

The role of IL-20 cytokine subfamily in the patomechanism kidney fibrosis

Ph.D. Thesis

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Budapest
2017

Introduction

The prevalence of chronic kidney diseases (CKDs) is estimated to be 8-16% worldwide. Currently, about 20-25 million patients need renal replacement therapy and their number is rapidly increasing. The most common etiologies of CKDs and renal fibrosis are diabetes mellitus (DM) and hypertension in the adult population and obstructive nephropathy in the childhood. Although the increasing number of patients the pathomechanism of CKD is not fully understood yet. Despite the clear unmet medical need there is no generally used drug to treat or hinder renal fibrosis. Identification of new target molecules in the pathomechanism of kidney fibrosis has far reaching beneficial consequences on the health care system.

In the past decade, the development of genomic technology has revolutionized the modern biological research and drug discovery. Functional genomic analyses by microarray technics enable researchers to perform comprehensive genetic analysis of several diseases including obstructive nephropathy and kidney fibrosis. This new molecular biological method has been widely used in gene discovery, biomarker determination, disease classification, and drug target identification.

Chronic kidney diseases irrespectively of their etiology always have an immunologically mediated inflammatory component, which shows strong correlation with the progression of fibrosis and the decline of renal function. Importance of the IL-20 subfamily of cytokines including IL-19, -20, and IL-24 has been proposed in different chronic inflammatory diseases based on its mediator role on inflammation and tissue remodeling. Indeed, members of the IL-20 subfamily have been suggested to facilitate the communication between the immune system and different type of epithelial cells. This unique biology is driven by the expression of their receptors by kidney epithelial cells

(IL20R α /IL20R β -t and IL22R α /IL20R β) a defining feature of this group of cytokines

As the final common pathway renal fibrosis is characterized by an excessive deposit of collagen-rich extracellular matrix (ECM), which disrupts the healthy structure of the kidney and leads to the reduction of renal function. The damaged kidney cells release danger signals and chemotactic stimuli, which trigger the rapid recruitment of immune cells. The infiltrating immune cells produce high levels of proinflammatory cytokines, growth factors, including tumor growth factor beta (TGF- β) which is a pleiotropic cytokine that has been established as a central mediator of ECM production of the myofibroblasts (MFs) which are the key effector cells of organ fibrosis. The platelet derived growth factor B (PDGF-B) is also an important profibrotic factor because PDGF-B stimulates the proliferation, differentiation MFs during kidney fibrosis. Tissue fibrosis occurs when the balance between the production and degradation of ECM components is shifted toward the increased production of collagen-I and -III-rich ECM deposition.

Matrix metalloproteinases (MMPs) are a family of extracellular zinc dependent endopeptidases. Although MMPs have long been considered to be primarily responsible for turnover and degradation of ECM components, they are now recognized as being responsible for mediating crucial functions such as cell migration, leukocyte activation, antimicrobial defense, chemokine processing. Furthermore, MMPs have both inhibitory and stimulatory roles in fibrosis.

The common, and widely used biomarker of myofibroblasts is the α -smooth muscle actin (α -SMA) which belongs to the gene family of actins isoforms. The different actin isoforms have a special role in the cell motility and intracellular transport

processes. Among these isoforms β -actin has a special importance as it is a widely used internal control in many molecular biological measurements. Although actin isoforms are encoded by different genes the similarity between them is significant. Indeed, the homology in the amino acid or nucleotide sequences of the different actin isoforms is over 90% making it a real challenge to selectively measure their expression. Due to the increasing importance of the fibroproliferative diseases the mRNA expression of α -SMA is frequently determined in thousands of experiments suggesting the importance of the issue. Unfortunately the improperly designed literary primer pairs significantly affect the results of PCRs measuring mRNA expression of α -SMA or β -actin.

The purpose of my PhD study was to provide a comprehensive analysis of molecular mechanisms potentially involved in the pathophysiology of congenital obstructive nephropathy and kidney fibrosis, thus contributing to the better understanding of the underlying molecular mechanisms.

