The design of a liver-selective form of interleukin-10

Rachmawati, Heni

Chapter - 5

A study on the antifibrotic effects of IL-10 in anti-Thy 1-induced glomerulosclerosis in rats

submitted

H.Rachmawati[1], L.Beljaars[1], C.Reker-Smit[1], H.I. Bakker[1], A.M.van Loenen-Weemaes[1], M.N.Lub-de Hooge[2], D.K.F, Meijer[1], K.Poelstra[1]

[1]Department of Pharmacokinetics and Drug Delivery, Groningen University Institute for Drug Exploration (GUIDE),University of Groningen

[2]Department of Nuclear Medicine, University Medical Center Groningen (UMCG)

Abstract

The progression of chronic renal failure represents one of the major challenges in nephrology as it leads to end stage renal insufficiency and requires long-term treatments. Effective antifibrotic drugs are yet to be developed to solve this problem. Interleukin-10 is a cytokine with potent antifibrotic and anti-inflammatory properties and for this reason is extensively studied. However, most studies focused on its anti-inflammatory effects, and IL-10 has a very short plasma half-life *in vivo*. This prompted the question whether a short iv treatment might have a prolonged effect on a more chronic process like sclerosis. Glomerulosclerosis was induced by administration of anti-Thy 1 IgG (5mg/kg) and normal rats received vehicle (PBS). Four days after induction of the disease, an iv injection of either rhIL-10 (8 µg/kg/day) or PBS (control) was given for 3 consecutive days. The animals were examined at day 7. Parameters of inflammation and fibrosis were assessed both at the protein levels by immunostaining and at the mRNA levels by real-time PCR techniques. Control rats, examined at day 7, showed many renal histopathological changes as compared to normal rats. Glomerular matrix expansion and inflammatory cell influx was seen and an increase in glomerular iNOS and α-SMA was found on the protein level, factors that were clearly attenuated by IL-10 treatment. In particular the decrease of MMP-13 levels between day 4 and day 7 was completely prevented by IL-10. In contrast, administration of IL-10 did not significantly reduce the mRNA levels for procollagen α1(1), α-SMA, and TGFβ-1. In conclusion, a short-term treatment with rhIL-10 after induction of anti-Thy 1 nephritic rats attenuated intraglomerular inflammation, and at the protein level also influenced the parameters reflecting matrix deposition and degradation. Despite in fact that IL-10 was shown to be effective in the inhibition of matrix deposition, it had no beneficial effect on proteinuria. We conclude therefore that despite a rapid clearance by the kidneys, IL-10 may be applicable for the treatment of a chronic disease like glomerulosclerosis with prominent effects on renal inflammation and fibrosis.

Introduction

Chronic glomerular diseases are accompanied by a number of characteristic features. Destruction of the glomerular integrity, followed by gradual decline in glomerular function, is most frequently associated with proliferation of resident glomerular cells and extensive matrix expansion. It is considered to be the final stage of several kidney diseases, leading to a progressive loss of renal function. The mechanism underlying this ECM accumulation is not fully understood yet. An imbalance between the synthesis and the degradation of ECM is the main cause of this process. Several components such as TGF-β, connective tissue growth factor (CTGF), reactive oxygen intermediates, matrix metalloproteinases and their inhibitors have been implicated in initiating or maintaining the sclerotic process.[1,2,3]

At present, fibrotic diseases in various organs, including glomerulosclerosis, are treated with immunosuppressant and/or cytotoxic drugs that all have serious systemic adverse effects.[4] Recombinant human interleukin-10 (rhIL-10) is a recently developed drug and it is intensively investigated as a therapeutic agent for chronic disease of different organs.[5-10] IL-10 exerts its antifibrotic activities either through direct effects on ECM modulating enzymes or through indirect effects by inhibition of the inflammatory process. A recent study reported that the administration of adenovirus-mediated IL-10 expression in the kidney could effectively prevent the development of glomerulosclerosis in animal models.[11,12] However, all these experimental treatments showed an effect after multiple injections or a prolonged IL-10 exposure following gene expression of IL-10. The local effect of IL-10 on the fibrotic process can not be discerned from chronic immunosuppressive effects. In particular, since IL-10 has a short half life in plasma and is rapidly cleared via glomerular filtration and accumulates in proximal tubular cells, we wondered whether iv administered IL-10 would have a local effect on this chronic glomerular process. We therefore tested the effect of iv rhIL-10 on the progression of glomerulosclerosis.

To induce glomerulosclerosis, we used anti-Thy 1 model in rats.[13] Intravenous injection of the anti-Thy 1 monoclonal antibody causes a severe mesangiolysis, complement activation and macrophage influx in the first day. This is followed by a marked mesangial repopulation, and severe extracellular matrix expansion which resembles human mesangial proliferative glomerulonephritis.

After 24 h, the influx of inflammatory cells already declines[14] and the accumulation of extracellular matrix in this model later on resolves, partly due to an increased expression of collagenases like MMP-13 and other proteases.[15] The progression of scarring, reflected by intraglomerular matrix expansion, in this experimental model peaks at day 10 and then very gradually decreases to normal levels.[16-19] Based on these reports we examined the effect of IL-10 administration from day 4 to day 6 on the fibrotic process and sacrificed the animals at day 7. In this time frame, initiation of disease has taken place, but the acute inflammation is already strongly diminished while, in contrast, the process of glomerulosclerosis is rapidly ongoing. In a previous study, IL-10 was administered before the onset of disease, and several studies have shown that progression of the disease can be prevented at the initiation phase.[14,20,21] We now focused on the phase following these events i.e. at the onset of glomerulosclerosis. The potential effects of IL-10 were examined by studying inflammatory and fibrotic parameters both at the protein and at the mRNA levels.

Materials and methods

Animals

Specific pathogen-free male Wistar rats (200 – 250 g), purchased from Harlan (outbred strain, Zeist, The Netherlands), were used in this study. The rats received a standard diet and were housed under standard laboratory conditions. The study as presented was approved by the Local Committee for Care and Use of Laboratory Animals and was performed according to strict governmental and international guidelines on animal experimentation.

