimulation (Figure 3A). Inhibition of the MEK kinase by U0126, however, led to an explicit reduction of cyclin A binding to CDK2 at all stimulation time points, whereas p27KIP1 binding was conserved (Figure 3A). Western blot analysis to detect CDK2 in cyclin E immunoprecipitates from MC demonstrated a weak binding of cyclin E to CDK2 in unstimulated and FCS-stimulated cells until 16 h of stimulation. Then, the CDK2 signal significantly increased, thereby demonstrating stimulation time point 24 h, as the one with the strongest binding between CDK2 and cyclin E (Figure 3B). Like the effect on the cyclin A/CDK2 complex, blocking the activation of ERK1/2, however, led to a significantly reduced cyclin E/CDK2 binding (Figure 3B).
Effect of MEK inhibition by U0126 on CDK2, cyclins A and E and p27KIP1 in experimental GN in rats

In vivo MEK blockade inhibits increased CDK2 phosphorylation and activation in total renal cortical tissue and isolated glomeruli of nephritic rats. Anti-phospho-CDK2 Western blot analysis, with cortical and glomerular lysates from normal rats, showed a weak CDK2 phosphorylation at Thr160 (Figure 4A and B, upper panels). Six days after disease induction, CDK2 phosphorylation clearly increased in the cortex and in the glomeruli of vehicle-treated animals with anti-Thy 1.1 GN. Treatment with 10 mg/kg, and more effectively with 100 mg/kg of MEK inhibitor U0126, however, reduced CDK2 phosphorylation at Thr160 in total cortical and glomerular tissues (Figure 4A and B, upper blot panels and densitometric analyses).

Compared to normal controls, total CDK2 expression did not change significantly in total cortical or glomerular fractions of vehicle-treated nephritic animals (Figure 4A and B, lower panels). Upon treatment with the high dose U0126, total glomerular CDK2 expression appeared slightly reduced (Figure 4B, lower panel).

The immunocomplex CDK2 kinase assay, using cortical lysates and Histon H1 as a substrate, confirmed the results of the anti-phospho-CDK2 Western blot analysis (Figure 4A and C). A strong induction of CDK2 kinase activity was noticed at day 6 of anti-Thy 1.1 GN (Figure 4C). Treatment with 10 mg/kg bw U0126 had no effect, however 100 mg/kg bw U0126 reduced CDK2 activity effectively (Figure 4C).
In vivo MEK blockade inhibits increased expression of cyclin A, cyclin E and p27KIP1 in cortical tissue and isolated glomeruli of nephritic rats. As demonstrated by Western blot analysis with cortical and glomerular lysates, induction of anti-Thy 1.1 GN led to an increased expression of cyclin A at day 6 in vehicle-treated rats, which was effectively reduced upon treatment with both dosages of U0126 (Figure 5A and B, upper panels and densitometric analyses). These results were confirmed by immunohistochemistry: staining of renal tissue for cyclin A and quantification of glomerular cyclin A-positive cells showed a 2.7 fold increase in vehicle-treated rats compared to normal controls. Inhibition of MEK, however, reduced glomerular cyclin A expression significantly, up to 46% (Figure 6). As shown in Western blot analysis, also cyclin E was significantly upregulated in glomerular and cortical tissue of vehicle-treated nephritic rats compared to normal controls (Figure 5A and B, middle panel and lower panel, respectively, and densitometric analyses). Treatment of nephritic rats with U0126 reduced the increased expression significantly in the cortex (Figure 5A) and less efficiently in the glomeruli (Figure 5B).

