**Fibrogenesis in progressive renal disease**
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# Chapter 4

## Alternatively spliced isoforms of fibronectin in immune-mediated glomerulosclerosis: the role of TGF-beta and IL-4

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## Abstract

Fibronectin (FN) is the main extracellular matrix component in glomerulosclerotic lesions. Different FN isoforms exists, which result from alternative splicing at the EDA and EDB regions of FN mRNA. Increased inclusion of EDA and EDB, which can be elicited by TGF-β, may be conducive to the development of glomerulosclerosis (GS). TGF-β and IL-4 have previously been shown to play a role in the development of GS.

We investigated the mRNA splicing patterns for EDA+ and EDB+ fibronectin *in vivo* in various experimental sclerotic glomerulopathies, *in vitro* in rat mesangial cells (MC) that were stimulated by TGF-β or transfected with IL-4, and in human kidney biopsies with GS from patients with various kidney diseases.

Glomerular FN mRNA demonstrated inclusion of both ED regions in rats with anti-Thy1 nephritis or chronic serum sickness and in mice with anti-GBM glomerulonephritis. Inclusion of both the EDA and EDB regions was associated with glomerular TGF-β expression. In contrast, in mice with Th2-mediated graft-versus-host disease, a model for lupus nephritis, the FN transcripts included neither the EDA, nor the EDB region, and renal TGF-β expression was absent. Compared to normal MC in culture, MC transfected with IL-4 produced lower amounts of FN and demonstrated less EDA inclusion, while MC that had been treated with TGF-β showed increased production of FN and more EDA inclusion. Renal biopsies from patients with renal diseases, except those taken from patients with lupus nephritis, showed higher TGF-β levels, higher FN levels, and more EDA inclusion than controls.

TGF-β might be a key player in the development of GS by inducing local FN production and alternative splicing of FN mRNA. In lupus glomerulonephritis, in which the involvement of TGF-β in GS is less prominent, Th2 cytokines such as IL-4 probably account for increased intrarenal collagen synthesis and subsequent FN accumulation from the circulation. We conclude that in lupus nephritis neither alternative FN splicing, nor a high transcription level of TGF-β appears to be a general prerequisite for the development of GS.

## Introduction

Injury to the kidney can result in chronic progressive renal failure with glomerulosclerosis, accompanied by excessive accumulation of extracellular matrix (ECM) components. Several studies in humans and animal models have shown increased deposition of fibronectin (FN) in glomerulosclerotic lesions (1-5). Accumulation of FN in the glomeruli may be caused by both increased local gene transcription and specific accumulation from the circulation (6).
The glycoprotein FN plays an important role in several biological processes including: maintenance of normal cell morphology, cell migration, cell differentiation, cell remodeling during embryogenesis, and wound healing (7-9). Three distinct splicing sites have been identified in the mRNA of the fibronectin molecule (10,11), which may be spliced-in or spliced-out in a tissue-specific and developmental stage-specific manner (12,13): the extra domain A (EDA), the extra domain B (EDB), and the type III connecting segment (IIICS or V) region. Increased inclusion of EDA-encoding fragments in fibronectin mRNA is observed in tissue during embryonic development (14) and at the margins of healing wounds (9,15), suggesting that the appearance of EDA+ and EDB+ fibronectin mRNA in the tissue is associated with a high rate of remodeling.

In the normal kidney, in both humans and rodents, the FN that contains the EDA domain is present in only small amounts in the mesangium, while the EDB containing FN is not detectable at the protein level. Alterations in the amount and localization of FN protein isoforms which contain the EDA and EDB domains have been found in both human diseases and experimental models (1,2,16-18).

A number of cytokines have been found to mediate the pathological process that leads to glomerulosclerosis. The cytokine TGF-β plays a causal role in the pathogenesis of glomerulosclerosis in the anti-Thy1 model by increasing the production of matrix molecules (19). TGF-β also affects splicing of FN (20,21). IL-4 may also play a role in the induction of quantitative and qualitative alterations in matrix composition, which occurs during the development of glomerulosclerosis. IL-4 expression is increased in various types of glomerulonephritis (22). *In vivo* studies showed that IL-4 plays a role in the development of glomerulosclerosis in GVHD mice (23).

