Role of basic fibroblast growth factor (FGF-2) in diabetic nephropathy and mechanisms of its induction by hyperglycemia in human renal fibroblasts

RADOVAN VASKO, MICHAEL KOZIOLEK, MASAMI IKEHATA*, MARIA PIA RASTALDI*, KLAUS JUNG**, HOLGER SCHMID†, MATTHIAS KRETZLER†, GERHARD ANTON MÜLLER and FRANK STRUTZ

Department of Nephrology and Rheumatology, Georg-August-University Goettingen,
**Department of Medical Statistics, Georg-August-University Goettingen,
†Medical Polyclinic, Ludwig Maximilian University, Munich, Germany, and *Fondazione D'Amico and Fondazione IRCCS Policlinico, Milan, Italy

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Corresponding address: R. Vasko, M.D.

Department of Nephrology and Rheumatology
Georg-August-University Goettingen
Robert-Koch-Str. 40
37075 Goettingen
Germany

Phone: +49-551-396331

Fax: +49-551-398507

E-mail: rvasko@inbox.com

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Abbreviations:

ANOVA – analysis of variance
BrdU – bromodeoxyuridine
BSA – bovine serum albumin
cDNA – complementary DNA
CDK – cyclin dependent kinase
CTGF – connective tissue growth factor
DMEM – Dulbecco’s modified Eagle’s medium
DN – diabetic nephropathy
DNA – deoxyribonucleic acid
DTT – dithiothreitol
EGTA – ethyleneglycoltetraacetic acid
ELISA – enzyme-linked immunosorbent assay
EMT - epithelial-mesenchymal transition
FCS – fetal calf serum
FGF-2 – basic fibroblast growth factor
FITC – fluorescein isothiocyanate
FSGS – focal segmental glomerulosclerosis
GAPDH – glyceraldehyde-3-phosphate dehydrogenase
HIV – human immunodeficiency virus
Ig – immunoglobulin
kb – kilobase
kDa – kilodalton
MAPK – mitogen-activated protein kinase
mRNA – messenger RNA
p27kip1 – kinase inhibitor protein 27 kDa
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PDGF – platelet-derived growth factor
PKC – protein kinase C
PMA - phorbol 12-myristate 13-acetate
PMSF – phenylmethanesulfonyl fluoride
RNA – ribonucleic acid
RNAi – RNA interference
RT-PCR – reverse transcriptase PCR
SDS – sodium dodecyl sulfate
SEM – standard error of mean
siRNA – small inhibitory RNA
SMA – smooth muscle actin
TGF – transforming growth factor
TPA - 12-\(O\)-tetradecanoyl phorbol-13-acetate
VEGF - vascular endothelial growth factor
Abstract

Basic fibroblast growth factor (FGF-2) plays a role in renal fibrogenesis though its potential implications for tubulointerstitial involvement in diabetic nephropathy are unknown. We evaluated the expression of FGF-2 in kidney biopsies from patients with diabetic nephropathy and studied the mechanisms of its induction in human renal fibroblasts under hyperglycemia. Tubulointerstitial expression of FGF-2 was significantly upregulated in diabetic nephropathy compared to control kidneys with a good correlation to the degree of the injury. Fibroblasts cultivated in high glucose displayed increased FGF-2 mRNA as well as protein synthesis and secretion when compared to normal glucose. Proliferation rates under hyperglycemia were significantly higher and could be almost completely inhibited by addition of a neutralizing FGF-2 antibody. Alterations in proliferation were associated with changes in p27kip1 expression. Hyperglycemia induced the expression of PKC-β1 and PKC-β2, however only inhibition of PKC-β1 but not PKC-β2 led to a significant decrease of FGF-2 levels. Relevance of the culture findings and functional association was corroborated by colocalisation of FGF-2 and PKC-β in human diabetic kidneys in vivo. High glucose stimulated fibronectin synthesis and secretion, which could be substantially prevented by inhibition of PKC-β1 and to a lesser extent by inhibiting the FGF-2. Expression of active phosphorylated form of p38 mitogen-activated protein kinase was upregulated under hyperglycemia, however its inhibition had no effects on FGF-2 synthesis. Our results implicate a role of FGF-2 in high glucose altered molecular signaling in pathogenesis of diabetic renal disease.
Introduction

