Role of CX3C-chemokine CX3C-L/fractalkine expression in a model of slowly progressive renal failure

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Abstract

Background. The chemokine/chemokine receptor pair CX3C-L/CX3C-R is suspected to play a role in renal fibrogenesis. The aim of this study was to investigate their function in an animal model of slowly progressive chronic renal failure.

Methods. Functional data were analysed in folic acid nephropathy (FAN) at different time points (up to day 142 after induction). Immunostaining for CX3C-L, CD3, S100A4, collagen type I, fibronectin, alpha-smooth muscle actin, Tamm-horsfall protein, aquaporin 1 and 2 as well as quantitative real-time PCR (qRT–PCR) for CX3C-L, CX3C-R and fibroblast-specific protein 1 (FSP-1) were performed. Additionally, regulatory mechanisms and functional activity of CX3C-L in murine proximal and distal tubular epithelial cells as well as in fibroblasts were investigated.

Results. CX3C-L/GAPDH ratio was upregulated in FAN 3.4-fold at day 7 further increasing up to 7.1-fold at day 106. The expression of mRNA CX3C-L correlated well with CX3C-R (R² = 0.96), the number of infiltrating CD3+ cells (R² = 0.60) and the degree of tubulointerstitial fibrosis (R² = 0.56) and moderately with FSP-1 (R² = 0.33). Interleukin-1β, tumour necrosis factor-α, transforming growth factor-β as well as the reactive oxygen species (ROS) H₂O₂ were identified by qRT–PCR as inducers of CX3C-L/fractalkine (FKN) in tubular epithelial cells. Functionally, CX3C-L/FKN chemoattracts peripheral blood mononuclear cells, activates several aspects of fibrogenesis and induces the mitogen-activated protein kinases in renal fibroblasts.

Conclusions. In FAN, there is a good correlation between the expression of CX3C-L with markers of interstitial inflammation and fibrosis which may result from upregulation by pro-inflammatory and pro-fibrotic cytokines as well as by ROS in tubular epithelial cells. The FKN system may promote renal inflammation and renal fibrogenesis.

Keywords: chronic renal failure; fibrosis; inflammation

Introduction

Tubulointerstitial fibrosis is a key component of the final common pathway in progressive chronic kidney disease and its extent is closely associated with the loss of renal function [1,2]. Whereas the early phases are potentially reversible if the underlying cause can be controlled, the later phases are often characterized by autonomous progression despite apparent resolution of the underlying disease [3,4].

In the past, there have been several reports on the role of chemokines and their receptors in progressive renal failure [5]. For example, in the induction phase of interstitial inflammation, chemokines regulate the influx of inflammatory cells into the tubulointerstitial space. However, chemokines do not only mediate interstitial inflammation but may have direct pro-fibrotic effects through receptors on parenchymal cells [6].

Another example represents the chemokine/chemokine receptor pair CX3C-L/CX3C-R [7,8]. In the kidney, expression of CX3C-L/fractalkine (FKN) has been described in glomerular and vascular endothelial cells, in glomerular and tubular epithelial cells as well as in mesangial and stromal interstitial cells [8–10]. CX3C-L/FKN has been shown to be induced by pro-inflammatory cytokines, including CX3C-L/FKN itself, by proteinuria in tubular epithelial cells, by lipopolysaccharides [11], advanced glycosylation end products [12], and the pro-fibrotic cytokine platelet-derived growth factor (PDGF) or fibroblast growth factor-2 (FGF-2) in endothelial, epithelial as well as in mesenchymal renal or non-renal cells [13–20]. CX3C-L/FKN induces adhesion in different subsets of mononuclear cells [21–23], mediates chemotactic effects in mesenchymal cells [7,24] and has proliferative and anti-apoptotic effects on epithelial, mesenchymal or neuronal cells [24–26]. Moreover, chemokine upregulates the expression of matrix metalloproteinases [25,27] as well as CXC-L8/IL-8 or CX3C-L/FKN itself, suggesting autocrine/paracrine properties [24,25]. In several animal models with glomerular or tubulointerstitial injury, the
inhibition of CX3C-L/FKN or its receptor has been demonstrated to mitigate the progression of tubulointerstitial fibrosis [8,28,29]. However, no study has evaluated the exact time course of CX3C-L/FKN in progressive tubulointerstitial injury.

Thus, we chose to investigate the time course of CX3C-L/FKN expression in the chronic folic acid nephropathy (FAN) mouse model, analysing at the same time the expression of markers of cellular infiltration or tubulointerstitial fibrosis. Folic acid induces dose-dependent nephrotoxicity in mice and rats, with the rapid appearance of folic acid crystals within renal tubules and subsequent acute tubular necrosis, followed by epithelial regeneration. Progressive renal cortical scarring leading to end-stage renal failure has been described in selected mice [30,31]. Furthermore, we wanted to identify the regulation and functional activity of CX3C-L/FKN expression in tubular epithelial cells and renal fibroblasts.