In the current study, the splicing pattern of the FN EDA and EDB regions at the mRNA level was examined *in vivo* in several experimental models of immune complex glomerulonephritis, and it was examined *in vitro* in rat mesangial cells that had either been stimulated by TGF-β or transfected with IL-4. These data were also compared with the mRNA levels for fibronectin, EDA+ and EDB+ fibronectin, and TGF-β in human kidney biopsies from patients with glomerulosclerosis.

## Materials and methods

### *In vivo* animal studies

*Animal models of nephritis-induced glomerulosclerosis.*
Anti-Thy-1 nephritis (αThy1) was induced in female Lewis rats as described elsewhere (24). Animals were sacrificed at day 2, 6, and 14. Chronic serum sickness (CSS) was induced in Wistar rats as has been described elsewhere (25). These rats developed a membranous glomerulopathy with focal and segmental glomerulosclerosis (26). The time points at which the proteinuria reached a level of 800 mg/24 hr was considered week 0 of the experiment for each rat. Animals were sacrificed at week –3, 0, 5, and 20. Anti-glomerular basement membrane nephritis (αGBM) was induced in 12-weeks-old female C57BL/10 mice through injection of rabbit anti-GBM antibodies (a generous gift of Dr. K.J.M. Assmann, Dept. of Pathology, University Hospital Nijmegen, The Netherlands). The antibody was prepared as described before (27). Animals were sacrificed at day 0 (1hr after injection), 5, and 14. Animals developed glomerulonephritis and glomerulosclerosis within 14 days. Lupus nephritis was induced with the chronic Graft-versus-Host disease (GVHD) model by injecting 8-week-old female (C57BL/10 x DBA/2) F1 hybrids with a single-cell suspension of DBA/2 donor cells, as described before (28). These mice developed immune-complex glomerulonephritis, which was followed by glomerulosclerosis 8 to 10 weeks after induction of the disease. Animal care and experimentation were in accordance with legislation on animal experiments as determined by the Dutch Veterinary Inspection.

*Glomerular RNA isolation*
Rat glomeruli were isolated with the differential sieving technique (29). Mouse glomeruli were isolated with iron-oxide perfusion followed by magnetic extraction (30). RNA was isolated with TRIzol® reagent (Invitrogen, Paisley, Scotland, UK) according to the instructions of the manufacturer.

*Reverse transcriptase-polymerase chain reaction (RT-PCR) for EDA and EDB fibronectin*
cDNA was prepared using AMV reverse transcriptase RT (Roche, Germany), according to the prescription of the manufacturer, and the PCR was performed as described (31). DNA fragments were analyzed by electrophoresis and the radioactivity of each PCR product was visualized with a Phosphor Imager 445 SI (Molecular Dynamics, Sunnyvale, CA).

*Immunohistochemistry*

Immunofluorescence studies were performed on frozen kidney tissue from rats with CSS and from mice with GVHD to detect the presence of TGF-β1. Cryostat sections were incubated for 30 minutes at room temperature with a polyclonal rabbit antibody against human TGF-β1 (32).

### *In vitro* studies

*Culture and transfection of rat MC*

Glomeruli were isolated from 10-weeks old Lewis/MAA rats by the differential sieving method (29) and MC were cultured in RPMI 1640 medium supplemented with 20% heat inactivated FCS. The full-length rat IL-4 cDNA sequence from the plasmid pBABE-IL4, a generous gift of Dr. Wesseling from the University of Rotterdam, was subcloned into the expression vector pHβApr-1, which contains a β-actin promoter and a neomycin-resistance gene. This construct was introduced *in vitro* into rat MC with Fugene (Roche, Germany). Transfectants were tested for IL-4 production with ELISA according to the instructions of the manufacturer (Pharmingen, CA). Mesangial cells that had been transfected with the empty vector pHβApr-1 were used as a control.

*Experimental design of in vitro studies with MC*

IL-4 and mock-transfected MC were grown to near confluence. After growth-arrest for 18 hours in RPMI medium without serum, the cells were kept in growth-arrest state for another for 24 or 48 hours and the supernatants were collected for analysis. Total RNA was extracted. Cell lysates were harvested from cells that had been incubated for 48 hours by adding 5 ml lysisbuffer (1% triton X-100, 62.5 mM EDTA and 50 mM Tris/PBS). As a positive control for the alternative splicing of FN mRNA, untransfected cells were also cultured with 100 pM TGF-ß (Sigma, St. Louis, MO).