Gammacamera imaging of IL-10 in normal rats

IL-10 was radiolabeled with ^{123}I using a standard method.[22] [^{123}I]IL-10 (5.10^5 cpm/rat) was intravenously injected to normal rats via the penile vein. The distribution of this radiolabeled cytokine was dynamically recorded using a gammacamera from 0 – 90 minutes with a frame rate of one total body scan per minute.[23]

Production, purification and affinity test of monoclonal anti-Thy 1 antibody

Monoclonal antibody against Thy-1 was produced *in vitro* as described previously[13] from a hybridoma cell line kindly provided by Dr. W.W. Bakker (Dept.of Pathology, University of Groningen, The Netherlands). The binding of this antibody to its antigen was checked on cryosections of healthy rat kidneys with an immunohistochemical staining method.

Induction of glomerulonephritis in rats

To induce glomerulonephritis (GN), rats received a single iv dose of anti-Thy 1 IgG (5 mg/kg), via the penile vein, under anaesthesia with 40% O_2:60% N_2O combined with 0.5% isoflurane.

Experimental protocol

The rats were divided into four groups: Group A was sacrificed at day 4 after induction of anti-Thy 1 GN (N = 4) that is, at the onset of treatment. Group B was treated with vehicle (PBS) from day 4 to day 6 and sacrificed at day 7 after induction of the disease (N = 6), and group C was treated with IL-10 from day 4 to day 6 and sacrificed at day 7 after induction of the disease (N = 6). Another control group of rats (group D, N = 4) received only PBS (normal rats). Recombinant human IL-10 (PeproTech EC Ltd., UK) was given as a single iv dose (8 μg/kg/day) via the penile vein for 3 consecutive days, starting at day 4 after the disease induction.

Analysis of samples

At day 7, all animals were sacrificed and blood, urine, serum and organs were collected. To study the pharmacological effects of IL-10 treatment, various parameters were examined using biochemical assays, immunohistochemical methods and quantitative real-time PCR (QRT-PCR) techniques.

Biochemical assays

Determination of nitric oxide levels in serum

Nitric oxide was determined in serum samples with a standard Griess reaction method.[24] The calculation of serum NO_x levels was corrected for

endogenous nitrite in the serum which was determined from the nitrite assay procedure.

Determination of urinary protein levels

Urine samples were collected from all rats at day 7 during the final 24 h of the experiment. Total urinary protein content was determined with a standard Bradford method according to the manufacturer's instruction (Biorad kit protein assay, Biorad, Herculas, CA, USA), with bovine serum albumin as a reference.

Renal morphology

Tissues for light microscopy were fixed overnight in 4% formalin in PBS and dehydrated in 70% ethanol. The tissues were embedded in paraffin and subsequently the sections (4 μm thick) were routinely stained with hematoxylin/eosin (HE) and with the periodic acid-Schiff (PAS) reagents.

Immunohistochemical staining

Kidney samples were snap frozen into isopentane (-80^{o}C) and immunostainings were performed on cryostat sections (5 μm thick). The following antibodies were used: mouse anti-ED1 IgG (Serotec, Oxford, UK), mouse anti-Thy1 IgG (isolated from culture media of a hybridoma cell line), rabbit anti-inducible Nitric Oxide Synthetase (iNOS, generous gift of Dr. H.Moshage, Univ. of Groningen) IgG, mouse anti-alpha smooth muscle actin (α-SMA) IgG (Sigma, St.Louis, USA) goat anti-collagen I, III, and IV IgGs (Southern Biotech, Birmingham, USA), and goat anti-matrix metalloproteinase-13 (MMP-13) IgG (Santacruz Biotechnology, CA, USA). Sections were routinely stained with peroxidase-conjugated rabbit anti-mouse IgG (RAMPO, Dako, Golstrup, Denmark) to check the presence of mouse anti-Thy 1 IgG that was administered to the rats.

After fixation with aceton, sections were rehydrated in PBS and then incubated for 1 h at room temperature with the indicated primary antibody. To block the endogenous peroxidase activity, the sections were incubated with 0.1% H_2O_2 in PBS for 20 minutes. Subsequently, the secondary antibodies (Dako, Golstrup, Denmark) were applied for 30 minutes. The immunoreactions were visualized with 3-amino-9-ethylcarbazole (AEC, Sigma Inc., St.Louis, USA) and sections were counterstained with hematoxylin (Fluka, Buchs, Switzerland).

Quantitation of immunostainings

The number of intraglomerular macrophages was quantified by counting the number of ED-1 positive cells in at least 50 glomeruli per rat. The sum of ED-1 positive cells was divided by the number of glomeruli counted.

The glomerular MMP-13 staining was graded semiquantitatively from 40 glomeruli of each rat using the following scale: 0 (no staining within glomeruli), 1 (1- 25%); 2 (26 – 50%); and 3 (> 50% of total glomerular area is positive). The score index per section was then calculated with the following formula:

$$\textbf{Score index} = [(n_{0\%} \times 0) + (n_{1\text{-}25\%} \times 1) + (n_{2\text{-}50\%} \times 2) + (n_{>50\%} \times 3)]/40$$

(n = number of glomeruli)

The glomerular iNOS staining was semiquantitatively graded. Sections which were positive for glomerular iNOS were marked as "+", whereas sections without glomerular iNOS staining were marked as "-".

The glomerular α-SMA, staining of mesangial cells with anti- Thy 1 IgG, and type I, III, and IV collagen stainings were quantified with image analyzing software, ImageJ (National Institutes of Health, USA) for at least 40 glomeruli per rat. To measure the percentage of stained area per glomerulus in a digital picture, each glomerulus was outlined separately and the intraglomerular area positively stained for a particular parameter was measured and related to the total glomerular area. Greyscale (8 bit) and threshold (0/80 for stained area and 0/255 for intraglomerular area) of stainings were kept constant for all stainings. For Thy1 (mesangium cells) staining, threshold was set up as 0/130 and 0/255.