Materials and methods

Experimental model

Animal protocols were approved by the local animals committee (9509.42505/01-04.99) and were conducted in conformity with the Guiding Principles in the Care and Use of Animals. Forty-day-old female CD1 mice were administered folic acid (200 mg/kg body weight) in vehicle (150 mM NaHCO3; pH 7.4) or vehicle-only by a single intraperitoneal injection. This folic acid dose reliably induces severe nephrotoxicity, as assessed by histological finding of grossly flattened renal epithelia after 72 h [31]. The mice were killed under CO2-narcosis and kidneys were harvested at days 3, 5, 7, 14, 21, 56, 84, 112 and 142 with three to five FAN (the experimental groups) and two vehicle (the sham groups) animals at each time point. Mice were sacrificed by neck fracture and kidneys were removed within minutes. Left kidneys were used for routine histology and immunohistochemistry (IHH)/immunofluorescence; right kidneys were used for real-time RT-PCR. In the latter case, organs were snap-frozen in liquid nitrogen. Proteinuria and blood urea nitrogen (BUN) were measured by standard routine methods.

Materials

Recombinant CX3C-L/FKN, interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), PDGF, epithelial growth factor (EGF), basic FGF-2 and transforming growth factor-β1 (TGF-β1) (the experimental groups) and two vehicle (the sham groups) animals at each time point. Mice were sacrificed by neck fracture and kidneys were removed within minutes. Left kidneys were used for routine histology and immunohistochemistry (IHH)/immunofluorescence; right kidneys were used for real-time RT-PCR. In the latter case, organs were snap-frozen in liquid nitrogen. Proteinuria and blood urea nitrogen (BUN) were measured by standard routine methods.

RNA isolation and quantitative real-time PCR

Total RNA from whole kidneys or cells was extracted as described previously [7] using the phenol–guanidine isothiocyanate reagent RNA-Be (Tel-Test, Friendswood, TX, USA). Oligo(dT)-primed reverse transcription was performed at 42°C for 50 min by heating the RNA at 70°C for 10 min. CX3C-L/FKN, fibroblast-specific protein 1 (FSP-1) and GAPDH PCR of reverse-transcribed RNA (3 μg) was performed using the following principles in the Care and Use of Animals. Forty-day-old female CD1 mice were administered folic acid (200 mg/kg body weight) in vehicle (150 mM NaHCO3; pH 7.4) or vehicle-only by a single intraperitoneal injection. This folic acid dose reliably induces severe nephrotoxicity, as assessed by histological finding of grossly flattened renal epithelia after 72 h [31]. The mice were killed under CO2-narcosis and kidneys were harvested at days 3, 5, 7, 14, 21, 56, 84, 112 and 142 with three to five FAN (the experimental groups) and two vehicle (the sham groups) animals at each time point. Mice were sacrificed by neck fracture and kidneys were removed within minutes. Left kidneys were used for routine histology and immunohistochemistry (IHH)/immunofluorescence; right kidneys were used for real-time RT-PCR. In the latter case, organs were snap-frozen in liquid nitrogen. Proteinuria and blood urea nitrogen (BUN) were measured by standard routine methods.

CD3, Col I, FN, α-sm actin, S100A4, AQ1/2 and THP

CD3, Col I, FN, α-sm actin, S100A4, AQ1, AQ2 or THP on one hand and α-sm actin, AQ1, AQ2 or THP on the other hand with FITC (green).
Quantitative real-time PCR from human kidney biopsies

The tubulointerstitial mRNA expression of CX3C-L/FKN was examined after the microdissection of kidney biopsy specimens from patients with tubulointerstitial fibrosis (fibrosis: n = 11) using quantitative real-time PCR and biopsy material from the European Renal cDNA Bank-Kroener Fresenius Biopsiebank and non-fibrotic, non-inflammatory biopsy specimens dedicated to variable primary or secondary nephropathies (no fibrosis; n = 9) as described previously [7]. Moreover, the acquired data were correlated with proteinuria.

Cell motility assay

FCS-starved MCT or NP-1 were left untreated (co) or stimulated with either IL-1β, TNF-α, TGF-β (each 10μg/mL) or 0.1mM H2O2 for 48 h. Thereafter, medium was replaced and, after another 24 h, supernatants were added in IMDM and the supernatants from the cell culture experiments were used as chemotactic agent in the presence or absence of CX3C-L/FKN neutralizing antibody (1:200) in the lower chamber.

Analysis of aspects of renal fibrogenesis

Renal fibroblasts were stimulated with CX3C-L/FKN and several aspects of renal fibrosis were investigated. This included analyses of cell viability by cell count, bromodeoxyuridine (BrdU) incorporation, FACS analysis for annexin V and propidium iodide, zymographic detection for matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9), ELISA for FN and Col I and PCR for α-sm actin. These methods were described in detail in our previous study [4].