Soluble fibronectin (s-FN) and cell-associated fibronectin (c-FN) was measured with an ELISA. In brief, microtiter plates were coated with goat-anti-human FN (Sigma, St. Louis, MO). Rabbit-anti-mouse FN (1:2500) was applied to detect the bound FN, and anti-rabbit-HRP (DAKO, Demark) was used as a second step. A serial dilution of mouse FN (Gibco BRL, MD) was used as a standard.

Dot blot analysis, as previously described (33), was performed to measure the amount of FN mRNA. The hybridized filters were analyzed with a Phosphor Imager 445 SI (Molecular Dynamics, Sunnyvale, CA).

TGF-β mRNA levels were measured with real-time PCR using an Abi Prism 7700™ (Perkin

and Elmer). GAPDH was used as a housekeeping gene. Primer and TaqMan® Probe sequences are summarized in Table 1. Details concerning the real-time PCR protocol have been described elsewhere (34).

## Studies on human biopsies

*Biopsy material*

The alternative splicing pattern of EDA and EDB FN was measured in human biopsies to compare the results obtained from the animal models and cultured mesangial cells. Twenty-four renal biopsies with glomerulosclerosis in at least 10% of the glomeruli were selected from the archives of the Pathology department at our hospital center. These biopsy specimens had been obtained from 24 patients (Table 2). As controls, renal tissue from five kidneys that had been obtained at autopsy, two biopsies from transplanted kidneys without histological abnormalities, and five cadaveric donor kidneys, not used for transplantation, were analyzed. These kidneys demonstrated normal function and histology. As a disease control, three transplanted kidneys with acute rejection were used as an additional group. Patient studies were approved by the LUMC ethical committee.

*RT-PCR for TGF-β, total fibronectin, EDA, and EDB*

RNA was extracted from the cortical tissue of frozen biopsy tissue from each patient with the aid of the Trizol® (Gibco BRL) method (35). Real-time PCR was used to quantify the mRNA levels of TGF-β, fibronectin, and the housekeeping molecule GAPDH. Primer and probe sequences can be found in Table 1.

**Table 1.** Primer and Taqman® probe sequences.

| Gene | Species | Forward primer | Reverse primer | Taqman® probe | Label |
|---|---|---|---|---|---|
| EDA | Rat/mouse | TTGATTTCTTTCATTGGTCCTGTCTT | AAACAGAAATGACCATTGAAGGTTTG | | |
| EDB | Rat/mouse | TGACATCAGAAGAATCAAAACCAGTT | TTACACTGTCAAAGATGACAAGGAAA | | |
| TGF-ß | Rat | CACCGGAGAGCCCTGGATA | TTCCAACCCAGGTCCTTCCT | ACTACTGCTTCAGCTCCACAGAGAAGAACTGC | TET |
| GAPDH | Rat | ACCACCAACTGCTTAGCCCC | CACAGCCTTGGCAGCACC | TGGAAGGGCTCATGACCACAGTCCA | TET |
| Fibronectin | Human | GGAGAATTCAAGTGTGACCCTCA | AGGCAACGTGTTACGATGATGGGAAGACAT | TGCCACTGTTCTCCTACGTGG | TET |
| TGF-ß | Human | CCCAGCATCTGCAAAGCTC | GTCAATGTACAGCTGCCGCA | ACACCAACTATTGCTTCAGCTCCACGGA | TET |
| GAPDH | Human | TTCCAGGAGCGAGATCCCT | CACCCATGACGAACATGGG | CCCAGCCTTCTCCATGGTGGTGAA | FAM |
| EDA | Human | AAACAGAAATGACTATTGAAGGCTTG | AAACAGAAATGACTATTGAAGGCTTG | | |
| EDB | Human | ATTACTGGTTATAGAATTACCACAACC | TAATATCAGAAAAGTCAATGCCAGTTG | | |

*Statistics*
Statistical analysis for comparison between groups was performed with unpaired Student's T-tests.