Although glomerular changes remain the main focus in investigation of diabetic renal disease, numerous studies have determined that tubulointerstitial involvement plays an important role, even in early stages of the disease (47). Diabetics with preserved tubulointerstitium have better kidney survival rates, despite variable degrees of glomerulosclerosis (4). Interstitial fibroblasts and proximal tubular cells, the two major cells in the tubulointerstitium, play pivotal role in tubulointerstitial pathology of diabetes. Both respond directly to glucose, communicate through paracrine mechanisms and regulate each other reciprocally (15, 16). Hyperglycemia associated molecular pathways involve many cytokines and growth factors which may cause alteration of renal function and damage. Particularly, important roles for transforming growth factor-β (TGF-β) (32) and connective tissue growth factor (CTGF) (43) have been described in diabetic nephropathy. In addition, angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2) were reported to contribute to the glucose-induced vascular dysfunction (34, 48). Nevertheless, the importance of their non-angiogenic effects in kidney remains unclear. FGF-2, one of the first described members of the FGF family, is a pleiotropic growth factor involved in developmental processes, wound healing, angiogenesis and tumorigenesis. We previously described its involvement in human renal fibrogenesis (37, 38) and in epithelial-mesenchymal transition (EMT) (39). FGF-2 is normally synthesized at low levels but its elevation was observed in various pathological conditions including diabetes. Increased serum levels of FGF-2 have been reported in diabetic women with retinopathy (14). Ogata et al. found upregulated expression of FGF-2 in peritoneal mesothelial cells and fibroblasts under hyperglycemia (27). Our current study investigated the role of FGF-2 in human diabetic kidney disease. We examined the expression of FGF-2 in kidney biopsies with diabetic nephropathy and
the correlation to the degree of tubulointerstitial injury. In vitro, we investigated FGF-2 expression in cultured human renal fibroblasts under hyperglycemia and the mechanisms implicated in the regulation of its expression.
**Materials and Methods**

*FGF-2 Expression in Human Kidney Biopsies*

Tubulointerstitial expression of FGF-2 was examined in kidney biopsies from patients with diabetic nephropathy (DN, n=20) using quantitative real-time PCR and material from the European Renal cDNA Bank (see appendix for participating centers). Donor kidneys (0-biopsies) and normal parts from tumour nephrectomies served as controls (n=12). Sample preparation and processing was performed as described previously (6). In brief, tubulointerstitial tissue was microdissected, RNA was isolated using Qiagen RNeasy mini columns, total RNA was reverse transcribed using random priming and RT-PCR was performed using 18S rRNA as a reference housekeeper and the standard curve method for quantification. FGF-2 primer and probes were designed to be cDNA specific and obtained from ABI Weiterstadt Germany using the human FGF-2 sequence (NM 002006). The severity of the tubulointerstitial lesions was assessed and semiquantitatively graded from 0 (absent) to 3 (severe damage). 1 indicates focal lesions involving ≤25% of the renal parenchyma and interstitium; 2 indicates moderate damage, affecting 26-49% of the parenchyma and interstitium and 3 indicates severe extensive tubulointerstitial changes and fibrosis involving ≥50% of the parenchyma and interstitium. A renal pathologist blinded for the clinical and experimental data performed the histopathologic analysis. The use of kidney biopsies for research purposes was approved by the local ethics committee of the University of Goettingen as well as the local ethics committees of the other participating centers, and written consent was obtained from all patients prior to biopsy.

*Immunohistochemistry for FGF-2*
Stainings were performed by indirect immunohistochemistry. Briefly, 5 μm thick acetone-fixed frozen sections were incubated with the primary antibody (rabbit anti-FGF-2, Abcam, Cambridge, UK), followed by fluorescent-labeled secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen, Italy). For double stainings, sections were incubated with the first primary mouse anti-PKC-ß1 or mouse anti-PKC-ß2 antibody (Santa Cruz), followed by AlexaFluo 488 goat anti-mouse antibody; after adequate washing, the procedure was repeated with the second primary rabbit anti-FGF-2 antibody followed by AlexaFluo 546 goat anti-rabbit antibody. Specificity of antibody labeling was demonstrated by the lack of staining after substituting proper control immunoglobulins for the primary antibodies. Slides were mounted with Fluorsave aqueous mounting medium (Calbiochem, Italy) and images were acquired by a Zeiss Axioscope 40FL microscope.

Slides were mounted with Fluorsave aqueous mounting medium (Calbiochem, Italy) and images were acquired by a Zeiss Axioscope 40FL microscope. The evaluation of the percentage of the interstitial area occupied by FGF-2 staining and the evaluation of interstitial fibrosis were performed by using appropriate macros (essentially formed by color threshold procedures and filtering) on images taken at 200x enlargement, avoiding glomeruli. The software was programmed to automatically calculate the percentage of the image resulting from the specific macro. Measurements were performed on an average of 6±1 images/biopsy.

**Cell Culture**

The human fibroblast cell line Tk-173 (obtained from a normal kidney) has been characterized previously (25). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL Ltd., Scotland) supplemented with 10% fetal calf serum (FCS, Gibco) and 2 mM L-
glutamine (PAA Laboratories, Austria). For experimental setup, cells were grown to subconfluence and subsequently made quiescent in serum free low glucose DMEM for 24 hours before changing to the experimental media (all serum free). DMEM with 5.5 mM D-glucose was used as a normal glucose medium, DMEM with 25 mM D-glucose as a medium with diabetic glucose concentration and DMEM with 5.5 mM D-glucose with addition of 19.5 mM D-mannitol served as osmotic control.

**FGF-2 mRNA Expression**

Cells were cultivated in media with normal or high glucose concentration as mentioned above for 24-72 hours and total RNA was extracted using RNA-Bee reagent (Tel-Test, Friendswood, TX, USA). FGF-2 PCR of reverse-transcribed RNA was performed as described previously (26). Products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Densities of the products were measured with Bio-Rad MultiImager scanner and evaluated with Bio-Rad Multi-Analyst software. FGF-2 bands were normalized for GAPDH.