Protein extraction, SDS–PAGE and immunoblotting

Western blot analyses of CX3C-L/FKN-stimulated renal fibroblasts for MEK-1, pMEK-1, ERK-1/2, pERK-1/2, c-Jun N-terminal kinase (JNK-1/2), pJNK-1/2, p38 MAPK, pp38 MAPK, p65 or β-actin were performed as described previously [4]. The p65 fragment of NF-κB was analysed in cytosolic and nuclear compartments separately [4].

Statistical analysis

Values are shown as the mean ± standard deviation (SD). Statistical analysis was carried out as indicated in the text using the Statistica programme version 7.1 (StatSoft, Tulsa, USA). Results with levels of P < 0.05 were considered significant.

Results

Time course of renal function, proteinuria and tubulointerstitial fibrosis in FAN

Functional data of BUN, proteinuria and the degree of renal fibrosis in sham-treated mice and FAN are summarized in Figure 1.

Table 1. Primer sequences and PCR conditions for qRT–PCR

| Primer sequence     | Annealing temperature (°C) | Cycles |
|---------------------|-----------------------------|--------|
| α-sm actin fw:      | 62.7                        | 38     |
| re:                 |                             |        |
| mCX3C-L/FKN fw:    | 58                          | 40     |
| re:                 |                             |        |
| mCX3C-R fw:        | 66                          | 40     |
| re:                 |                             |        |
| hCX3C-L/FKN fw:    | 57                          | 38     |
| rv:                 |                             |        |
| mFSP-1 fw:         | 62.7                        | 40     |
| rv:                 |                             |        |
| GAPDH fw:           |                             |        |

Time course of CX3C-L/FKN-, CX3C-R- and FSP-1-mRNA expression

In sham-treated animals, the mRNA quotient for CX3C-L/FKN-GAPDH was 10.4 ± 4.1 (× 10e−3 copies). In FAN, the quotient increased starting from 18.0 ± 11.2 (× 10e−3 copies) at day 3 to the first peak at day 7 with 41.7 ± 29.5 (× 10e−3 copies) (3.4-fold compared to sham). Thereafter, levels varied and reached a second peak at day 112 [82.4 ± 22.1 (× 10e−3 copies); 7.1-fold compared to sham]. Figure 2A summarizes the time course of the CX3C-L/GAPDH quotient.

In sham-injected animals, the mRNA ratio of CX3C-R/GAPDH was 6.8 ± 1.7 (× 10e−3 copies). In FAN, the ratio was robustly increased starting from 14.6 ± 11.8 (× 10e−8 copies) at day 3 with the first peak at day 7 with 43.0 ± 23.1 (× 10e−8 copies) (5.2-fold compared to sham) and elevated expression level at day112 up to 86.9 ± 23.1 (× 10e−8 copies) (6.1-fold compared to sham). Figure 2B summarizes the time course of the CX3C-R/GAPDH quotient.

FSP-1 is an S100A4 protein constitutively expressed in the cytoplasm of tissue fibroblasts [37]. It identifies fibroblasts and tubular epithelium undergoing epithelial–mesenchymal transition (EMT) and is critically related to the progression of renal diseases [38]. Thus, we investigated the time course of this protein in our model. In sham-treated animals, the mRNA quotient of FSP-1/GAPDH was 12.8 ± 4.5 (× 10e−8 copies). In FAN, the ratio was increased, peaking at day 14 [44.3 ± 30.1 (× 10e−8 copies); 3.6-fold compared to sham]. Thereafter, FSP-1/GAPDH decreased, but was slightly elevated at the end of the observation period [day 142 = 27.0 ± 28.1 (× 10e−8 copies); 2.0-fold compared to sham]. Figure 2C summarizes the time course of the FSP-1/GAPDH ratios.

Expression of CX3C-L/FKN in human kidney-derived fibrotic and non-fibrotic nephropathies

In order to further corroborate the relevance of the data obtained in the animal model, we additionally analysed the tubulointerstitial CX3C-L/FKN expression in human biopsies. The total expression of CX3C-L/FKN mRNA was significantly upregulated in biopsies from fibrotic kidneys compared to non-fibrotic nephropathies (1.6-fold) by quantitative real-time PCR (P = 0.041 comparing no fibro-
sis vs fibrosis) (Figure 3). There was only a very minor correlation between the mRNA quotient CX3C-L/GAPDH and the daily amount of proteinuria ($R^2 = 0.3483$).

**Immunohistochemical expression and distribution of CX3C-L/FKN**

In sham-treated animals, CX3C-L/FKN was constitutively expressed in the endothelial cell layer of arterioles and arteries (Figure 4A). No additional cellular expression of CX3C-L/FKN could be detected. To confirm the specificity of CX3C-L/FKN immunostaining, we used a rabbit isotype control antibody, which did not show any immunoreactivity (Figure 4B).