## Results

### *In vivo* animal studies

*Development of renal disease*
After induction of αThy1 nephritis, proteinuria started to develop at day 3. Mesangial cell proliferation and mesangial matrix expansion were visible after 6 days, and mesangial sclerosis was observed at day 14 (36). Induction of CSS has been shown to result in the development of membranous nephropathy (26). At the most severe stage of CSS (week 0) protein excretion of the rats reached a level of 800 mg/24 hours. In the following weeks, the protein levels gradually decreased to 400 mg/ 24 hours. In week 15 mesangial matrix expansion and thickening of the GBM were observed. At a later stage (from week 20) the development of focal and segmental glomerulosclerosis was observed. Induction of αGBM nephritis in mice leads to the development of proteinuria, mesangial cell proliferation, and finally glomerulosclerosis on day 14. GvHD, a model for human lupus nephritis, was induced in F1 hybrid mice, leading to the development of class V lupus nephritis. Albuminuria started to develop in week 4. From week 6 onwards, mice showed glomerular hypercellularity and membranous nephritis, leading to the development of

**Table 2**. Characteristics of controls and patient groups

| Diagnosis | No. | Age (years) [b] | Sex (f/m) | Serum creat (μM) [b] |
|---|---|---|---|---|
| Normal controls | 12 | 50.0 ± 22.8 | 7/5 | 87 ± 20 |
| Acute transplant rejection | 3 | 49.7 ± 7.8 | 1/2 | 377 ± 340 |
| Lupus nephritis | 3 | 36.3 ± 9.1 | 3/0 | 90 ± 37 |
| *Other chronic diseases* | | | | |
| IgA nephropathy | 7 | 49.6 ± 11.4 | 1/6 | 200 ± 110 |
| FSGS [a] | 5 | 45.8 ± 9.9 | 2/3 | 489 ± 412 |
| Diabetic nephropathy | 5 | 63.2 ± 5.1 | 1/4 | 378 ± 338 |
| Proliferative GN | 4 | 61.5 ± 18.9 | 2/2 | 266 ± 100 |
| Total | 21 | 54.19 ± 13.2 | 6/15 | 318 ± 262 |

[a] FSGS: Focal and segmental glomerulosclerosis, GN: Glomerulonephritis
[b] Data are presented as means ± SD