**FGF-2 Immunoblot**

Cells were washed twice with ice-cold PBS and lysed in buffer containing 0.4% sodium deoxycholate, 1% Igepal C-630, 1.9% EGTA, 10 mM Tris (pH 7.4) and 100 μg/mL phenylmethylsulfonyl fluoride. After 15 min incubation on ice, cells were detached, lysates were collected and clarified by centrifugation, and the supernatants were used for the experiments. Equal amounts of total cellular protein were suspended in Laemmli sample buffer and boiled for 5 minutes. SDS-polyacrylamide gel electrophoresis was performed on 15% gel, followed by transfer to nitrocellulose membrane (Amersham Biosciences, England) by electroblotting. Non
specific binding was blocked with 5% nonfat dried milk in PBS/0.1% Tween 20 at 4°C overnight and the blots were incubated with the primary anti-FGF-2 antibody (Becton Dickinson, Germany), followed by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham). Visualisation was performed with a chemiluminescence detection system (ECL Plus; Amersham) according to the manufacturer’s instructions. Nuclear extracts were prepared according to Marui et al. (21). Briefly, after incubation, cells were washed twice with ice-cold PBS, and lysed in buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF and 0.1% Igepal C-630 by incubating on ice for 15 minutes. Lysates were centrifuged at 3500 rpm for 4 min and supernatants stored as cytosolic fraction. Nuclear pellets were washed with buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, PMSF 1 mM and next incubated in buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA and 1 mM PMSF on ice for 30 minutes. After centrifugation at 14000 rpm for 10 min, supernatants were collected and stored as nuclear extracts.

**Assay for FGF-2 Secretion**

Cells were seeded in 24-well culture plates (3x10⁴/well) and cultivated for 24-72 hours. Supernatants were collected and FGF-2 concentration was determined using an enzyme-linked immunosorbent assay (R&D Systems, USA) according to the protocol submitted by the manufacturer. The amount of secreted FGF-2 was corrected for cell numbers and expressed in pg/10³ cells. The assay determines the concentration of free mature FGF-2. No cross-reactivity with other members of FGF family was observed. The intraassay and interassay coefficient of variation was 6.7% and less than 15% respectively (sensitivity for FGF-2 was less than 3 pg/mL).
**Fibronectin Expression**

Fibronectin protein synthesis and expression was examined with immunoblot and ELISA respectively. Cells were cultivated in media with normal or high glucose as mentioned above for 24-72 hours. For inhibitory studies, a neutralizing FGF-2 antibody (10 μg/mL, R&D Systems, USA) or a specific PKC-β1 inhibitor, or both were added to high glucose medium. Immunoblot was performed using a rabbit anti-fibronectin antibody (Sigma, Germany) as described above. ELISA of supernatants was performed as described previously (35) and the results were corrected for cell numbers.

**Proliferation Studies**

Proliferation studies were performed using bromodeoxyuridine (BrdU) incorporation assays (Biotrak ELISA, Amersham) and cell counts. Briefly, cells were incubated in 96-well plates containing DMEM with the usual supplements for 24 hours. After 24-hour starvation, experimental media were added. For the inhibitory studies, a neutralizing FGF-2 antibody in a concentration of 10 μg/mL or a non-relevant IgG immunoglobulin, were added to high glucose medium. Media were replaced by the same media but with added BrdU four hours prior to measurement. Assay procedure was performed according to the manufacturer’s instructions and optical densities were measured photometrically at 450 nm. In order to confirm true cell proliferation, cell numbers were determined using a Neubauer chamber. Furthermore, expression of p27\(^{kip1}\) protein was examined in cells cultured in normal and high glucose and after addition of a neutralizing FGF-2 antibody to high glucose by immunoblot using the anti-p27\(^{kip1}\) antibody (Becton Dickinson) as described above.
Immunofluorescence for PKC-β1 and PKC-β2

Cells were grown to subconfluence on chamber slides (Lab-tek) and starved for 24 hours. Next, cells were cultured in normal or high glucose media for 48 hours. Afterwards, the media were removed, cells washed 3 times with PBS and fixed with methanol/acetone. Cells were washed 3 times with PBS, permeabilized with 0.1% Triton X-100 for 1 minute and blocked with 1% BSA in PBS for 1h. Cells were incubated with PKC-β1 or PKC-β2 antibody (Santa Cruz, USA) for 1h at 37°C, washed 3 times with PBS, and incubated with secondary FITC conjugated antibody (Jackson Laboratories) for 1h at 37°C. Finally, cells were mounted by Vectashield-mounting medium (Vector Laboratories) and visualisation was performed using an Olympus IX10 fluorescence microscope.

PKC-β1 Expression

Cells were cultivated in normal and high glucose for 24-72 hours and the expression of PKC-β1 was examined by immunoblot using an anti-PKC-β1 antibody (Santa Cruz, USA). The effect of FGF-2 was studied by addition of its neutralizing antibody to high glucose medium.