The p27KIP1 protein was upregulated in the cortex and in isolated glomeruli of vehicle-treated nephritic controls at day 6 after disease induction (Figure 5A and C, lower panel and upper panel, respectively, and densitometric analyses). Treatment with U0126 reduced p27KIP1 expression in the total renal cortex and in the isolated glomeruli (Figure 5A and C). By using a second mouse monoclonal p27KIP1-specific antibody, the results in glomerular lysates were confirmed (Figure 5C, middle panel). Furthermore, the specificity of the polyclonal p27KIP1 antibody was confirmed by preincubation with its corresponding immunization peptide, in which case no signal was obtained (Figure 5C, lower panel). Finally, immunohistochemistry showed a 1.5 fold upregulation of the portion of glomerular p27KIP1-positive cells in vehicle-treated nephritic rats at day 6 compared to normal controls (Figure 7A, B and D). Treatment of nephritic rats with high dose U0126 led to a slight, nonsignificant reduction of glomerular p27KIP1-positive cells (Figure 7C and D). The difference between Western blot analysis and immunohistochemistry may be due to intracellular reductions of p27KIP1 expression upon treatment with U0126 which is detectable by densitometry, but which may be missed by the observer who counts positively stained cells only.

Glomerular cell proliferation, apoptosis and p27KIP1 expression in the course of experimental GN in rats

Glomerular mitoses, glomerular apoptotic (TUNEL-positive-) cells and glomerular p27KIP1-positive cells were counted at days 0, 4, 7, 9 and 14 following induction of anti-Thy 1.1 GN in rats. Whereas glomerular cell proliferation was maximal around day 7 after disease induction, glomerular apoptotic cells as well as p27KIP1-positive cells increased simultaneously until day 9 (Figure 9). At day 14 all parameters were nearly at the level of the normal day 0-controls.

Discussion

We previously identified ERK and the ERK-activating kinase MEK as central regulators of cellular growth in proliferative experimental GN [2,3]. Now, we focused on the mechanisms, by which ERK regulates growth, in particular on the regulation of cell cycle proteins by MAP kinases in rat mesangial cells in vitro and in kidneys of rats with mesangioproliferative GN in vivo. We analysed the proteins important for the progression through the late G1 restriction point, namely CDK2, cyclins E/A and p27KIP1.

The major finding of the present study was that MAP kinase activation is responsible for the renal activity of CDK2 not only in cultured mesangial cells but that this can also be demonstrated in mesangioproliferative GN in vivo and that this regulation occurs at several levels. First, we investigated the effects of the MEK inhibitor, U0126, on cell cycle proteins in rat mesangial cells in vitro. In stimulated cells, we detected a pronounced increase of CDK2 activity, upregulation of the expression of cyclins E and A, reduced expression of CDK inhibitor p27KIP1, the increased assembly of cyclin A-CDK2 and cyclin E-CDK2 complexes and finally a reduction of p27KIP1-CDK2 complexes. These findings are consistent with those of other studies, showing that stimulation of mesangial cells with PDGF-B, aldosterone, bFGF or FCS lead to a decrease of p27KIP1 expression, an increase of cyclin A expression and an increase of CDK activity [23–26]. Using the MEK inhibitor U0126, we now show that these changes in cell cycle proteins of mesangial cells are mainly due to the activated MAP kinase pathway. This is in accordance with in vitro data from mesangial cells, showing that blockade of MEK abolished growth factor-induced reduction of p27KIP1 expression [14], and is in accordance with data from fibroblasts, epithelial cells and tumor cells, showing that the activated MAP kinase cascade is required for p27KIP1 downregulation and S phase entry [16,18,27]. A study in fibroblasts showed that ERK regulates the Thr160 phosphorylation of the CDK2-cyclin-E