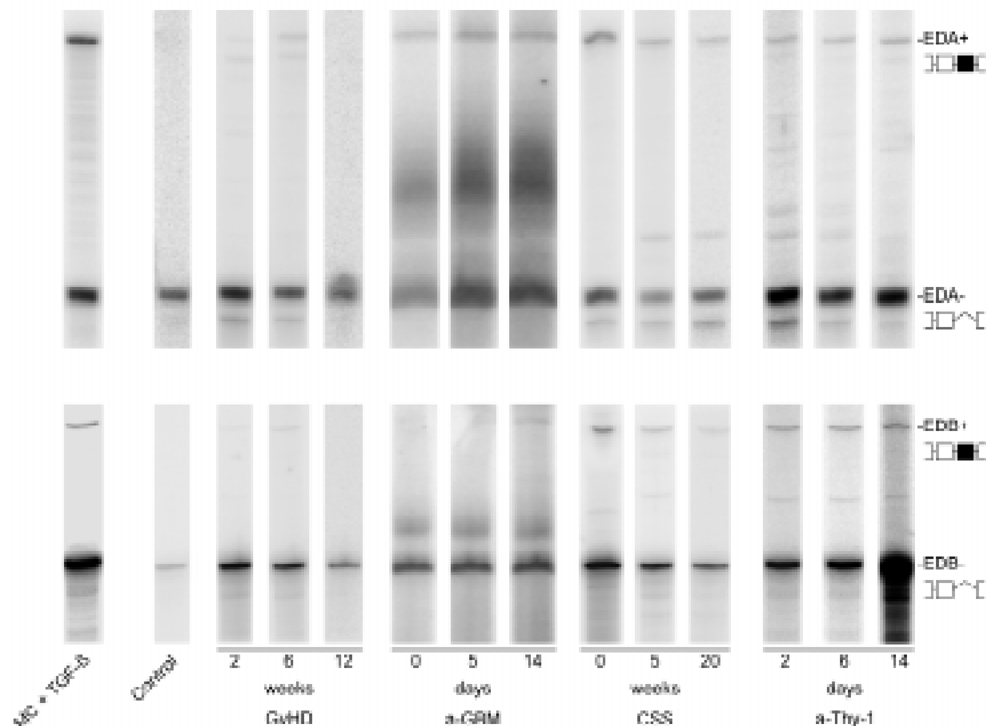


**Figure 1.** *Inclusion and exclusion patterns of the EDA and EDB domains in FN mRNA. Upper panel of the figure shows RT-PCR results for in- or exclusion of the EDA domain in FN mRNA, while the lower panel shows the results for the EDB region. A control sample of mouse mesangial cells cultured in the presence of TGF-β showed inclusion of both the EDA and the EDB domains in the FN mRNA, while normal mice did not express the oncofetal domains EDA and EDB. Induction of αGBM gave rise to an inclusion of the EDB domain in the FN mRNA at day 14, and an inclusion of EDA at days 0, 5, and 14. Induction of GvHD in mice did not lead to an inclusion of EDA or EDB region. Rats with αThy1 or CSS expressed both the oncofetal FN domains EDA and EDB.*

focal and segmental glomerulosclerosis from week 8 onwards.

*Splicing patterns of FN mRNA in experimental models of glomerulonephritis*

We performed an RT-PCR, with primers flanking the EDA or EDB region, on glomerular mRNA isolated from mice and rats at several time points after induction of the disease. The 526 bp amplification product corresponded to FN mRNA, which included the EDA region, while the 256 bp product corresponded to EDA-negative FN mRNA. Inclusion of the EDB region in the FN mRNA corresponded to the 640 bp product, and the 367 bp amplification product represented EDB-negative FN mRNA. Figure 1 shows the results of the RT-PCR experiments. A control sample of mouse mesangial cells that had been cultured in the presence of 100 pM TGF-β showed inclusion of the EDA and the EDB domains in the FN mRNA. Normal mice did not

express the oncofetal domains EDA and EDB. Induction of αGBM nephritis resulted in inclusion of the EDA domain in the FN mRNA on days 0, 5, and 14 and in inclusion of EDB on day 14. However, induction of GvHD in mice had no effect on the splicing pattern of FN mRNA (no inclusion of EDA and EDB). Induction of CSS or αThy1 in rats also resulted in oncofetal EDA and EDB-positive FN mRNA in glomeruli. These results are summarized in Table 2.

*Immunohistochemistry for TGF-ß*

In the rat model of CSS, TGF-β positive cells were observed in the glomerulus for 2 to 3 weeks prior to the most severe stage of the disease (Figure 2A). These TGF-β positive cells were not ED1 positive. The role of TGF-β in αThy1 disease and αGBM nephritis has been extensively described in the literature. Border et al. (19) have shown that TGF-β plays a role in the development of αThy1 disease. An important role for TGF-ß has also been described in anti-GBM nephritis (37,38). In the GVH model in the mice, no positive cells for TGF-β could be detected at any stage of the disease. Fig. 2B shows the results for week 6.

Table 3. Overview of the involvement of TGF-ß and the FN EDA/B splicing pattern in the different animal models, cultured mesangial cells, and human biopsies.

| | TGF-ß | EDA + | EDB + |
|---|---|---|---|
| *Animal model:* | | | |
| GVHD | = | = | = |
| α-GBM | ↑ | ↑ | =/↑ |
| α-Thy-1 | ↑↑ | ↑ | ↑ |
| CSS | ↑ | ↑ | ↑ |
| *Mesangial cells:* | | | |
| IL-4 overexpr.[1] | ↓ | ↓ | = |
| Exogenous TGF-ß (100 pM)[2] | = | ↑ | ↑ |
| *Human biopsies:* | | | |
| Acute rejection | ↑ | ↑ | = |
| Lupus nephritis | = | = | = |
| Other chronic diseases | ↑ | ↑ | = |

[1]IL-4 transfected rat mesangial cells. [2]TGF-ß means rat mesangial cells stimulated with TGF-ß. ↑ indicates increased expression compared to controls; ↓ indicates decreased expression compared to controls; = indicates no differences compared to controls.