Objectives

Our objectives were the following:

- To analyze the gene expressional changes and identify the relevant functional groups and molecular pathways involved in the patomechanism of CON
- To determine the localization, function and role of the revealed molecules in the pathophysiology of CON
- To investigate the role of the IL-20 cytokine subfamily in the urether obstruction induced kidney fibrosis
- To evaluate the IL-20R β dependent alterations in the fibrotic kidney
- To investigate the effect of IL-24 on the profibrotic cytokine production
- To design a specific primer to detect α -SMA and β -actin, and to evaluate the specificity the of the literally used α -SMA and β -actin primers

Methods

***In vivo* experiments**

The animal model of unilateral obstructive nephropathy was performed on newborn Wistar rats, and 7-8 week old male C57BL/6J mice (wild type and/or IL-20R β KO). After standard midline laparotomy then the left ureter of animals in the UUO group was isolated by blunt dissection and completely ligated using fine suture material. Sham-operated control animals underwent identical surgical procedure without occlusion of the left ureter. In case of newborn rats ten days and in case of mice seven days after the initiation of UUO left kidneys were surgically removed.

***In vitro* experiments**

Human embryonic kidney (HEK-293) and human proximal tubular epithelial (HK-2) cell lines were cultured. HEK-293 and HK-2 cells were plated into 6-well tissue culture dishes (5×10^5 cells/well) than incubated in serum-free medium for 24 hours. Cells were treated either with 1nM recombinant human (rh) rhTGF- β or 0.4 nM rhPDGF-B or 2 nM rhIL-24 or 25 μ M H₂O₂ for 24 hours (N=6 well/treatment group). Control cells were treated with vehicle only.

Microarray analysis

To identify the differentially expressed genes in the pathomechanism of congenital obstructive nephropathy, microarray analysis was performed on the kidney samples of neonatal rats underwent unilateral ureteral obstruction. Bioinformatics analysis was carried out to identify the relevant genes, functional groups and pathways involved in the pathomechanism of CON. After reactome pathywas analysis we allowed to find interactions and pathways which are similar in

species *Rattus norvegicus* and *Homo sapiens*. Cytoscape software was used to visualize the results on a network graph.

Histology

Tubular injury was evaluated on hematoxylin-eosin and periodic acid-Schiff stained kidney sections. Masson's trichrome staining was used to evaluate tubulointerstitial fibrosis and Sirius red staining to evaluate collagen accumulation. MMP-12 and IL-24 localization was determined with specific DAB staining. The cellular expression of IL-20R β was investigated by fluorescent immunohistochemistry.

Western blotting

Western blot method were used on kidney samples of wild type and IL-20R β KO mice to determine the protein amount of α -SMA after unilateral urethral obstruction. Bands of interest were corrected for GAPDH housekeeping protein.

Measurements of mRNA levels

Total RNA was isolated from frozen kidney samples of newborn rats and also from the HEK-293 and HK-2 cells. Quantitative real-time PCR were used to detect mRNA levels. The results were normalized against GAPDH as a housekeeping gene.

Design and alignment of α -SMA and β -actin specific primers

The sequences of mouse α -SMA and β -actin primers were designed by Primer3web software considering the significant overlap between the mRNA sequences of different actin isoforms. Literary mouse α -SMA and β -actin specific primer pairs were selected from different high quality papers. To investigate the specificity of the self designed and literary primer pairs artificial α -SMA, β -actin, γ -cyto-actin, and γ -smooth-actin DNA templates were used. RT-PCR products were then separated by electrophoresis on 2% agarose gel.

Flow cytometry

The cells of singlecell suspension generated from the kidney samples of newborn rats as well as rhIL-24 treated HK-2 cells were used. After permeabilisation, cells were incubated with MMP-12, IL-24, TGF- β , and PDGF-B specific antibodies. Cells were subsequently washed and incubated with fluorescent dye conjugated secondary antibodies. The negative controls were incubated only with the secondary antibody. Tenthousand cells were collected and results were analyzed using the BD FACSDiva Software.