**Fig. 2.** Time courses of CX3C-L/FKN (A), CX3C-R (B) and FSP-1 (C) in FAN examined with quantitative real-time RT–PCR. Kidneys from FAN were harvested, mRNA isolated and qRT–PCR was performed. Results are expressed as CX3C-L/GAPDH (A), CX3C-R/GAPDH (B) and FSP-1/GAPDH (C) ratios. Mean ± SD of all sham animals was 10.4 ± 4.1 (A), 6.8 ± 1.7 (B) and 12.8 ± 4.5 (C). *$P < 0.05$ compared to all sham animals using the Kruskal–Wallis test followed by the Mann–Whitney U-test for comparison.

**Fig. 3.** Tubulointerstitial expression of CX3C-L/FKN mRNA in biopsy material from patients with fibrotic kidneys (Fibrosis) examined with quantitative real-time RT–PCR after tissue microdissection. Secondary non-fibrotic nephropathies served as controls. Results are expressed as the CX3C-L/GAPDH ratio. Mean values ± SD are shown. *$P < 0.05$ compared to No Fibrosis using the t-test to the log-transferred data.
In FAN animals, we found a *de novo* expression of CX3C-L/FKN within the peritubular as well as glomerular capillaries, in mesangial cells and predominantly in tubular epithelial cells. In endothelial cells of arteries or arterioles, CX3C-L/FKN was upregulated, peaking at day 14, although a low-degree constitutive expression was detectable even at day 3. In tubular epithelial cells, CX3C-L/FKN upregulation persisted to the end of the observation period. Figure 4C–F displays the representative expression of CX3C-L/FKN at different stages of FAN. In the interstitium, CX3C-L/FKN was also discretely upregulated throughout the study period, whereas the cellular source could not be further defined.

In DIF staining, we found co-expression of CX3C-L/FKN with AQ1 (Figure 4H) and, to a lesser extent, AQ2 (Figure 4I), but sparsely with THP (Figure 4J). Moreover, in progressive stages, we found a co-expression of CX3C-L/FKN and α-sm actin or S100A4 in selected tubular ep-

![Fig. 4.](https://academic.oup.com/ndt/article-abstract/25/3/684/1914187)

**Fig. 4.** Time course of expression of CX3C-L/FKN expression in FAN and DIF staining of CX3C-L/FKN (red staining signal) and either AQ1 (H), AQ2 (I), THP (J) or α-sm actin (K) (all green staining signal) in FAN. Sham (A) and FAN mice (B–F) were investigated at day 3 (C), 7 (D), 21 (E) or 82 (F). CX3C-L/FKN was analysed by IH. B displays the negative control. In FAN, *de novo* expression of CX3C-L/FKN within peritubular capillaries was detectable even at day 3 (C) lasting throughout the phase of tubulointerstitial fibrosis (F). CX3C-L/FKN was found upregulated within tubular epithelial cells in phases characterized by interstitial inflammation (D) as well as in the phase of tubulointerstitial fibrosis (E and F). In addition, increased expression could be found within the mesangium, glomerular capillaries, BC and within the interstitium. G1–3 show the negative control using goat/rabbit IgG as the primary antibody. In FAN, we found a co-expression of CX3C-L/FKN (H1–K1, red staining signal) with AQ1 (H, small arrow) but also tubular segments with exclusive CX3C-L/FKN expression and, to a lesser extent, a co-expression within AQ2-expressing tubules (I3, small arrow). Moreover, some tubular segments sparsely co-expressed CX3C-L/FKN and THP (J3), whereas others showed positivity for CX3C-L/FKN alone (small arrow). In K and L, we found a co-expression of α-sm actin or S100A4, respectively, and CX3C-L/FKN in selected tubular epithelial cells (small arrow) and in interstitial cells (big arrow). Magnification ×200 (G1–3), ×400 (A–D, H–J), ×800 (K–L, inlets). After immunohistochemical staining of FAN, kidneys’ expression of tubular CX3C-L/FKN (M) was evaluated semiquantitatively as described in the Materials and methods section. Mean values ± SD throughout the time course are shown. Mean ± SD of all sham animals was 0.07 ± 0.23. *P < 0.05 compared to all sham using Bonferroni–Holmes adjustment.
 epithelial cells, respectively (Figure 4K and L). In tubular epithelial cells, we found robustly increased expression peaking twice at day 5 as well as at day 84, remaining elevated until the end of the observation period. Figure 4M summarizes the semiquantitative analysis of the time course within the tubules.

**Immunohistochemical expression of CD3, S100A4, FN and Col I**

The extent of renal interstitial inflammation was studied by staining for CD3 as an exemplary marker for CX3C-R-expressing inflammatory cells. In sham-treated animals, no relevant infiltration with CD3+ cells could be detected. In FAN, we found increased amounts of CD3+ cells starting at day 3 with increasing numbers until day 21 and remaining elevated throughout the observation period. A representative example is shown in Figure 5A and B. S100A4 belongs to the S100 family of proteins and has been implicated in the progression of fibrosis. Several of the S100A4 effects described resemble the processes that occur during EMT [39]. In sham-treated animals, no relevant S100A4 expression was found (Figure 5C). In FAN, we detected an upregulation within tubular epithelial and interstitial cells (Figure 5D).