Quantitative real-time PCR

The quantitative real-time PCR (QRT-PCR) was performed to assess the most important gene expression levels associated with the disease.

mRNA isolation

Total RNA was extracted from samples of the renal cortex with a mini column according to the manufacturer's instruction (RNeasy® mini kit, Qiagen Sciencer, Maryland USA). mRNA concentrations were determined with NanoDrop® (ND-1000 UV-Vis spectrophotometer, NanoDrop Technology,USA). Integrity of mRNA samples was checked by electrophoresis on a 2% agarose gel and the

absence of cDNA in the samples was verified by performing a PCR on the mRNA samples using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) while omitting the reverse-transcriptase step. cDNA was prepared from 1 μg of total RNA in a 25 μL of total volum with RT-PCR system according to the manufacturer's instruction (Promega Inc., USA).

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed with a high-throughput real-time PCR system (ABI 7900HT sequence Detection System, Applied Biosystems, CA, USA).

Table I. Rat oligonucleotide primers used for the analysis of genes by quantitative real time-PCR

Genes	Nucleotide sequences
α-SMA	Upstream: 5′-GACACCAGGGAGTGATGGTT-3′
	Downstream: 5′-GTTAGCAAGGTCGGATGCTC-3′
TGFβ-1	Upstream: 5′-CAACAACGCAATCTATGACAAAA-3′
	Downstream: 5′-AGTAGTTGGTATCCAGGGCTCTC-3′
Procollagen α1(1)	Upstream: 5′-AGCCTGAGCCAGCAGATTGA-3′
	Downstream: 5′-CCAGGTTGCAGCCTTGGTTA-3′
GAPDH	Upstream: 5′-CCATCACCATCTTCCAGGAG-3′
	Downstream: 5′-CCTGCTTCACCACCTTCTTG-3′

The PCR mixture (total volume of 20 μL) contained 1.25 μL cDNA, primers (1 μM concentration of each primer, table I), and 2x SybrGreen master mix (Applied Biosystem). An initial denaturing step at 95°C for 10 minutes was followed by 40 cycles of 95°C for 15 seconds, 56°C for 15 seconds, and 72°C (a measuring step) for 40 seconds. Each measurement was performed in triplicate. A single product was confirmed by checking the dissociation curve at the end of the PCR reaction. Data were analyzed with the SDS software 2.1 (Applied Biosystems). The calculation of gene expression levels was normalized to GAPDH as a house

keeping gene. The fold induction was counted relative to the mRNA levels of the indicated gene in normal kidneys.

Statistical analysis

Data were presented as mean ± SD of all rats per group or as individual data after real time PCR measurements. All data were subjected to an unpaired, two-tailed distribution student t-test. Differences were considered significant at $p<0.05$.

Results

In vivo distribution of IL-10

Using a gammacamera technique, we recorded the biodistribution of [^{123}I]IL-10 in normal rats after iv administration.

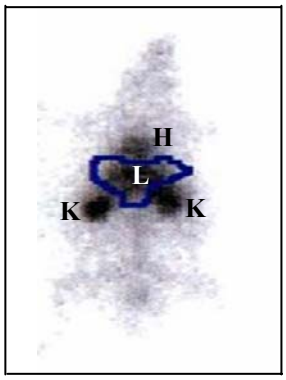


Fig.1. A gammacamera analysis showed a high level of [^{123}I]IL-10 in the kidneys (K) after iv injection of a tracer amount of radiolabeled IL-10. L: liver, H:heart.

The result showed that immediately after injection, that is within one minute, high levels of IL-10 were found in the kidney and remained high up to the end of experiment (60 min).

Analysis of renal histology

Renal histology was examined on paraffin sections stained with PAS and HE. Glomerular changes were examined on day 4 and 7 after administration of anti-Thy 1 IgG. On day 4, mesangiolysis was seen in many glomeruli. In some glomeruli, the mesangiolysis led to capillary microaneurysmal baloon formation.

There was also marked mesangial hypercellularity and the beginning of mesangial matrix expansion. This result is in line with previous studies.[16,25] On day 7, microaneurysmal balooning was still present and mesangial cell proliferation was more evident as compared to day 4. There was a difference between IL-10-treated rats and rats receiving vehicle after the anti-Thy 1 injection. Mesangial matrix expansion in a focal and segmental pattern was found on day 7 in control nephritic rats, whereas in IL-10-treated rats this was slightly reduced. Occasionally, degeneration of complete glomeruli was seen, both in IL-10-treated and control nephritic groups.

NO generation in serum after anti-Thy 1 IgG administration

The serum NO_x levels of normal rats were very low (7.3 ± 1.3 μM). Four days after induction of anti-Thy 1 GN, a significant increment in serum levels of NO_x was observed ($p<0.05$, table II). These NO_x levels did not further increase between day 4 and day 7 in all groups.

Urinary protein excretion

Proteinuria was markedly increased in control GN group as well as in IL-10-treated group, as compared to normal rats. There was no significant difference observed between these two groups (table II).

Table II. The effects of IL-10 treatment on serum NO_x levels and protein in urine of rats after GN induction with anti-Thy 1 antibody

Parameters	Normal rats	Group A*	Group B	Group C
NO (μM)	7.27 ± 1.3	25.0 ± 10.1	32.9 ± 16.8	27.2 ± 17.6
Proteinuria (mg/24 h)	20.2 ± 8.5	nd	137.4 ± 29.4	128 ± 31.1

*Group A: 4 days after GN induction; group B= 7 days after GN induction; group C= 7 days after GN induction and receiving IL-10 treatment. nd : not determined. Data are presented by mean ± SD of 6 rats per group. *N = 4*

Studies on the effects of IL-10 administration on anti-Thy 1-induced glomerulosclerosis

The effects of IL-10 on anti-Thy 1-induced glomerulosclerosis were studied by examining the changes in glomerular proteins corresponding to inflammatory or fibrotic processes. Parameters reflecting the inflammatory process were ED-1 and iNOS. In addition, changes in mesangial cell phenotype associated with the

disease such as proliferation, activation, and matrix modulation were assessed by staining for Thy 1, α-SMA, collagen I, III, and IV and matrix metalloproteinase (MMP-13), respectively.

The effect of IL-10 on the number of glomerular ED1 positive cells

Determination of ED-1 positive cells in glomeruli represents the number of intraglomerular macrophage infiltration. In normal rats, glomerular ED-1 staining was negative. Compared to normal rats, the average number of ED-1 positive cells per glomerulus showed a significant increase at day 4 (group A, 8.45 ± 1.57) and remained constant up to day 7 (group B, 7.63 ± 2.47). Treatment with IL-10 significantly reduced the number of ED-1 positive cells within glomeruli compared to control nephritic rats at day 7 (3.48 ± 1.2, $p<0.05$). Surprisingly, the number of ED-1 positive cells after IL-10 treatment was even lower than in group A (fig.2, $p<0.05$).