Statistical analysis

The statistical evaluation of real-time RT-PCR and flow cytometric results were performed by GraphPad Prism 6.01 software (GraphPad Software Inc., La Jolla, CA, USA). After testing normality with Kolmogorov-Smirnov test Mann-Whitney U-test or unpaired, two-tailed t-test was used to determine the differences between two groups. . Multiple comparisons and possible interactions were evaluated by one-way ANOVA followed by Bonferroni post-hoc test. $p \leq 0.05$ was considered as statistically significant. Values were expressed as mean+SD.

Results

Microarray analysis of congenital obstructive nephropathy

Our genome-wide analysis of congenital obstructive nephropathy performed on newborn rat kidneys revealed 880 differentially expressed genes following complete UUO. Enrichment analysis of these genes resulted in GO terms and molecular pathways associated mainly with the immune response, inflammation, apoptosis and proliferation which confirms that developmental abnormalities trigger chronic renal inflammation leading to the excessive deposition of ECM components and consequent renal fibrosis.

Validation of the microarray results

To validate our microarray measurements real-time RT-PCRs were performed. Based on the microarray results eight genes were selected for validation. mRNA expression of MMP-3, MMP-7, MMP-12, IL-19, IL-24 and renin, clusterin and IL-1 β was studied. Moreover, MMP-12 and IL-24 were selected for further validation on protein level by flow cytometric measurement. In accordance with the results of our microarray analysis, we found significantly elevated mRNA and protein expression of each genes selected for validation in the kidney of newborn rats after 10 days of UUO compared to sham-operated controls. We also investigated the renal localization of MMP-12 and IL-24 and we found strong immunopositivity in the renal tubular epithelial and glomerular cells of newborn rats underwent urether obstruction. To the best of our knowledge this is the first study demonstrating the increased mRNA expression and protein level of IL-20 cytokine subfamily member IL-24 in relation to congenital obstructive nephropathy.

Effect of profibrotic molecules on the expression of MMP-12 and IL-24

Based on our microarray results we found reasonable to investigate the role of the well-known determinative molecules of organ fibrosis on the synthesis of MMP12 and IL-24. Our *in vitro* experiments demonstrated that while rhPDGF-B treatment or oxidative stress increased the expression of MMP-12 of HEK-293 or HK-2 cells, treatment with rhTGF- β , the strongest known inducer of ECM deposition inhibited its synthesis. Furthermore, we found that rhTGF- β , rhPDGF-B or H₂O₂ may inhibit or do not alter the synthesis of IL-24 suggesting rather a negative correlation between IL-24 and the main profibrotic factors.

The role of IL-24 in the pathomechanism of congenital obstructive nephropathy

Epithelial cells are major expression sites for IL-24 receptor complexes. The biological effect of IL-24 mainly dependent on the expression of the IL-20R β receptor subunit, and our results demonstrated its expression on HEK293 embryonal kidney and HK-2 proximal tubular epithelial cells. These results suggest that renal epithelial cells are targets of IL-24 in the kidney. Based on literally data IL-24 specifically modulate innate immune pathways. Therefore, we investigated the effect of IL-24 on pro-inflammatory cytokines including IL-1 β , IL-6 and TNF- α in renal HEK-293 and HK-2 cells and we found that IL-24 decreased the expression of IL-6 in HEK-293 embryonal kidney epithelial cells. These results suggest that the IL-24-induced downregulation of IL-6 expression in embryonal kidney epithelial cells may contribute to the suppression of inflammatory responses in the obstructed developing kidney. Our microarray analysis identified MMP-3 and -7 as the most upregulated genes in our animal model of CON. The regulatory mechanisms and factors which mediate their expression during

kidney fibrosis are less studied. The IL-24 expression was remarkably increased in our microarray analysis therefore we investigated the effect of IL-24 on the expression of MMP-3 and -7 in HEK-293 and HK-2 cell lines. In the present study we found that IL-24 significantly decreased the expression of MMP-3 in the renal epithelial cells indicating the potential role of IL-24 in the extracellular matrix remodeling.