Col I and FN have been shown to be two predominating components that are deposited within the interstitial extracellular matrix during renal fibrosis [3]. In sham-injected animals, there were only negligible amounts of FN or Col I detectable within the interstitium. Conversely, in FAN animals, we found an increase of FN within the interstitial space beginning as early at day 3 with increasing amounts through the observation period. Col I deposition was upregulated first at day 5, peaking at day 84 and remaining elevated until day 142. However, although FN and Col I expression tended to increase in FAN, statistical significance (P < 0.05 compared to sham) was achieved for Col I only on day 84. Figure 5E–H shows representative examples and Figure 6A and B summarizes the results of semiquantitative analyses after IH in sham and FAN animals.

**Expression of CX3C-L/FKN, CX3C-R or FSP-1 correlates poorly with proteinuria, but highly with tubulointerstitial fibrosis and each other**

Next, we correlated the number of copies of mRNA ratios of CX3C-L/GAPDH and CX3C-R/GAPDH. Not unexpected, the expression highly correlated with $R^2 = 0.9627$. The number of mRNA copies of CX3C-L/GAPDH correlated well with the degree of interstitial fibrosis ($R^2 = 0.5576$) and modestly with the mRNA FSP-1/GAPDH ($R^2 = 0.3317$). We found a good correlation with an $R^2$-value of 0.5986 after comparison of mRNA CX3C-L/GAPDH and the sum of CD3 positively stained cells.

Comparing the amount of proteinuria with the copies of mRNA CX3C-L/GAPDH demonstrated a poor correlation with $R^2 = 0.101$.

**CX3C-L/FKN is upregulated in response to pro-inflammatory and pro-fibrotic cytokines as well as the reactive oxygen species H$_2$O$_2$**

To examine whether CX3C-L/FKN could be upregulated by pro-inflammatory or pro-fibrotic cytokines in tubular epithelial cells, FCS-starved proximal (MCT) or distal (NP-1) tubular epithelial cells were left untreated (co) or treated for 24 h with 10 ng/mL of either the pro-inflammatory cytokines IL-1β or TNF-α or the pro-fibrotic cytokines PDGF, epidermal growth factor (EGF), basic FGF-2 or TGF-β. Additionally, H$_2$O$_2$ (0.1 mM) was used as a reactive oxygen species (ROS) stimulus under the same conditions.

In MCT cells, the CX3C-L/GAPDH coefficient increased significantly after stimulation with IL-1β (401.6 ± 76.6%), TNF-α (292.2 ± 152.9%), TGF-β (210.9 ± 82.3%) or H$_2$O$_2$ (203.3 ± 42.0%) in qRT-PCR (all $P < 0.01$ compared to co using the Mann–Whitney U rank test adjusted for multiple choice comparison). Conversely, incubation with PDGF, EGF or FGF-2 did not change CX3C-L/GAPDH expression significantly. In NP-1 cells, IL-1β (150.3 ± 33.5%), TGF-β (139.7 ± 13.5%), or H$_2$O$_2$ (179.5 ± 38.6%) could be identified as inductors of CX3C-L/GAPDH (all $P < 0.01$ compared to co using the Mann–Whitney U rank test adjusted for multiple choice comparison) though to a lesser degree compared to MCT cells, whereas TNF-α, PDGF, EGF or FGF-2 did not change CX3C-L/GAPDH expression significantly. These results are summarized in Figure 7.

**Supernatants of IL-1β, TNF-α, TGF-β (each 10 ng/mL) or 0.1 mM H$_2$O$_2$-stimulated MCT and NP-1 chemoattract PBMCs via CX3C-L/FKN**

Supernatants of untreated (co = 100%) or IL-1β, TNF-α, TGF-β or H$_2$O$_2$-stimulated MCT and NP-1 in presence or absence of specific CX3C-L/FKN-blocking antibody were used as chemoattractants in migration assay.

We found a significant enhanced migration rate of PBMCs using supernatants of IL-1β, TNF-α, TGF-β or H$_2$O$_2$-stimulated MCT as chemoattractants ($P < 0.01$ using the Mann–Whitney U rank test adjusted for multiple comparison). Upregulation was up to 540 ± 113% in IL-1β, 718 ± 185% in TNF-α, 195 ± 62% in TGF-β and 218 ± 50% in H$_2$O$_2$. Administration of CX3C-L/FKN-blocking antibody in the lower chamber partially, but significantly ($P < 0.05$) abolished the rate of migrated cells to 350 ± 76% in IL-1β, 477 ± 127% in TNF-α, 136 ± 54% in TGF-β and 145 ± 41% in H$_2$O$_2$.
Fig. 5. Expression of CD3 (A, B), S100A4 (C, D), FN (E, F) and Col I (G, H) in sham (A, C, E, G) and FAN (B, D, F, H). In sham, there were no CD3+ cells detectable (A) and there was only a sparse expression of S100A4 (C), FN (E) and Col I (G), whereas in FAN the number of CD3+ cells was increased (B). S100A4 was found strongly upregulated within tubular epithelial as well as interstitial cells (D) and FN (F) and Col I (H) within the interstitial space in FAN. Magnification ×400, inlet (D, H) negative control omitting the primary antibody ×100.
stimulated NP-1 cells, we found similar data (not shown). The results are summarized in Figure 8.