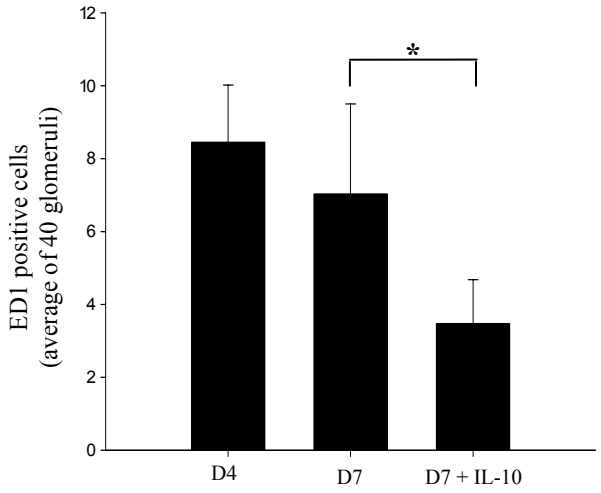


Fig.2. The effect of IL-10 treatment on the glomerular macrophage accumulation. The number of ED-1 positive cells within glomeruli was significantly reduced by IL-10 as compared to the untreated GN group at day 7 (D7) after anti-Thy 1 injection. The values are the means of four rats at day 4 (D4) and of six rats at day 7 (D7) ± SD. *$p<0.05$.

The effects of IL-10 on the glomerular staining for iNOS, the messangial cell marker (Thy 1), α-SMA, MMP-13, type I, type III, and type IV collagen

Inducible nitric oxide synthetase (iNOS), α-SMA, type I and type III collagen, and MMP-13 were not constitutively expressed in the glomeruli, except for collagen type IV which was present in normal glomeruli. At day 4 after

induction of anti-Thy 1 IgG, iNOS and type III collagen were not detected in glomeruli, whereas moderate increases in type I and type IV collagen, α-SMA and MMP-13 were found. At day 7 after induction of disease, however, a marked increase in iNOS, α-SMA, type I, III, and IV collagen was noted, while MMP-13 staining was clearly reduced in comparison to nephritic rats at day 4.

In particular for iNOS, expression was not present in all rats. Four out of six nephritic rats contained iNOS positive cells in the glomeruli. The iNOS staining was detected along the capillary loops of glomeruli (fig.3). In the IL-10-treated group, 2 out of 6 rats had iNOS positive in glomeruli and the staining was weaker than in the control nephritic rats at day 7. Despite this reduced glomerular iNOS expression, IL-10 this did not significantly influence the NO_x levels in serum (table II).

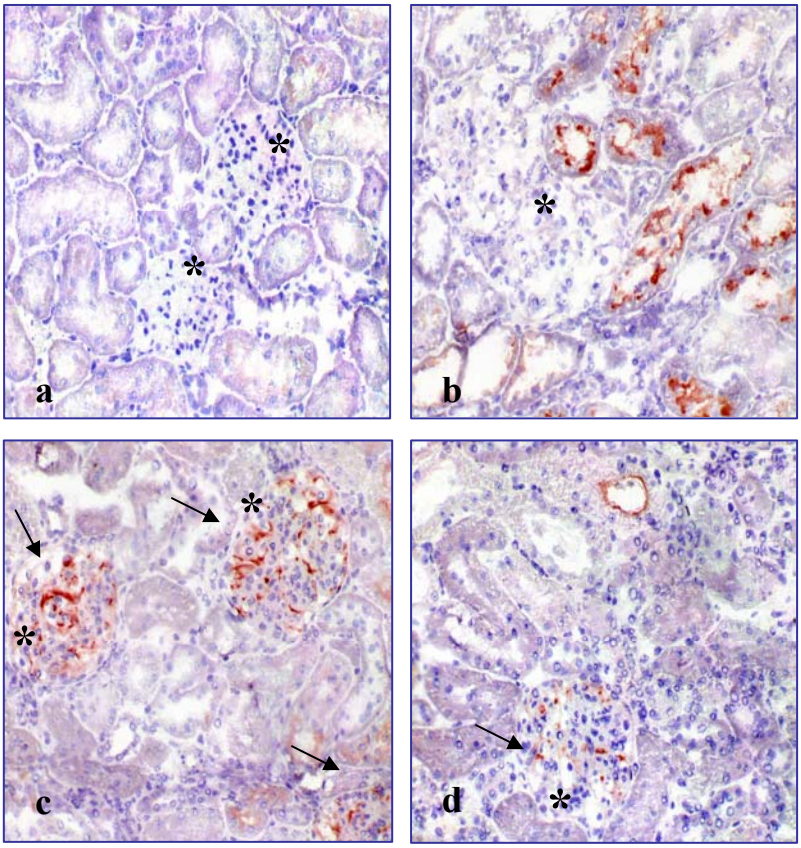
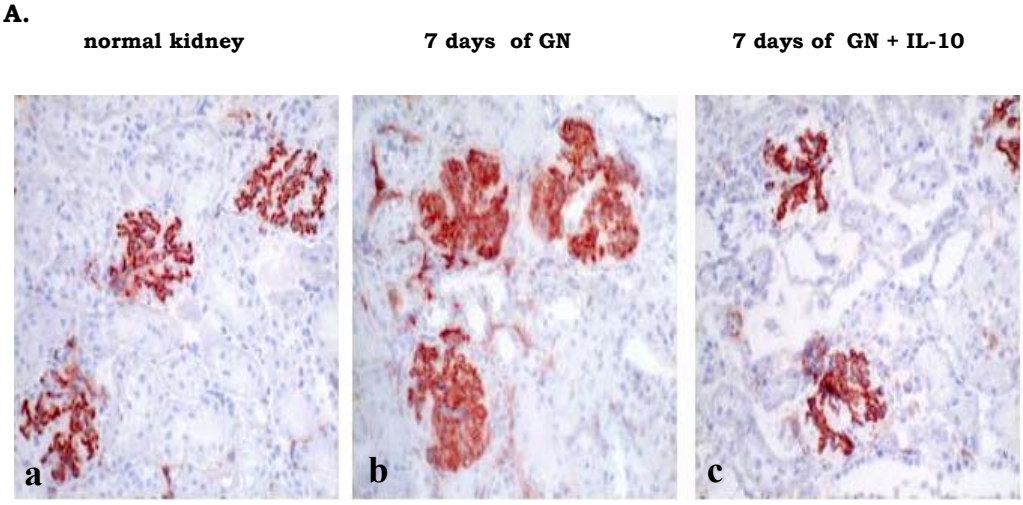