PKC Inhibition Using Calphostin C

Cells were grown to subconfluence and then made quiescent in serum free medium. Raising concentrations of calphostin C (Calbiochem, USA) were added to experimental media, cells grown in medium without inhibitor served as control. The concentration range of calphostin (25-200 nM) was chosen due to specific inhibition of PKC and no toxic effects on cell viability in
preliminary experiments. Total RNA was extracted and FGF-2 mRNA expression was examined using RT-PCR as described above.

**PKC-β1 and PKC-β2 Inhibition**

Cells were cultivated under hyperglycemia with addition of beta-1 and beta-2 inhibitory concentrations (21 nM and 5 nM, respectively) of a specific PKC-β inhibitor (4-anilino-1H-pyrrole-2,5-dione, Calbiochem) (41) for 24-72 hours. We chose this inhibitor since it is the only one commercially available with specificity for beta isoforms, which was reported by previous studies. Total cellular protein was extracted and immunoblots for PKC-β1 and FGF-2 were performed as described above.

**PKC-β1 Inhibition using RNA Interference**

RNA interference was applied to selectively suppress the expression of PKC-β1. Specific siRNA targeted to the coding region of PKC-β1 was created using GenBank and synthesized by Ambion siRNA service. BLAST search of the target sequence was performed to prevent unwanted silencing of genes which contain an identical sequence. Cells were seeded into 6-well culture plates (2x 10^5/well) and transfected with siRNA using RNAi transfection reagent (RNAiFect, Qiagen). After transfection and cultivation in high glucose media for 24-72 hours, RNA and protein were extracted. FGF-2 mRNA and protein expression was examined by RT-PCR and immunoblot as described above. Cells transfected with a control non-silencing siRNA (Qiagen, Germany) and cells incubated with transfection reagent alone served as controls.

**p38 MAPK Expression**
Cells were cultivated in normal glucose, high glucose and in mannitol supplemented medium for 24-72 hours and the expression of p38 and phosphorylated p38-MAPK was examined by immunoblot using anti-p38 and anti pp38 MAPK antibodies (Santa Cruz). For inhibitory studies, a specific p38 MAPK inhibitor (10 µM SB203580, Calbiochem) was added to high glucose medium.

**Cell Viability**

Cell viability was assessed by the trypan blue exclusion assay. Potential differences between cells cultivated in the experimental media were examined. Furthermore, to exclude toxicity, cell viability was examined in studies using the neutralizing FGF-2 antibody and in experiments with PKC and p38 MAPK inhibitors.

**Statistical Analyses**

All experiments were repeated at least three times and results are presented as mean values ± standard error of mean. Data sets were tested for normal distribution by quantile-quantile-plots and the differences between the groups were analyzed using t-test. FGF-2 expression and serum creatinin were compared between the three distinct groups of kidney damage by Kruskal-Wallis tests. Wilcoxon rank-sum tests were used for subsequent pairwise comparisons. All analyses were performed with the software R or SPSS. P values <0.05 were considered statistically significant.
Results

FGF-2 Expression in Biopsies from Diabetic Patients

A marked upregulation of FGF-2 was detected within the tubulointerstitium of diabetic patients (Table 1) compared to 0-biopsies and normal parts from tumor nephrectomies (Figure 1A, 3.34-fold and 1.78-fold respectively, p=0.004). Statistical analysis revealed a significant relationship between FGF-2 expression (Figure 1B, p=0.003) as well as serum creatinine levels (Figure 1C, p=0.029) and the degree of the tubulointerstitial injury. Pairwise comparisons yielded a significant difference of FGF-2 expression between all three damage scores and a significant difference of serum creatinine levels between damage score 1 and 3. We are aware of the limited number of studied subject and do not want to jump to conclusions about the causation of the association between FGF-2 and damage, based only of these clinical parameters. In order to prove the causative role of the FGF-2/damage association, further studies with a large number of subjects would be required. Immunofluorescence revealed different staining patterns in control and diabetic kidney (Figure 1D); control kidneys were negative, whereas FGF-2 was expressed in tubulointerstitial compartments of patients with diabetic nephropathy (Table 2 and 3). We know from our earlier studies that a large number of FGF-2 positive cells in the interstitial space are fibroblasts though macrophages may express the protein as well.

FGF-2 Expression in Cell Culture

Fibroblasts cultured under hyperglycemia showed significantly higher FGF-2 expression, both at mRNA and protein levels compared to normoglycemia. RT-PCR and immunoblot demonstrated similar patterns of FGF-2 expression with a maximum at 48 hours (Figure 2A, 302.2 ± 13.88% and Figure 2B, 270.39 ± 17.02% respectively). After 48 hours, mRNA and protein levels
decreased but still remained increased under hyperglycemia. Mannitol did not have significant effect though there was a slight increase of FGF-2 at 24 hours. FGF-2 secretion, measured by ELISA, was also upregulated in high glucose, peaking at 72 hours (Figure 2C, 150.85 ± 4.89%, 5.05 ± 0.16 pg/10^3 cells), however without significant differences at 24 hours. Since the mechanisms of FGF-2 secretion are complex and not fully understood, we examined its protein levels separately in cytosolic and nuclear fraction. We found a different pattern of subcellular FGF-2 distribution at 24 hours with predominant nuclear localization under hyperglycemia (Figure 2D). This may represent a nuclear autoinduction of FGF-2 under hyperglycemia, a process previously described in vascular smooth muscle cells (2). A fraction of the nuclear FGF-2 may also arise from translocation from cytosol in high glucose. We observed an analogous translocation of the p65 but not the p50 subunit of NF-kB under hyperglycemia (data not shown).