CX3C-L/FKN upregulates CX3C-L/FKN expression and has modest effects on renal fibrogenesis in renal fibroblasts

To further analyse the relevance of the CX3C-L/CX3C-R pair, we investigated the effects of CX3C-L/FKN on CX3C-R-expressing renal fibroblasts [7] regarding potential autoinduction and aspects of fibrogenesis. CX3C-L/FKN-stimulated Tk173 and 188 fibroblasts dose-dependently increased the CX3C-L/GAPDH mRNA levels with a maximum of 312.6 ± 61.8% in Tk173 and 335.8 ± 130.4% in Tk188 (both \( n = 3, P < 0.01 \)) (Figure 9A).

In a first set of functional experiments, we observed a dose-dependent effect of increasing doses of CX3C-L/FKN on cell growth. Counted cells increased up to 243 ± 59% of controls after 48 h stimulation with 20 ng/mL CX3C-L/FKN in Tk461, but only modestly with 125 ± 5% in Tk173 and 127 ± 11% in Tk188 fibroblasts (all \( n = 3, P < 0.05 \); not shown). We further analysed whether this effect is due to changes in cell proliferation, apoptosis or necrosis rates. The proliferation rate increased dose-dependently up to a maximum of \( 160 \pm 28\% \) \((n = 3, P < 0.01)\) in Tk461, \( 231 \pm 92\% \) in Tk173 and \( 224 \pm 30\% \) in Tk188 fibroblasts after stimulation with CX3C-L/FKN 20 ng/mL (all \( n = 3, P < 0.01\); Figure 9B). Conversely, the apoptosis rate was decreased to \( 59 \pm 38\% \) in Tk461, \( 71 \pm 8\% \) in Tk173 and unchanged with \( 120 \pm 35\% \) in Tk188 (all \( n = 5; P < 0.05 \) for Tk461 and Tk173; not shown) as evaluated by FACS analysis for annexin V. Although necrosis rates tended to be lower, it failed to reach significance in all three cell types (\( n = 5; \text{n.s.} \)).

To further characterize the effects of CX3C-L/FKN on renal fibroblasts, Tk461 were stimulated with increasing, physiologically relevant doses of CX3C-L/FKN and activity of MMP-2 and MMP-9 were determined by zymography. MMP-9, but not MMP-2, was significantly induced by CX3C-L/FKN up to \( 157 \pm 41\% \) of the control (\( n = 3, P < 0.05 \)). Determination of FN and Col-I synthesis in CX3C-L/FKN-stimulated fibroblasts revealed no significant change (not shown).

Activation of fibroblasts to myofibroblasts is accompanied by the expression of \( \alpha \)-sm actin. To analyse the influence of CX3C-L/FKN on this aspect, we stimulated Tk461 dose-dependently with CX3C-L/FKN as described above, and \( \alpha \)-sm actin expression was detected by RT–PCR. However, we found no effects in any investigated dose (Figure 9C).

CX3C-L/FKN stimulates MAPK phosphorylation and a shift of NF-kB from cytosol to nucleus

To examine the mechanisms of the effects of CX3C-L/FKN, the influence of CX3C-L signal transduction pathways in renal fibroblasts was investigated. Therefore, immunoblot analyses for cytosolic MEK-1, p38 MAPK, JNK-1/2 and ERK-1/2 and, additionally, for their phosphorylated forms were performed. Moreover, a potential
shift of NF-κB from cytosol to nucleus was additionally investigated by immunoblots for p65. There was no influence of CX₃C-L/FKN on the expression of MEK-1, p38 MAPK, JNK-1/2, ERK-1/2 or the cytosolic fraction of p65. Conversely, there was a time- and dose-dependent increase of the phosphorylated forms of mitogen-activated protein kinase (MAPK)-kinase MEK-1 and all MAPKs investigated and of the nuclear p65. The strongest increase was found after 4h of incubation with CX₃C-L/FKN 20 ng/mL, reaching a peak value with 245 ± 96% in pMEK-1 (n = 3; P < 0.05), 331 ± 93% in pp38 MAPK (n = 3; P < 0.05), 277 ± 45% in pJNK-1 (n = 3; P < 0.05), 338 ± 176% in pJNK-2 (n = 3; P < 0.05), 323 ± 116% in pERK-1, 255 ± 85% in pERK-2 (n = 3; P < 0.05) and 143 ± 29% in the nuclear fraction of p65 compared to the controls (n = 3; P < 0.05). Representative blots and results are summarized in Figure 10A–F. These effects were specific since pre-incubation with a CX₃C-R-blocking antibody partially abrogated MAPK phosphorylation (Figure 10G) and administration of the MEK-1 and ERK-1/2 inhibitor PD98059 partially inhibited CX₃C-L/FKN autoinduction (Figure 10H).