Increased binding of cyclin A, cyclin E and CDK inhibitor p27KIP1 to CDK2 in the cortex of nephritic rats is reduced by inhibition of MEK. As shown by Western blot analysis, binding of cyclin E, cyclin A and p27KIP1 to CDK2 increased markedly upon induction of experimental GN at day 6 after disease induction. When nephritic animals were treated with the MEK inhibitor U0126, binding of all three proteins to CDK2 was reduced, thereby showing the more prominent effect with high dose U0126 (Figure 8).
Fig. 5. Expression of cyclin A, cyclin E and p27Kip1 in vivo in (A) renal cortical tissue and (B) and (C) glomerular tissue of normal rats and rats with mesangio proliferative glomerulonephritis at day 6 after disease induction, treated with or without U0126 (Western blot analysis). Densitometric analyses of each parameter are shown in the graphs. (C) p27Kip1 signals were comparable by using different antibodies; upper panel: polyclonal rabbit antibody; middle panel: monoclonal mouse antibody. Lower panel: preincubation of the polyclonal antibody with specific blocking peptides blunted the p27Kip1 signal; graph: quantification of p27Kip1 signals obtained with the polyclonal antibody. Tissues of all rats were analysed as described above. Representative results are shown. *P < 0.05 vs normal control rats without treatment, #P < 0.05 vs nephritic rats treated with vehicle only. AU, arbitrary units.
complex which is required for CDK2 activity [17], and in insulin-like growth factor-stimulated osteoblasts, the MEK/ERK pathway was demonstrated to activate CDK2 [28].

Next, we studied the role of the MEK/MAP kinase pathway activation in vivo. Consistent with our in vitro results, untreated nephritic rats exhibited an increased phosphorylation of CDK2 at Thr160 in the cortical and glomerular protein fraction, increased renal cortical CDK2 activity, increased expression of cyclins E and A and an increased assembly of CDK2-cyclin E and CDK2-cyclin A complexes. MEK inhibition blunted these changes efficiently and strongly reduced renal CDK2 activity. Surprisingly, using two different

Fig. 6. Immunohistochemical detection of glomerular cyclin A expression. (A) Normal rat, showing one glomerular cell with cyclin A expression in the nucleus (arrow). (B) Nephritic rat treated with vehicle only, showing two cyclin A-positive glomerular nuclei (arrows) and (C) a nephritic rat treated with 100 mg/kg U0126, showing one cyclin A-positive cell. (D) Quantification of glomerular cyclin A-positive cells. Nephritic tissues were from day 6 after disease induction. *P < 0.05 vs normal control rats without treatment, #P < 0.05 vs nephritic rats treated with vehicle only. Magnification 600×.

Fig. 7. Immunohistochemical detection of glomerular p27Kip1 expression. (A) Normal rat, showing about half of the nuclei stained for p27Kip1. (B) Nephritic rat treated with vehicle only, showing an increase in the number of p27Kip1 positively stained nuclei. (C) Glomerulus of a nephritic rat treated with 100 mg/kg U0126, showing a slightly decreased number of p27Kip1 positively stained nuclei compared to the nephritic control (B). (D) Quantification of glomerular p27Kip1-positive cells. Nephritic tissues were from day 6 after disease induction. *P < 0.05 vs normal control rats without treatment. Magnification 600×.
antibodies in the Western blot analyses and immunohistochemistry, p27KIP1 was upregulated and showed increased binding to CDK2 in glomerular and renal cortical tissue of nephritic animals. This observation is in contrast to findings of Shankland et al. [29], who noted a decreased p27 KIP1 expression during the mesangioproliferative phase of anti-thymocyte plasma-induced glomerulonephritis in rats. Additionally, in renal biopsy specimens of patients with IgA nephropathy, a decrease of glomerular p27KIP1 expression was observed in proliferative and sclerosing lesions [30]. Therefore, why do we detect an increase of p27KIP1 in our model? Upregulation of glomerular p27KIP1 expression has been shown to lead to cell hypertrophy [13] whereas proliferation is associated with a decrease in p27KIP1 levels [8]. However, Ophascharoensuk et al. posed a new role for p27KIP1, namely shielding tissues from inflammatory damage, particularly against apoptosis [31], and Hiromura et al. [32] showed that p27KIP1−/− mesangial cells and fibroblasts have elevated rates of apoptosis which can be blocked by recombinant p27KIP1. In experimental GN, the phase of mesangial cell proliferation is accompanied by high levels of glomerular apoptosis [33], and we described a massive induction of apoptosis within hours and mild increase of glomerular apoptosis up to 10 days following induction of anti-Thy 1.1 GN [2]. These data were now confirmed and supplemented in a glomerulonephritis time course experiment in the present study. Additionally, in our first report on U0126 intervention in experimental GN, we detected a 5.3-fold increase of glomerular apoptotic cells at day 6 in the nephritic controls compared to non-nephritic rats [3]. Our present data now show that the expression of glomerular p27KIP1 during anti-Thy 1.1 GN follows the extent of glomerular apoptosis in this model. One could therefore speculate that the cortical and glomerular increase of p27KIP1 expression in our nephritic controls reflects an attempt to protect cells from overshooting apoptosis in this phase of the disease. Another aspect which might explain the findings concerning p27KIP1 expression in our model, is that the time course and the pathophysiology of the OX-7 antibody induced anti-Thy 1.1 GN is different from the one in the study of Shankland et al. [29], who used anti-thymocyte plasma for inducing GN. Interestingly, this is supported by a study of Daniel et al. [34], who also detected an upregulated glomerular p27KIP1 in the acute phase of the OX-7-induced anti-Thy 1.1 GN. Finally, the unexpected p27KIP1 expression profile in our intervention study is also in contrast to the in vitro findings. However, one has to consider that, albeit being in a proliferative phase, the in vivo situation with many different cell types playing regulatory roles is much more complex.