**Proliferation Studies**

High glucose induced a time-dependent increase in bromodeoxyuridine incorporation with a maximum at 72 hours (Figure 3A, 221.85 ± 12.12%), which correlated to the peak of FGF-2 secretion. Addition of a neutralizing FGF-2 antibody to high glucose decreased (Figure 3A, max. 72h, 56.12 ± 6% compared to high glucose), whereas addition of a non-relevant immunoglobulin (control) caused no significant changes in bromodeoxyuridine incorporation. At the same time, cell counts demonstrated that bromodeoxyuridine incorporation rates were paralleled by changes in absolute cell numbers (Figure 3B, 199.06 ± 11.81%). Cell number peaked at 96 hours under hyperglycemia, although the additional increase after 72 hours was only minimal. Thus, as expected, the peak in cell number was later than the peak in bromodeoxyuridine incorporation. Cells cultured in high glucose displayed decreased expression
of p27kip1 protein compared to cells grown in normoglycemia (Figure 3C, max. 24h, 31.09 ± 6.75%), which correlated with increased proliferation rates under hyperglycemia. Addition of a neutralizing FGF-2 antibody reversed the depression of p27kip1 in high glucose.

**Protein Kinase C Immunofluorescence**

Immunofluorescence of fibroblasts cultured for 48 hours demonstrated upregulated expression of PKC-ß1 with a perinuclear staining pattern and vesicular appearance under hyperglycemia (Figure 4B) compared to normoglycemia (Figure 4A). PKC-ß2 showed preferentially a nuclear staining pattern with granular appearance. Staining pattern of PKC-ß2 was also augmented in high glucose (Figure 4D) compared to normal glucose medium (Figure 4C).

**Protein Kinase C Expression**

PKC-ß1 showed sustained increased expression under hyperglycemia with a maximum at 72 hours (Figure 5A, 321.48 ± 36.38%) compared to normal glucose. Addition of a neutralizing FGF-2 antibody to high glucose gradually decreased the expression of PKC-ß1 in the cells.

**Protein Kinase C Inhibition**

In preliminary experiments, we examined the expression of FGF-2 under hyperglycemia in the presence of calphostin C, a potent specific nonselective PKC inhibitor. Cells were incubated with different concentrations of calphostin C for 48 hours, where the maximal upregulation of FGF-2 had been observed in our previous experiments. Calphostin C dose dependently reduced the FGF-2 mRNA levels compared to medium without inhibitor (Figure 5B, dose range 25 - 200 nM, percent of control: 91.23±3.72 - 36.04±4.43%). Next, we selectively inhibited the ß1- and
ß2-isoform of PKC and examined the expression of FGF-2. Inhibition of PKC-β1, but not PKC-β2 caused downregulation of FGF-2 protein (Figure 5D, max. 48h, 34.34 ± 6.64% compared to high glucose) with repression pattern similar to that of PKC-β1 (Figure 5C, max. 48h, 37.33 ± 6.22% compared to high glucose).

**Specific Inhibition of PKC-β1 by RNA Interference**

Owing to the important role of the PKC-β1 of in the pathogenesis of diabetic complications, we further investigated functional involvement of this isoform using RNA interference. RT-PCR and immunoblot confirmed efficient repression of endogenous PKC-β1 synthesis with siRNA (Figure 6A). Cells transfected with PKC-β1 specific siRNA showed a robust decrease of hyperglycemia induced FGF-2 mRNA (Figure 6B, max. 24h, 19.45±3.75%) and protein (Figure 6C, max. 24h, 25.21±6.07%) levels, which paralleled the repression pattern of PKC-β1 (Figure 6A, max. 24h, 57.09±6.05). The gradually normalizing expression of PKC-β1 and FGF-2 reflects the regeneration of cellular synthetic apparatus resulting from consecutive degradation of the inhibitory (but non-lethal) dose of siRNA. The disproportionate raise of FGF-2 protein compared to FGF-2 mRNA at 72 hours may reflect the significant raise of FGF-2 mRNA levels between 24-48 hours (almost 2.4-fold) and as mentioned above, the subsiding effect of the siRNA. Cells transfected with a control non-silencing siRNA and cells incubated with transfection reagent alone showed no differences in FGF-2 and PKC-β1 expression. To confirm functional relevance of the reciprocal FGF-2/PKC-β regulation observed in cell culture experiments, we performed stainings for FGF-2 and PKC-β1/PKC-β2 in human diabetic kidneys. Double immunofluorescence revealed colocalisation of FGF-2 and PKC-β in vivo and confirmed so the relevance of our functional findings (Figure 7).
**p38 MAPK Expression**

Fibroblasts cultivated under hyperglycemia demonstrated constantly increased amounts of the active phosphorylated p-p38 MAPK (Figure 8B, max. 24h, 163.07±27.93%). Expression of the total p38 MAPK showed a delayed upregulation at 72 hours (Figure 8A, 150.22±9.20%). Even so, addition of a specific p38 MAPK inhibitor to high glucose medium did not affect the expression of FGF-2, making it unlikely that p38 MAPK is directly involved in the FGF-2 induction.