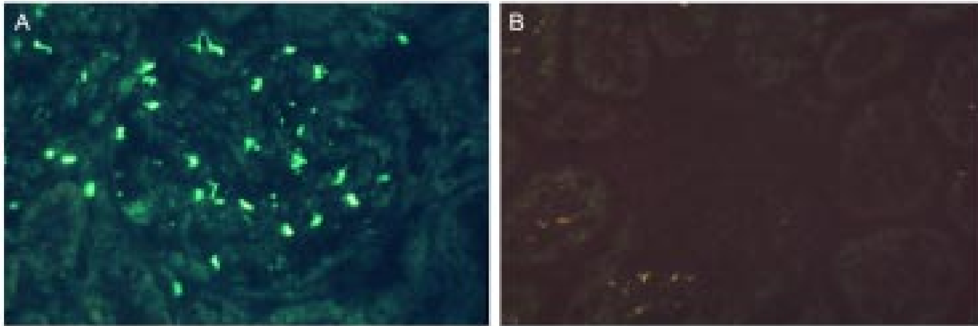


**Figure 2.** Expression of TGF-β1 positive cells in rats with CSS. Immunohistochemical staining of kidney sections for TGF-β1 showed expression of positive cells 2 and 3 weeks prior to the most severe stage of the disease (week 0)of CSS (Fig. A). Figure B shows a kidney section stained for TGF-ß six weeks after induction of GVHD.

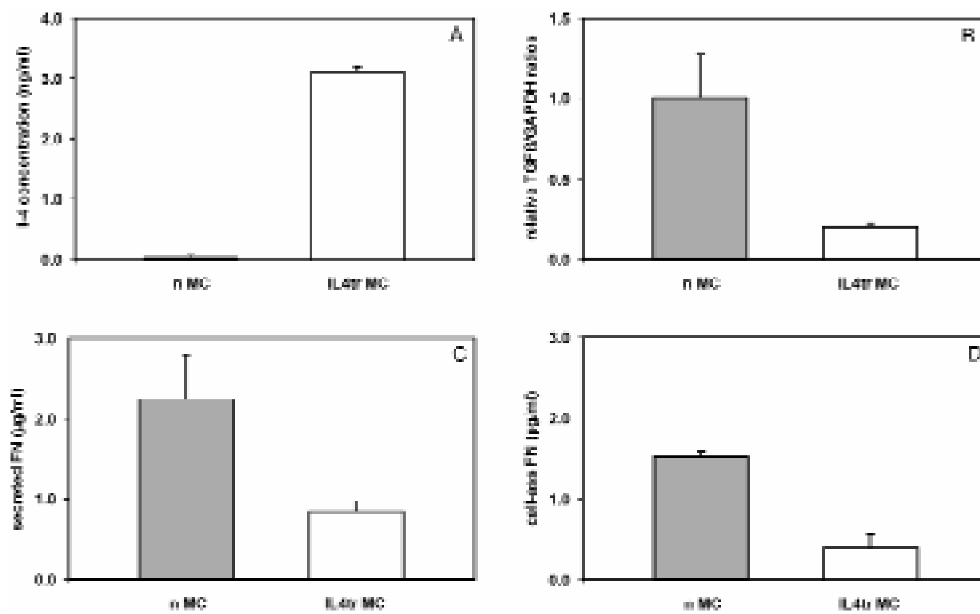


**Figure 3.** Measurements on cultured mesangial cells. Normal mesangial cells (nMC) cultured for 24 hours showed no production of IL-4 as measured by ELISA. IL-4 transfected mesangial cells (IL4tr MC) showed an increased production of the cytokine as compared to normal mesangial cells (A) (P<0.01). Mesangial cells were cultured for 24 hours and TGF-β1 mRNA levels were measured by RT- PCR. IL-4 transfected mesangial cells (IL4tr MC) showed a significant decrease in TGF-β1 mRNA production as compared to normal mesangial cells (nMC) in culture (B) (P<0.01). The production of secreted-FN (C) and cell-associated FN (D) of mesangial cells in culture was measured by ELISA. IL-4 transfected cells showed a significant decreased production of both secreted and cell-associated FN, as compared to normal mesangial cells (P<0.01).

### *In vitro* studies

*IL-4 overexpression in mesangial cells*
Rat mesangial cells were transfected with IL-4 to investigate the role of IL-4 in the alternative splicing of FN. Normal mesangial cells in culture do not produce detectable amounts of IL-4 measured by ELISA, whereas mesangial cells transfected with the full-length rat IL-4 gene show an increased production of IL-4 (Fig 3A). Incubation of normal rat B cells with culture supernatant from IL-4 transfected cells resulted in an increased expression of MHC class II antigens (data not shown), indicating that the produced IL-4 is biologically active.