Fig. 8. Binding of cyclin A, cyclin E and p27KIP1 to CDK2 in renal cortical tissue of normal rats and rats with mesangioproliferative glomerulonephritis at day 6 after disease induction, treated with or without U0126. Immunoprecipitation of CDK2 was followed by Western blot analysis. 'IP-CDK2', Immunoprecipitation with anti-CDK2 antibody. Tissues of all rats were analysed as described above. Representative results are shown. Densitometric analyses of each parameter are shown in the graphs. *P < 0.05 vs normal control rats without treatment, #P < 0.05 vs nephritic rats treated with vehicle only, §P < 0.05 vs nephritic rats treated with 10 mg/kg U0126. AU, arbitrary units.
than the in vitro situation with only one cell type and synchronized proliferation. Our data show, that one has to be cautious to simply extrapolate from in vitro data to complex in vivo events. Linking ERK activation in vivo to the regulation of the cell cycle may be of considerable therapeutic interest. Indeed, recent studies demonstrated improved renal function by blocking CDK2 activity with the purine analogue, Roscovitine, in experimental GN [35,36]. In addition, we have described that blocking CDK2 is safe in renal disease with podocyte damage, where concerns were raised that inhibition of the cell cycle might reduce the adaptive response of podocytes to injury and might therefore potentially even aggravate the course of the disease [35,36].

In summary, this is the first study to show that MEK inhibition in experimental GN markedly reduces mesangial cell proliferation via decreasing the glomerular and renal cortical cyclin E, -A and CDK2 overexpression as well as the formation of their complexes. The unexpected regulation of p27KIP1 in the in vivo model merits further investigation. Activated proteins of the MAP kinase cascade, therefore, have a key role not only in vitro but also in vivo in the downstream activation of cell cycle proteins and identify pharmacologic interference with the activation of ERK as a potential novel therapeutic approach to proliferative renal disease.

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Conflict of interest statement. None declared.

References
12. Mahimainathan L, Ghosh-Choudhury N, Venkatesan BA, Danda RS, Choudhury GG. EGF stimulates mesangial cell mitogenesis via PI3-kinase-mediated MAPK-dependent and

![Fig. 9. Glomerular mitoses, apoptosis and p27KIP1 expression in the course of anti-Thy 1.1 mesangioliprotective GN. d, day following disease induction. *P < 0.05 vs normal rats (d0), $P < 0.05 vs normal rats (d0) and nephritic rats at d14, #P < 0.05 vs normal rats (d0) and nephritic rats at d4 and d14.](image-url)


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