The role of IL-20 cytokine subfamily in the pathophysiology of kidney fibrosis

Our microarray analysis revealed the potential role of IL-19 and IL-24 in the pathomechanism of obstructive nephropathy. Therefore, we investigated the renal expression of IL-19,-20,-24 and their receptor IL-20R β in the mouse model of unilateral urethral obstruction induced kidney fibrosis. Our results showed that mRNA expression of IL-19, IL-24 and their receptor IL-20R β were increased in the obstructed kidney. To the best of our knowledge this is the first study demonstrating the increased mRNA expression IL-24 in relation to tissue remodeling of the kidney.

The role of IL-20 subfamily on the obstruction induced extracellular matrix production

We investigated the urether obstruction induced, IL-20R β dependent alterations on the synthesis of ECM in the kidney samples of wild type and IL-20R β KO mice. The remarkable finding in this study is that IL-20R β deficiency lead decreased expression of collagen amount in the obstructed IL-20R β KO mice than in obstructed wild type mice. We also found that lack of IL-20R β significantly decrease the amount of myofibroblast in the fibrotic kidney after the onset of UUO compared to wild type mouse. These data suggesting the central role of IL-20 subfamily on the profibrotic growth factors

production and myofibroblast proliferation during the pathomechanism of kidney fibrosis.

Effect of IL-24 on the TGF- β and PDGF-B expression of tubular epithelial cells

Epithelial cells are the main effectors of kidney fibrosis and were described as the main target of IL-20 cytokine subfamily. Therefore, we investigated the *in vitro* effect of IL-24 on the kidney proximal tubular epithelial cells. We found that IL-24 treatment induce the TGF- β , PDGF and production of the HK2 cells. As above mentioned these profibrotic cytokines play central role of the “core pathway” of the kidney fibrosis. These data are consistent with our previous data demonstrates that lack of the IL-24 receptor can directly influence the ECM production and number of the renal fibroblasts *in vivo*.

Design and alignment of α -SMA and β -actin specific primers

The homology in the amino acid or nucleotide sequences of the different actin isoforms is over 90% making it a real challenge to selectively measure their expression. Therefore, in the present study we located our primers to those nucleotide sequences which show the greatest possible difference from other actin isoforms thus maximizing the chance of specific priming. These real-time PCRs resulted in specific α -SMA PCR product amplification similarly to the separation of the PCR products by gel electrophoresis resulted in one discrete band with the expected product length.

Specificity of literary primer pairs used to determine the expression of mouse α -SMA or β -actin

Investigating the biological relevance of the nonspecific primer binding the template specificity of three-three randomly chosed literary mouse α -SMA and β -actin primer pairs were tested *in vitro*. We found that all primer pairs amplified both mouse α -SMA and β -actin specific artificial DNA templates, as well. The separation of the PCR products by gel electrophoresis also confirmed this cross-reaction. These results suggest that cross-reaction between β -actin and α -SMA primer pairs may lead to false experimental outcome and conclusions.

Conclusions

1, Microarray analysis was performed on the on the animal model of congenital obstructive nephropathy which revealed 880 differentially expressed transcripts, associated mainly with the immune homeostasis. Our results confirms that developmental abnormalities trigger chronic renal inflammation.

2, The most up-regulated genes were MMPs and members of IL-20 cytokine subfamily, including MMP-3, MMP-7, MMP-12, IL-19 and IL-24.

3, We demonstrated that IL-24 mediates the expression of IL-6 and MMP-3 in congenital obstructive nephropathy.

4, In accordance with our microarray results we found elevated level of IL-20 cytokine subfamily (IL-19,-20,-24) members in the fibrotic kidney.

5, We demonstrated that lack of the IL-20 subfamily receptor resulted in decreased number of myofibroblast, and directly influence the ECM production in the obstructed kidney.

6, Our *in vitro* results showed that IL-24 increases the TGF- β and PDGF-B production of renal epithelial cells.

7, We developed set of carefully designed mouse α -SMA-specific primer pairs and PCR conditions to determine the expression of α -SMA, the most important biomarker of MFs. Furthermore, our investigations give an explanation of the expressional variability of the housekeeping gene β -actin observed in different experiments.

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