**Discussion**

After the inhibition of CX₃C-L/FKN or its corresponding receptors, respectively, a significant reduction of infiltrating sites and a reduction of irreversible, chronic tubulointerstitial damage has been achieved in several models of progressive glomerular and tubulointerstitial diseases [8,13,28]. However, no data exist on the time course of CX₃C-L/FKN expression in progressive tubulointerstitial disease and the role of tubular CX₃C-L/FKN expression.

We detected the expression of CX₃C-L/FKN, CD3, S100A4, Col I, FN, AQ1, AQ2, THP and α-sm actin by immunostaining in FAN as well as CX₃C-L/FKN, CX₃C-R and FSP-1 by qRT–PCR. Our follow-up investigations lasted from day 3 to 142 after induction. However, our study had some limitations as we were not able to detect monocytes/macrophages and CX₃C-R by immunostaining despite using different sample processing methods, staining protocols, antigens or antibodies. Moreover, the earliest stadium investigated was day 3 after induction as we focused our investigations on the progression phase of tubulointerstitial fibrosis.

In IH, we found that CX₃C-L/FKN was constitutively expressed within the endothelial cell layer of arteries and arterioles in a low amount, but not within the other compartments. This is in concordance with a previously published mouse model in which anti-CX₃C-L/FKN immunoreactivity was detected mainly on endothelial cells throughout the kidney in sham-operated mice [8]. In FAN, we found a rapid de novo CX₃C-L/FKN expression within the microvascular endothelial cell layer, suggesting a role in attracting inflammatory cells into the damaged tissue [40]. This is in accordance with previous in vitro [41] and in vivo data on renal inflammation [9,29] and further supported by the fact that CX₃C-R-expressing lymphocytes [7] were found to correlate very well with the amount of mRNA CX₃C-L/FKN in our model.

Next, we found an upregulation within the tubules, peaking first at day 5 which lasted until the end of the observation period at day 142. So far, previous animal studies focused on early changes in expression with the longest observation period of 14 days after induction [8]. In DIF, we found a double labelling with AQ1 and, to a lesser extent, AQ2, but only sparsely with THP, suggesting a CX₃C-L/FKN expression predominantly in proximal tubules. Several studies demonstrated an upregulation of CX₃C-L/FKN within the tubules in inflammatory diseases, e.g. acute tubulointerstitial rejection in humans [14,42]. Otherwise, and in accordance with our observation, an upregulation has also been observed in...

**Fig. 8.** Number of migrated cells using supernatants of stimulated MCT as chemoattractant. MCT were left untreated (co) or stimulated as described in the Materials and methods section. Supernatants of the stimulated cells were used in presence or absence of CX₃C-L/FKN-blocking antibody (Ab) as chemoattractants for PBMCs in Boyden chamber assay. Migrated cells using supernatant of untreated cells (co) were defined as 100%. Results are shown as mean values ± SD in percentage of co (n = 6). *P < 0.05 compared to unstimulated co using the Mann–Whitney U rank test adjusted for multiple comparison. Intragroup comparison (no antibody vs CX₃C-L/FKN-blocking antibody) was tested using the adjusted rank test.
slow-declining progressive fibrotic and sclerotic renal and non-renal diseases, e.g. chronic allograft nephropathy [43], prolonged mesangial proliferative glomerulonephritis [44], chronic pancreatitis [45], pulmonary hypertension [46], chronic liver injury [47], systemic sclerosis [40] and atherosclerosis [24]. Corroborating the hypothesis of an involvement of the CX₃C-L/CX₃C-R system in renal fibrosis, we found an expression of CX₃C-L/FKN in tubular epithelial cells putatively undergoing tubular epithelial transition, demonstrated by a double labelling with the mesenchymal marker α-sm actin or S100A4, respectively, and, moreover, by our findings in human fibrotic and non-fibrotic nephropathies. In previous studies, tubular upregulation of CX₃C-L/FKN was induced by protein overload [15], TNF-α [48] or IFNγ stimulation [49]. However, we found no good correlation with proteinuria in FAN and only a minor correlation in humans. Otherwise, our in vitro analyses identified the pro-inflammatory cytokines IL-1β and TNF-α, the pro-fibrotic cytokine TGF-β as well as the ROS H₂O₂ as the main inductors of CX₃C-L/FKN in murine proximal and distal tubular epithelial cells in vitro. These data suggest a role of mediators of the early inflammation, but also of mediators expressed during the progression phase of renal scarring. This was not unsuspected, since ROS are known to mediate an upregulation of several chemokines in tubular epithelial cells [50,51], although that had not been described for CX₃C-L/FKN. In rat mesangial cells, an upregulation of CX₃C-L/FKN in response to the pro-fibrotic cytokines FGF-2 or PDGF has been described [20], but not to TGF-β. However, these differences may depend on the kind of investigated cells and species.