**Fibronectin Expression**

Hyperglycemia strongly induced fibronectin protein synthesis in the cells (Figure 9A, max. 72h, 283.33±23.33%). Addition of a neutralizing FGF-2 antibody or a PKC-ß1 inhibitor substantially prevented this increase with a more profound effect using the PKC-ß1 inhibitor. Simultaneous addition of both, neutralizing FGF-2 antibody and PKC-ß1 inhibitor, did not exhibit significant additive effects on fibronectin protein synthesis compared to PKC-ß1 inhibition alone. This more profound effects of PKC-ß1 inhibition can be explained by the more significant and general role of this kinase in cellular signaling. The secretion of fibronectin under hyperglycemia did not differ significantly at 24 hours and only slightly increased at 48 hours compared to normal glucose. However at 72 hours, we observed a prominent augmentation of fibronectin secretion under hyperglycemia (Figure 9B, max. 72h, 181.67±10.14%), which could be almost entirely prevented by addition of the PKC-ß1 inhibitor. Inhibition of FGF-2 alone resulted in only a small decrease of fibronectin secretion, which suggests only a minor role for FGF-2 in the process of fibronectin secretion. There is a lot of convincing data supporting the key role of TGF-ß in
extracellular matrix production in diabetic and non-diabetic kidney disease. Our experimental data presented in Figure 9 demonstrate only one “particular selected and analyzed” pathway, which cannot be naturally separated from the complex cytokine cellular network in vivo.
Discussion

FGF-2 has been proposed to have an important function in the morphogenesis of the embryonic kidney with abundant expression in epithelial cells of the branching ureteric bud. In adult human kidney, FGF-2 expression has been described in glomerular endothelial, parietal, and visceral cells, tubular epithelial cells and interstitial fibroblasts (5, 11, 37). Our data demonstrated an upregulated expression of FGF-2 in diabetic nephropathy *in vivo*. Expression of FGF-2, as shown previously in eye and heart, is regulated transcriptionally and thus its protein levels usually correlate to the amount of mRNA synthesized. To exclude a passive trapping of FGF-2 in renal parenchyma, we examined expression of both, FGF-2 mRNA and FGF-2 protein within the tubulointerstitium. We were able to demonstrate concurrently increased FGF-2 mRNA and protein levels, which together with the cell culture findings suggest a transcriptional character of FGF-2 regulation in kidney. Expression of FGF-2 correlated to the degree of tubulointerstitial injury and serum creatinine levels. Increased interstitial expression of FGF-2 was previously reported in IgA nephropathy and focal segmental glomerulosclerosis (FSGS) with more prominent expression in the later, reflecting more severe degree of the injury (33). In hyperglycemic conditions, fibroblasts showed upregulated FGF-2 mRNA as well as protein synthesis and secretion *in vitro*. Secretion of FGF-2 correlated with proliferation rates of the cells, suggesting a direct stimulation by autocrine and/or paracrine action as described previously by our group (37). Proliferation of fibroblasts is a recognized mechanism in renal pathology (22, 36). FGF-2 has been described to induce proliferation of tubular epithelial (31), mesangial (9, 10), and glomerular parietal cells (7, 40) as well as of renal fibroblasts (37, 38). Diabetic milieu per se causes alterations of cell cycle control mechanisms resulting in subsequent proliferation abnormalities of the cells. Though most renal cells are arrested in the G1-phase of the cell cycle
after actively leaving G0-phase, selected cell populations such as fibroblasts may undergo sustained proliferation in diabetes (44). The cyclin kinase inhibitor p27^Kip1 reduces the activity of cyclin E or A, and regulates the progression from a quiescent phase into the G1 and S-phase of the cell cycle. In general, growth factors which prevent cell growth cause an increase in p27^Kip1 levels. Increased expression of p27^Kip1 causes the mesangial cells to undergo hypertrophy instead of hyperplasia, one of the earliest morphological abnormalities seen in diabetes (45, 46). There is only sparse data about FGF-2 and p27^Kip1 interactions in fibroblast cells. In pulmonary fibroblasts, FGF-2 induced proliferation was not accompanied by changes of p27^Kip1 levels (17). In our study, the decreased expression of p27^Kip1 in hyperglycemia could be substantially prevented by inhibition of FGF-2 suggesting a potential role of p27^Kip1 in FGF-2 stimulated proliferation. Hyperglycemia causes activation of various isoforms of protein kinase C, although the beta isoform appears to be preferentially activated in diabetes. In the kidneys of Sprague-Dawley diabetic rats, both PKC-β1 and PKC-β2 were upregulated (29), whereas in spontaneously hypertensive diabetic rats, increase of the delta but not the beta isoform was noted (28). We observed increased perinuclear staining with vesicular appearance of PKC-β1 and increased nuclear staining with granular appearance of PKC-β2 under hyperglycemia. The staining patterns of PKC-β reported in the literature are not consistent. The majority of studies reported membrane accumulation of PKC-β1 upon stimulation, however these studies were mostly performed in non-fibroblast cell lines. In mouse embryonic fibroblasts, TPA stimulation caused nuclear translocation of PKC-β1 (3). Treatment of monocytes with interferon-gamma resulted also in nuclear translocation of PKC-β1 but not of PKC-β2 (23). Photomicrographs of the mesangial cells in the work of Kumar et al. revealed perinuclear accumulation of PKC-β1 upon PMA stimulation (19). Thus, it is evident that the activation pattern of PKC-β is not
uniform and seems to be cell type and stimulus dependent. The FGF-2 gene promoter contains a PKC-responsive region controlling its expression (24). In our work, inhibition of the PKC-ß1 but not PKC-ß2 repressed the synthesis of FGF-2. On the other hand, neutralization of FGF-2 attenuated the expression of PKC-ß1 induced by hyperglycemia. We observed a good colocalisation of FGF-2 and PKC-ß1 expression in human diabetic kidneys in vivo and confirmed so the relevance of cell culture findings. These results demonstrate for the first time a reciprocal signaling between FGF-2 and PKC-ß1 in human diabetic nephropathy. Altered p38 MAPK expression is a feature in human and experimental diabetic nephropathy (1). Even though hyperglycemia stimulated expression of the active phospho-p38 MAPK in our cells, specific inhibition of this kinase did not have effect on FGF-2 levels. Thus, p38 MAPK seems to act as a downstream mediator of FGF-2 rather as a direct regulator of its expression (20).