In IL-4 overexpressing mesangial cells, TGF-β1 mRNA levels were 5 times lower (P<0.01) than those in normal mesangial cells (Fig. 3B) The production of soluble fibronectin (s-FN) and cell-associated fibronectin (c-FN) was significantly reduced in IL-4 transfected mesangial cells (Fig. 3 C and D). The amount of total FN mRNA that was measured with dot blot analysis showed a significant decrease in cultured cells that had been transfected with IL-4 in comparison to mock-transfected mesangial cells (Fig.4A). IL-4 overexpression also influenced the splicing pattern of FN mRNA. After 24 hours of culture a significant decrease was seen in EDA+ FN mRNA levels. There was no significant effect of IL-4 over-expression on EDB+ FN mRNA levels (Figs. 4B and C).

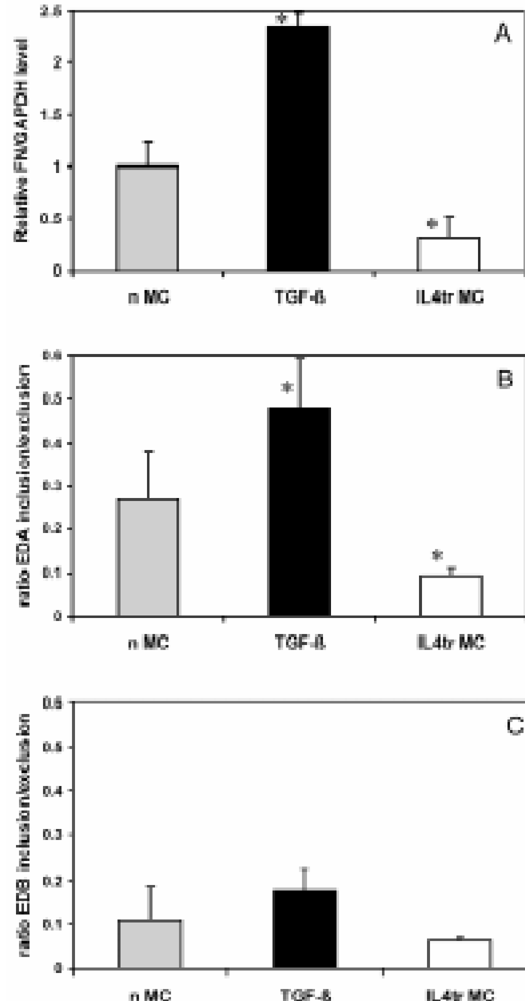


**Figure 4.** FN mRNA measurement on cultured mesangial cells. The amount of FN mRNA produced during 24 hours culture of mesangial cells was measured by dot blot analysis. IL-4 transfected cells produced significant lower levels of FN mRNA(A) while TGF-β stimulated MC showed an increase. Relative levels of FN mRNA containing the EDA and EDB domains were determined by rt-PCR. There was a significant decrease in the production of EDA+ FN mRNA in IL-4 transfected mesangial cells compared to normal mesangial cells in culture. TGF-ß stimulated MC showed a significant increase in EDA inclusion. The relative amounts of EDB positive FN mRNA remained unchanged both in IL-4 transfected and in TGF-β stimulated MCs (*P<0.05 compared to normal MCs).

## Studies on human biopsies

*mRNA levels in normal and diseased tissue*

To validate the results obtained from the animal models, TGF-β mRNA levels, fibronectin mRNA levels, and the EDA+/EDA- mRNA ratio were measured in renal cortical tissue. Based on the findings of the animal studies, where the GVH model was the only model showing no increase of TGF-β and no inclusion of EDA at any time point of the disease, the patients with lupus nephritis were considered a separate group for the analysis. The other chronic renal disease entities were treated as one group. The relative TGF-β mRNA levels were 1.0 ± 0.4 for the controls, 2.2 ± 1.0 ($P<0.05$) for the patients with acute rejection, 1.1 ± 0.7 (P = NS) for the patients with lupus nephritis, and 1.7 ± 0.7 ($P<0.05$) for the patients with other chronic renal diseases (Figure 5A). The relative fibronectin mRNA levels were 1.0 ± 0.5, 2.5 ± 1.4 ($P<0.01$), 2.1 ± 2.1 (P = NS), and 3.1 ± 2.3 ($P < 0.05$), respectively (Figure 5B). The percentage of EDA+ mRNA in these groups were 22.8 ± 13.3, 41.3 ± 6.5 ($P < 0.05$), 19.4 ± 13.3 (P = NS), and 34.0 ± 11.8 ($P < 0.05$), respectively (Figure 5C). The percentage of EDB+ mRNA was less than 5 % in all groups (data not shown).