Fig.3. The effect of IL-10 on the glomerular iNOS staining in nephritic rats, 7 days after anti-Thy1 injection. iNOS staining was not positive in the glomeruli (*) of normal rats (a) nor in nephritic rats at day 4 after induction of the disease (b). A marked increase of glomerular iNOS staining (arrow) was found in nephritic rats at day 7 after induction of the disease (c) and this was attenuated by IL-10 treatment (d). Original magnification: x200. GN : glomerulonephritis.

A.



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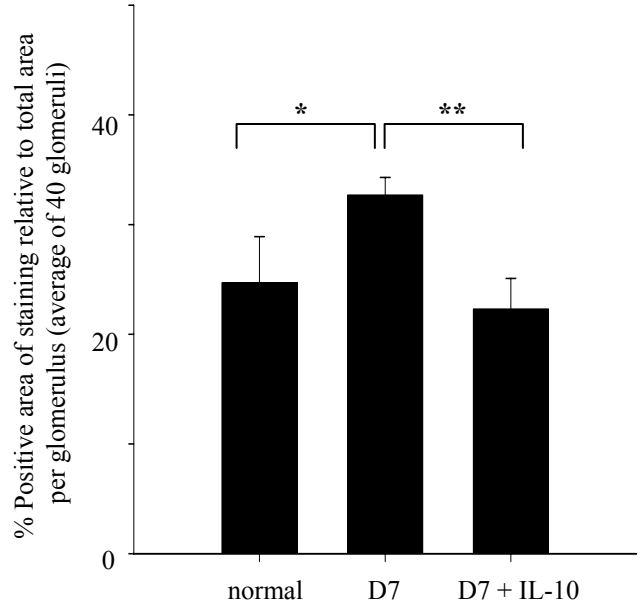


Fig.4. (**A**) Light microscopy of Thy 1 staining. At day 7 after injection of anti-Thy 1 IgG, the staining markedly enhanced (b) as compared to normal kidney (a), indicating mesangial cell repopulation. Treatment with IL-10 attenuated the increment of Thy 1 staining (c). Original magnification: x200. (**B**) Quantification analysis of Thy 1 immunostaining as measured with ImageJ software from at least 40 glomeruli of each rat. IL-10 treatment caused a significant inhibitory effect on Thy 1 staining as compared to untreated rats (D7). *$p<0.05$; **$p<0.01$.

Assessment of mesangial cells proliferation, performed by Thy 1 staining, revealed an increase in this cell number in control nephritic rats at day 7 (group C) as compared to normal rats (fig.4). This increment was accompanied by an upregulation of α-SMA staining.

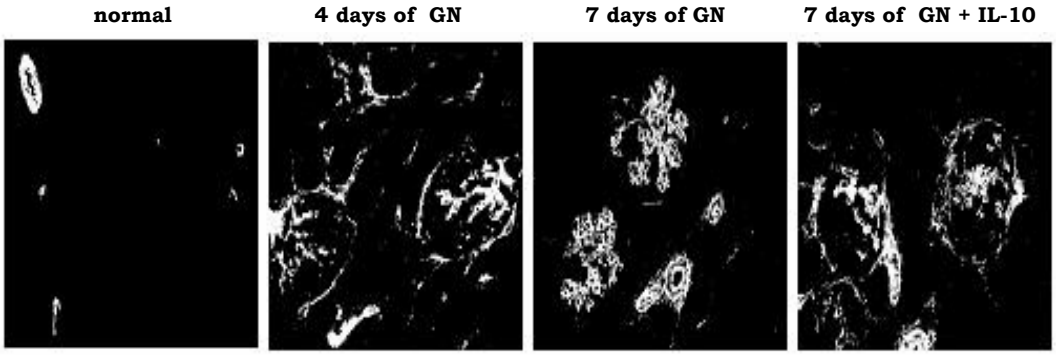
α-SMA staining was weak in glomeruli of rats at day 4 after induction of disease (group A). In contrast, a significant staining was found at day 7 in a pattern corresponding to the anti-Thy 1 staining in group C. In IL-10-treated rats, staining for anti-Thy 1 at day 7 was reduced compared to control nephritic rats (fig.4). This was accompanied with a decrease in glomerular α-SMA positive cells (fig.5). Quantitative analysis of this staining with ImageJ software revealed a reduction of 32% ± 8.4% and 23% ± 11.2% ($p<0.05$) for Thy 1 and α-SMA respectively, as compared to rats receiving no treatment (fig.4 and 5). The amount of this α-SMA protein as determined by ImageJ software in the IL-10-treated group was similar to that in rats at day 4 after induction of the disease (group A). This indicates that IL-10 prevented the increment in α-SMA staining from day 4 to day 7 of nephritis.

In normal rats, type IV collagen was found along the basement membrane of capillary loops and a small amount of this matrix protein was seen in the mesangial area. The staining intensity for type IV collagen was clearly enhanced at day 7 in untreated nephritic rats (fig.6). This enhancement was not seen in rats treated with IL-10. As shown in figure 7, IL-10 inhibited this deposition of this type of collagen by 32% ± 19% ($p<0.05$), and type IV collagen deposition at day 7 in the treated group was similar to the staining at the onset of treatment (day 4).