So how FGF-2 may be involved in the pathogenesis of diabetic nephropathy? Our previous studies demonstrated that TGF-ß1 stimulated proliferation of renal fibroblasts is mediated in part by induction of FGF-2 (38). Conversely, FGF-2 stimulates the secretion of preformed latent TGF-ß1 (30). Mesangial cells produce and release FGF-2 after injury, which may stimulate their proliferation in an autocrine fashion (9, 13). FGF-2 stimulation of extracellular matrix production, as shown in our present study may have long-term deleterious effects in progressive renal diseases. Treatment of rats with FGF-2 resulted in focal segmental glomerulosclerosis (18) and augmented the podocyte injury in membranous nephropathy (12). Conversely to the deleterious effects in chronic renal disease, there are several reports of possible protective role of FGF-2 in acute ischemic renal injury (8). This can be explained by an acute character of the renal damage and by FGF-2 induced expression of different morphogens also involved in early kidney development (42). The healing effects of topical application of FGF-2
in diabetic ulcers are equivocal and mediated mainly through stimulation of new dermis and vessel formation.

In summary, we demonstrated an upregulated expression of FGF-2 in diabetic nephropathy with a good correlation to the degree of renal injury. This in vivo finding was confirmed by increased expression in human renal fibroblasts grown in hyperglycemic conditions in vitro. Increased expression of FGF-2 stimulates fibroblasts proliferation and to a small degree also extracellular matrix production and therefore may play a role in the promotion of diabetic tubulointerstitial injury. Our results provide a novel association between FGF-2 and the established link of hyperglycemia – PKC-β - diabetic complications, and implicate a role of this growth factor in high glucose altered molecular signaling in diabetic kidney disease.
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Appendix

European Renal cDNA Bank participating centers:

Figure and Table Legends:

Figure 1

Expression of basic fibroblast growth factor (FGF-2) within the tubulointerstitium of diabetic kidneys examined with quantitative real-time PCR after tissue microdissection from renal biopsies. (A) Tubulointerstitial expression of FGF-2 mRNA was significantly up-regulated in biopsies from patients with diabetic nephropathy compared to controls (0-biopsies and tumor nephrectomies). Results are expressed as FGF-2/18S rRNA ratio. * p < 0.05 versus controls (B) FGF-2/18s RNA expression with respect to the severity of tubulointerstitial damage (C) Serum creatinine levels with respect to the severity of tubulointerstitial damage (D) displays FGF-2 immunohistochemistry of the tubulointerstitial space in a normal (control) and a diabetic kidney (diabetic). Magnification 400x

Figure 2

Basic fibroblast growth factor (FGF-2) expression is significantly induced by hyperglycemia but not by mannitol compared to normal glucose in human renal fibroblasts. (A) FGF-2 mRNA expression detected by RT-PCR and expressed relative to GAPDH. (B) FGF-2 protein synthesis examined by immunoblot and expressed relative to β-actin. (C) FGF-2 protein secretion detected by enzyme-linked immunosorbent assay. The results were corrected for cell numbers. (D) FGF-2 protein subcellular distribution examined by immunoblot separately in cytosolic and nuclear fractions. There is a disproportional distribution of FGF-2 at 24 hours in high glucose. * p < 0.05, ** p < 0.01 versus normal glucose