In our previous study, we have shown an upregulation of CX₃C-R in human tubular epithelial cells as well as a de novo expression in renal fibroblasts in progressive renal diseases mediated by oxidative stress. Thus, we chose this fibroblast model to further investigate potential profibrotic effects downstream [7]. Stimulation of CX₃C-R-expressing renal fibroblasts with CX₃C-L/FKN enhanced their migration in vitro [7], which might be provided by an upregulation of matrix metalloproteinase. Moreover, we found effects on cell viability and an MEK-1-/ERK-1/2-dependent autoinduction, but not on matrix synthesis or transformation to myofibroblasts. Furthermore, tubular CX₃C-L/FKN caused an enhanced migration of PBMCs in the migration assay. Taken together with this new data, we speculate that the CX₃C-L/CX₃C-R system may be an autoinductive system upregulated in progressive renal diseases which may enhance fibrosis by an increased invasion of mononuclear cells and also by an increased migration and proliferation of fibroblasts to the place of injury. This hypothesis is corroborated by several previous findings:

1. Lu and co-workers have demonstrated that blockade of CX₃C-R failed to block serum creatinine increase in a cisplatin-induced acute renal failure model within the early phase (day 3 after induction) but without investigation of later stadiums [52],
2. Furuichi and colleagues have shown that CX₃C-L/FKN-dependent fibrosis was not an early but a late event during an ischaemia–reperfusion injury model of mouse kidneys [8],
3. Feng et al. have demonstrated that CX₃C-L/FKN inhibition was less effective at the early stage than at the late stage in crescentic glomerulonephritis [28] as it ameliorated the progression of lupus nephritis in MRL/lpr mice [29].

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Conflict of interest statement. None declared.
Fig. 10. Influence of CX₃C-L/FKN on phosphorylation of MAPKs in human renal fibroblasts. Human renal fibroblasts were incubated with 20 ng/mL CX₃C-L/FKN for 0, 1, 4 and 12 h. In Tk461 primary human renal fibroblasts, CX₃C-L/FKN increased the phosphorylation of MEK-1 (A), p38 MAPK (B), ERK-1/2 (C) and JNK-1/2 (D) in a time-dependent manner. Representative blots are shown with β-actin as a control of equal loading. Activation of Tk461 resulted in an increased shift of p65 fragment of NF-κB from cytosol (white columns) to nucleus (grey columns) (E). Dose-dependent increase on MAPKs phosphorylation after incubation with rising doses of CX₃C-L/FKN for 12 h (F). A blockade of CX₃C-R with a specific antibody resulted in an inhibition of MAPKs phosphorylation (G). Inhibition of the MEK-1 and ERK-1/2 inhibitor PD98059 resulted in decreased autoinduction after incubation with CX₃C-L/FKN in RT–PCR (H). (A)–(D), (G) Values are given in percentage of positive control and are the mean of three independent experiments. *P < 0.05 vs baseline and **P < 0.05 vs positive control expression using Student’s t-test.
Fig. 10 (Continued)
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CX3C-L/FKN in folic acid nephropathy
Bradykinin and high glucose promote renal tubular inflammation

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Abstract

Background. The role of the kallikrein–kinin system in diabetic nephropathy remains controversial.

Methods and Results. High-glucose (HG) super-induced interleukin (IL)-6, CCL-2, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF) and B2K receptor (B2KR) mRNA in cultured proximal tubular epithelial cells (PTEC), whereas bradykinin (BK) upregulated IL-6, CCL-2 and TGF-β mRNA. HG activated mitogen-activated protein kinase (MAPK) p42/44 and protein kinase C (PKC) signals, whereas BK only activated PKC. Tubular expression of these mediators and tissue kallikrein 1 (KLK1) was confirmed in human diabetic kidney biopsies. Inhibition of MAPK p42/44 by PD98059 partially reduced HG-induced IL-6, CCL-2 and TGF-β expression. The B2KR blocker, icatibant, downregulated HG-induced MAPK p42/p44 but not HG-induced PKC activation and partially reduced both HG- and BK-induced IL-6, CCL-2 and TGF-β secretion. HG stimulated expression of KLK1 and low-molecular-weight kininogen (LMWK) and its downstream effects were attenuated by aprotinin (tissue kallikrein inhibitor). The peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, rosiglitazone, attenuated HG-induced PKC but not HG- or BK-induced MAPK p42/44 activation and reduced HG-stimulated VEGF, along with IL-6, CCL-2 and TGF-β secretion. Rosiglitazone plus icatibant further reduced these effects of HG.

Conclusions. In conclusion, HG stimulates tubular proinflammatory, profibrotic and angiogenic signals, which is partly mediated through BK via MAPK signalling and partly through PKC independent of BK. The potential therapeutic role of complementary B2KR blockade and PPAR-γ activation deserves clinical investigation.

Keywords: bradykinin; chemokines; diabetic nephropathy; high glucose; kallikrein

Introduction

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease (ESRD) in developed countries where type 2 diabetes mellitus (T2DM) has already reached epidemic proportions by the beginning of this millennium [1]. The