Type I collagen was clearly noted in mesangial hypercellular lesions of rats with Thy 1 nephritis both at day 4 and day 7 (fig.6), whereas type III collagen was only occasionally found in glomeruli. There was no significant effect of IL-10 on type I and type III collagen stainings. To study to what extent IL-10 influenced matrix remodelling, we also examined the glomerular expression of a matrix-degrading enzyme. MMP-13, one of matrix metalloproteinases, is the most important MMP since it degrades fibrillar collagens (type I and III).[26] MMP-13 is expressed at a very low level in healthy glomeruli. Twenty four hours after induction of anti-Thy 1 nephritis, MMP-13 expression was clearly induced (data not shown). In contrast, MMP-13 expression in a later stage of the disease (day 4)

appeared to be low, corresponding with reduced matrix degradation activity.[26,27] This MMP-13 expression was slightly further declined at day 7.

A.



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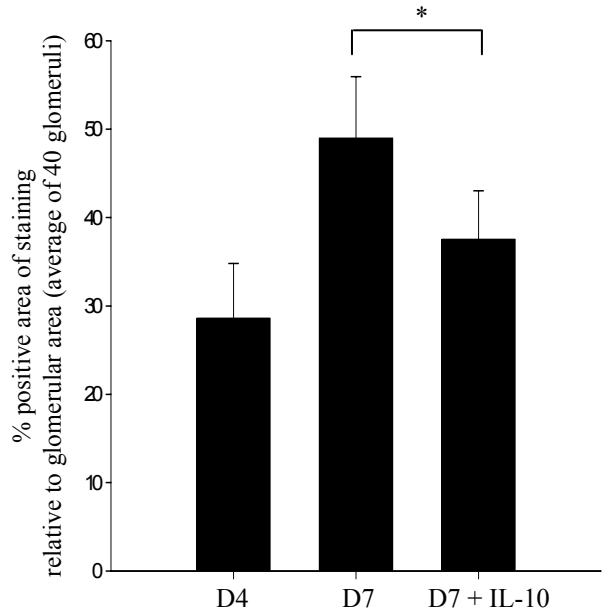


Fig.5. (**A**) Light microscopy of alpha smooth muscle actin (α-SMA) staining in kidney sections. α-SMA was not detectable in the glomeruli of normal rats and only observed in vascular muscle cells (a). At day 4 after induction of anti-Thy 1 nephritis, α-SMA was strongly expressed in the mesangial area and occasionally around periglomeruli (b). The glomerular α-SMA staining was markedly enhanced at day 7 of nephritis (c) and this enhancement was significantly attenuated by IL-10 treatment (d). Original magnification: x200. (**B**) A quantitative analysis with ImageJ software shows a significant reduction in the glomerular α-SMA staining in IL-10-treated rats as compared to untreated nephritic rats (D7). *$p<0.05$.

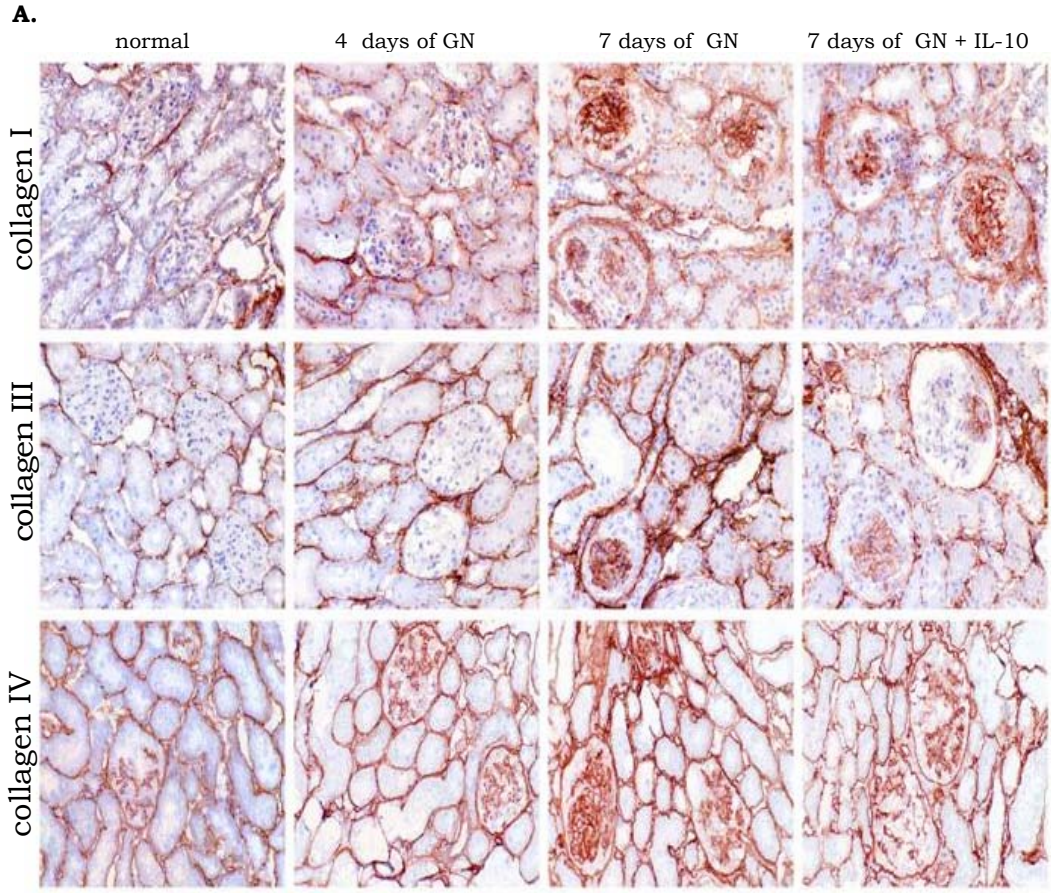
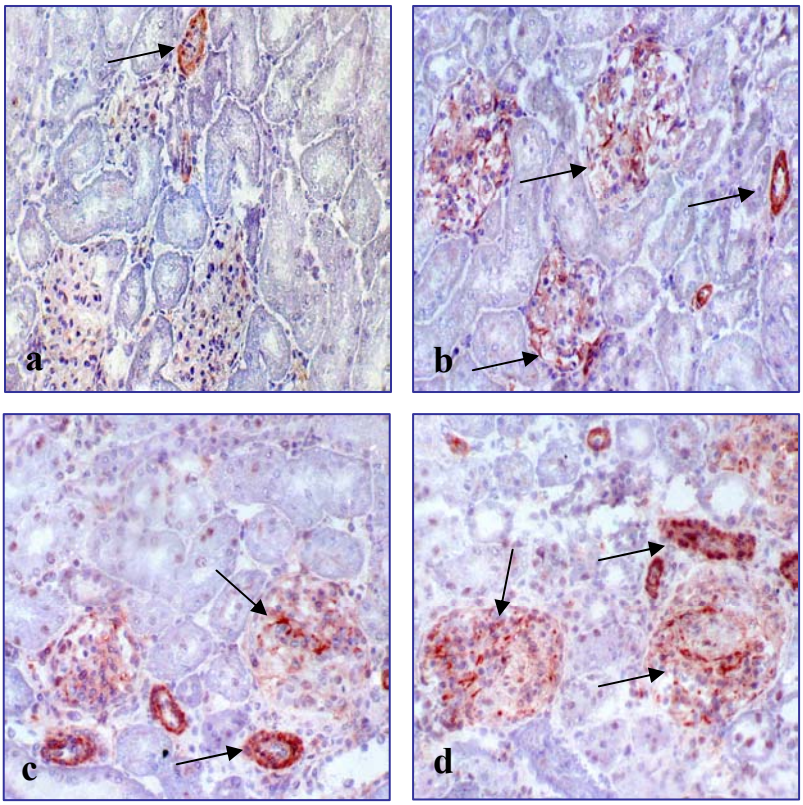