Figure 3

Bromodeoxyuridine incorporation (A) and cell counts (B) examined in human renal fibroblasts. Cells grown under hyperglycemia showed constantly higher bromodeoxyuridine incorporation as well as proliferation rates compared to normal glucose. Addition of a neutralizing FGF-2 antibody to high glucose decreased, whereas addition of a non-relevant immunoglobulin resulted in no significant changes in cell proliferation. Fibroblasts cultured in normal glucose at 24 hours were chosen as control. For the experiments with neutralizing antibody and non-relevant immunoglobulin, cells cultured in high glucose were chosen as
control. * p < 0.05, ** p < 0.01 versus control cells (C) Expression of p27\textsuperscript{kip1} protein in human renal fibroblasts examined by immunoblot. Cells cultured under hyperglycemia repressed the expression of p27\textsuperscript{kip1} compared to normal glucose. Addition of a neutralizing FGF-2 antibody to high glucose increased the expression of p27\textsuperscript{kip1} protein. * p < 0.05 versus normal glucose

Figure 4

Immunofluorescence showed augmented expression with a perinuclear pattern and vesicular appearance of PKC-β1 and a preferentially nuclear pattern with granular appearance of PKC-β2 in human renal fibroblasts under hyperglycemia. Immunostaining for PKC-β1 (A, B) and PKC-β2 (C, D) in cells cultured in normal (A, C) and high glucose (B, D) media for 48 hours. Magnification: 400x.

Figure 5

(A) Expression of PKC-β1 protein examined by immunoblot was increased under hyperglycemia compared to normal glucose. Addition of a neutralizing FGF-2 antibody to high glucose resulted in repression of PKC-β1. (B) FGF-2 mRNA expression examined by RT-PCR in cells cultivated for 48 hours under hyperglycemia with different concentrations of calphostin C. Calphostin C caused dose-dependent down-regulation of FGF-2 mRNA. The results represent the percentages of FGF-2 mRNA expression without addition of inhibitor (= 100%). * p < 0.05, ** p < 0.01 versus control. (C) (D) PKC-β1 and FGF-2 protein expression in human renal fibroblasts under hyperglycemia with addition of β1- or β2-selective concentration (21 nM and 5 nM, respectively) of a specific PKC-β inhibitor. Inhibition of PKC-β1, but not PKC-β2 repressed the synthesis of FGF-2 which paralleled the repression pattern of PKC-β1. Expression was examined by immunoblot and displayed relative to β-actin protein. * p < 0.05 versus high glucose

Figure 6

Inhibition of PKC-β1 with siRNA showed robust decrease of hyperglycemia induced FGF-2 mRNA and protein levels in human renal fibroblasts, which paralleled the repression pattern of PKC-β1.

Part (A) displays the effects on PKC-β1 protein synthesis in cells cultivated under hyperglycemia for 24 to 72 hours after transfection with a PKC-β1 specific and a control non-
silencing siRNA. The results represent the percentages of control cells. * P < 0.05 versus control cells. Parts (B) and (C) display the FGF-2 expression in cells cultivated under hyperglycemia for 24 to 72 hours after transfection with PKC-β1 specific siRNA. (B) FGF-2 mRNA expression was detected by RT-PCR and expressed relative to GAPDH. (C) FGF-2 protein expression was detected by immunoblot and expressed relative to β-actin protein. * p < 0.05, ** p < 0.01 versus control cells.

**Figure 7**

Double immunofluorescence demonstrating the coexpression (yellow) of FGF-2 (red) and PKC-β1 or PKC-β2 (green) in two human diabetic kidneys. Magnification x630.

**Figure 8**

Expression of p38 mitogen-activated protein kinase (MAPK) in fibroblasts cultivated in normal glucose, high glucose and in mannitol supplemented medium for 24 to 72 hours examined by immunoblot. (A) Expression of the total p38 MAPK showed a delayed increase at 72 hours in high glucose (B) Cells cultured under hyperglycemia showed constantly increased levels of the active phosphorylated form of p38 MAPK. * p < 0.05 versus normal glucose (C) Addition of a specific p38 MAPK inhibitor SB203580 to high glucose medium did not affect the expression of FGF-2. Cells cultured in normal glucose were chosen as control (= 100%).

**Figure 9**

Fibronectin expression in human renal fibroblasts cultured in normal or high glucose media, and under hyperglycemia with addition of a neutralizing FGF-2 antibody or a PKC-β1 inhibitor. (A) Fibronectin protein synthesis examined by immunoblot and expressed relative to β-actin protein. (C) Fibronectin protein secretion detected by enzyme-linked immunosorbent assay. The results were corrected for cell number. * p < 0.05, ** p < 0.01 versus normal glucose.

**Table 1**
Expression of FGF-2 within the tubulointerstitium examined with quantitative real-time PCR in kidney biopsies from twenty patients with diabetic nephropathy. Serum-creatinine levels and tubulointerstitial damage score distribution within the patient group.

Table 2
Results of the immunohistochemical stainings for FGF-2 in diabetic kidneys. Diabetic glomerulopathy was classified as not present (-), diffuse (d), nodular (n) or mixed (m).

Table 3
Characteristics of the patients whose kidneys were biopsied and stained for FGF-2 according to age, sex, duration of diabetes, creatinine (mg/dl), proteinuria (g/24h), and HbA1c.
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