Fig.6. (A) The effects of IL-10 treatment on the glomerular staining of type I, type III, and type IV collagen. At day 4 after anti-Thy 1 administration, type I and type IV collagen in the glomeruli increased, whereas type III collagen did not differ from normal rats. In contrast, at day-7, these collagens markedly increased in a focal and segmental pattern. The increase of these stainings was reduced by IL-10 treatment. Original magnification x200. (**B)** Quantitative analysis of glomerular type IV collagen staining with image analyzing software, ImageJ, in at least 40 glomeruli per rat. The level of glomerular type IV collagen increased from day 4 to day 7. IL-10 significantly attenuated this staining nearly to the levels of rats at day 4 after the disease induction (D4). *p<0.05.

A.



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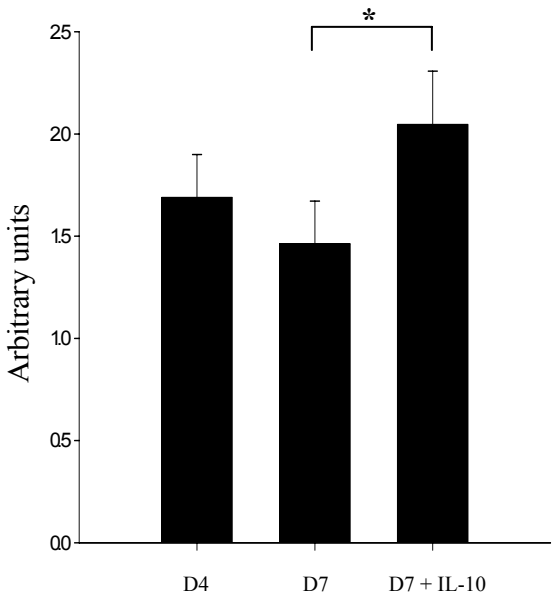

Fig.7. The effect of IL-10 on the glomerular staining of MMP-13 (arrow). (**A)** MMP-13 is expressed in low level in the glomeruli of normal rats (a). At day 4 after anti-Thy 1 administration, glomerular MMP-13 staining significantly increased (b), but decreased at day 7 (c). IL-10 increased the level of glomerular MMP-13 staining (d) as compared to untreated nephritic rats (c). Original magnification x200. (**B)** The scoring index of glomerular MMP-13 shows that IL-10 significantly enhanced glomerular MMP-13 staining as compared to untreated nephritic rats (d versus c).*$p<0.05$.

Yet, the MMP-13 expression in the IL-10-treated rats did not significantly change in comparison to rats at day 4 but was significantly higher than in untreated nephritic rats at day 7 (fig.7). This result suggests that one mechanism by which IL-10 might suppress the progression of disease is through the preservation of glomerular MMP-13 level.

The effect of IL-10 on gene expression for procollagen α1(1), α-SMA and TGFβ-1

We performed real-time PCR to analyze the expression of the most relevant genes during glomerulosclerosis, quantitatively. Real-time PCR was performed to analyze mRNA levels for procollagen α1(1), α-SMA, and TGFβ-1 (fig.8). As compared to normal rats, the mRNAs levels for most parameters studied, significantly increased after the disease induction. At day 7 of nephritis, the mRNAs levels did not increase any further compared to the mRNA levels at day 4. However, in IL-10-treated nephritic rats, expression of all these genes did not significantly change in comparison with the untreated nephritic group.

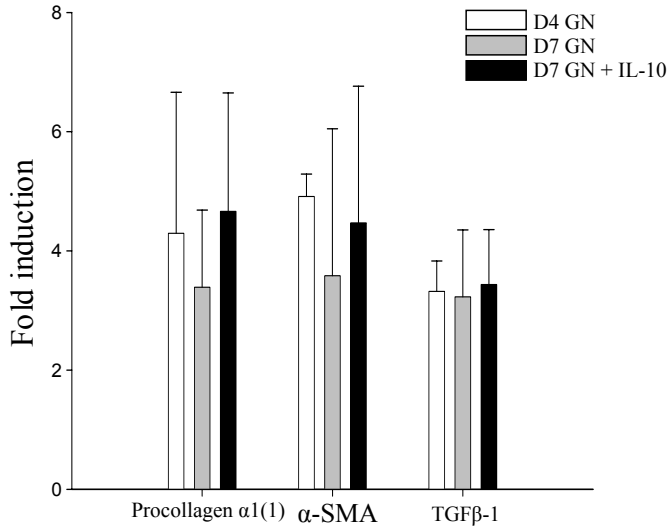

Fig.8. mRNAs expression of some genes in the renal cortex measured by quantitative real-time PCR techniques. Fold induction as depicted on the y-absis was calculated from mRNA level of the same genes in the normal renal cortex.

Discussion

In this report, we demonstrate an effect of IL-10 on the process of glomerulosclerosis induced by anti-Thy 1 IgG. IL-10 is a small protein which is rapidly cleared by the kidney after iv injection, as reflected by our gammacamera imaging (fig.2). Immediately after injection, a high amount of [^{123}]IL-10 distributed within one minute to the kidneys and remained present in this tissue up to the end of experiment (60 min). This may be beneficial when IL-10 is used to treat various diseases in the kidney, yet accumulation probably reflects uptake in tubular cells after glomerular filtration of this low molecular weight protein. So, we wondered whether a daily iv injection would have an effect on a chronic disease like glomerulosclerosis. In this study, we found potent effects of IL-10 treatment in nephritic rats.

Progressive glomerulonephritis (glomerulosclerosis) leading to renal insufficiency can be rapidly induced by a single iv injection of anti-Thy1 monoclonal antibody.[13,14,28] A severe glomerular damage, characterized by a moderate focal and segmental pattern of microaneurysmal ballooning, followed by enhanced mesangial repopulation, and scar formation was already detected on day 4. We used this model to study the effects of IL-10 on the development of glomerular fibrogenesis. We started treatment at day 4, to avoid any interference with the initiation phase of the disease. At this time point, mesangiolysis, complement activation and neutrophils influx has fully taken place and is already diminishing whereas the sclerotic process is rapidly ongoing.

Histopathological analysis revealed that a progressive glomerular fibrotic process had occurred 7 days after anti-Thy 1 injection, as indicated by high expression of type I, III, and IV collagen, and α-SMA. This glomerular damage corresponded with persistent accumulation of macrophages as represented by ED-1 positive cells which remained high to day 7. It is well known that intraglomerular macrophage accumulation is one of the crucial events during GN in animals and man.[29-32] Macrophages are able to produce high amounts of cytokines and growth factors that modulate mesangial cell proliferation and ECM production. It is reported that macrophages located in the mesangium participate in the progression of glomerulonephritis by inducing phenotypic changes of mesangial cells and enhancing the ECM production.[32] Our study shows that a short-treatment with IL-10 significantly reduces macrophage recruitment within

glomeruli. Considering the role of macrophage in the progression of disease, this is highly relevant.

The inhibitory effect of IL-10 on the glomerular iNOS expression found in our study (fig.3) might be due to this suppression of macrophage accumulation, since macrophages in the kidneys are known to produce iNOS *in vitro* and in certain experimental models.[30,31]

The disease is associated with an increased staining for Thy 1, reflecting mesangial cell proliferation at day 7. This increase was also inhibited by treatment with IL-10. This may be via indirect effects of IL-10 on intraglomerular macrophage accumulation or via direct effects on mesangial cells itself.[33] The ability of IL-10 to inhibit macrophage recruitment and activation *in vivo* was demonstrated in a macrophage-mediated passive anti-glomerular basement membrane antibody induced model of glomerulonephritis in rats.[30] Direct inhibitory effects of IL-10 on mesangial cell proliferation were also found in another study in the anti-Thy 1 model.[28] *In vitro* studies showed that IL-10 inhibited both macrophage and mesangial cells, making it likely that both indirect and direct effects on mesangial cell occur *in vivo*.[34] The IL-10 receptor is present within the mesangial area within normal glomeruli[22] and also in anti-Thy 1 GN at day 7 (data not shown) and is supposed to mediate the pharmacological activities of exogenous IL-10.

Induction of proliferation and activation of mesangial cells in this glomerular disease leads to enhanced α-SMA expression and an increase in the production of collagens, as also observed in the present study. Enhanced type I, III, and IV collagen expression was found within glomeruli, next to the α-SMA positive cells. This confirmed the result of Kaneko, *et al.*[35] Consistent with a reduction in mesangial cell proliferation induced by IL-10, we observed a reduced expression of α-SMA, type I, III, and IV collagen at the protein level. Quantitative evaluation of sections revealed a significant reduction in these parameters. Staining intensity of IL-10-treated rats at day 7 was similar to the staining at day 4, that is, at the onset of treatment. This indicates that IL-10 inhibited the progression of disease. The glomerular matrix expansion is likely to be a net result of an imbalance between synthesis and degradation of these components. In our study, an upregulation of type I, III, and IV collagen in glomeruli was not accompanied by an upregulation of glomerular MMP-13. MMP-13, an interstitial

collagenase-3, is a very potent enzyme involved in the degradation of interstitial collagens like type I and III collagen.[26] As compared to group A (day 4 after disease induction), levels of glomerular MMP-13 in group B (day 7 after disease induction) clearly decreased. However, in IL-10-treated rats, MMP-13 level did not decline but even increased above the level at day 4. Based on this observation, the reduction in the glomerular matrix expansion after IL-10 treatment may be partly due to an upregulation of MMP-13 expression, thereby increasing the collagenolytic activity.[36-38]

A markedly increase in the mRNA levels for the most relevant genes associated with the fibrotic process at day 4 also clearly reveals that the progression of the disease had already started at this time point. At day 7, when further scarring took place, the mRNA levels for procollagen α1(1), α-SMA, and TGFβ-1 did not increase any further as compared to day 4. This persistence levels for these genes was also found in the IL-10-treated rats. We therefore conclude that the effect of the treatment of this short-lived cytokine is notable on many parameters at the protein level, but the effect on the examined gene expression levels is no longer present, 24 h after the last injection.

In summary, in this study we demonstrate that a short treatment with recombinant human IL-10 is able to prevent the progression of glomerulosclerosis in the anti-Thy 1 model. Significant effects of IL-10 treatment on ED-1, iNOS, Thy1, α-SMA, type IV collagen, and MMP-13 stainings in the glomeruli indicate a role for IL-10 in controlling the most relevant factors involved in the glomerular disease process. Despite that IL-10 was effective as to almost all of the parameters that eventually attenuate matrix deposition, it had no beneficial effect on proteinuria. The inhibition of the fibrotic process evidently does not lead to attenuation of the damage of the glomerular filtration barrier. In conclusion, notwithstanding its rapid clearance by the kidneys, IL-10 may be considerable useful as a therapeutic protein to treat a chronic disease like glomerulosclerosis. The relevance of this result and applicability of IL-10 in this disease needs to be extended using various dose regiments as well as additional animal models.

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