A correlation between the fibronectin mRNA level and the EDA+/EDA- mRNA ratio ($r = 0.48$, $P < 0.005$) was observed. The TGF-β mRNA level did not correlate with either the fibronectin mRNA level or the EDA+/EDA- mRNA ratio.

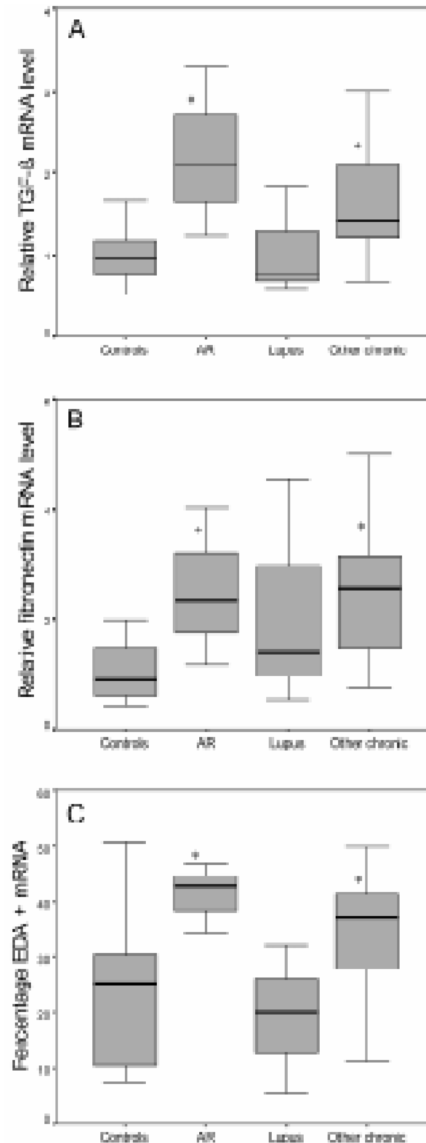


**Figure 5.** Relative mRNA levels in patients. (A) TGF-β mRNA levels, (B) fibronectin mRNA levels, and (C) the percentage EDA+ FN mRNA in renal cortical tissue from normal controls (n = 12), from patients with acute transplant rejection (n = 3), from patients with lupus nephritis (n = 3), and from patients with chronic renal disease (n = 21). Data are represented as means ± standard error of the mean. (*$P<0.05$ compared to controls)

## Discussion

Most chronic human kidney diseases are characterized by an accumulation of ECM resulting in glomerulosclerosis and tubulo-interstitial fibrosis. The concept has been proposed that the development of these lesions, which is accompanied by progressive loss of kidney function, results from a final common pathway in a manner that is independent of the original etiology (1). However, the initiating mechanisms which precede to this final common pathway may vary. In patients with glomerulosclerosis the presence of EDA positive FN in the glomerulus has been demonstrated (1). It remains to be established whether all glomerulosclerotic lesions require the involvement of the same inducing and mediating factors. Since matrix proteins can modify the behavior of mesangial cells (39), the presence of particular FN isoforms in the mesangial matrix, as well as their amount and persistence during glomerular injury, may determine the evolution of either healing or scarring processes.

The aim of the present study was to investigate the splicing patterns of the EDA and EDB regions of FN in various glomerulopathies, and to elucidate the role of TGF-β1 and IL-4 in this process. The *in vivo* studies showed that splicing of FN mRNA is differentially regulated in αThy1, CSS, αGBM, and GVHD. Both acute and chronic disease in rats showed inclusion of the EDA and EDB regions. An increase in EDA and EDB containing FN isoforms was only observed in the acute model of αGBM disease in mice. TGF-β might be responsible for the presence of FN isoforms that contain EDA. Previous studies have described that TGF-β is capable of inducing EDA+ fibronectin synthesis (13,16,20,40,41). We presented data showing the presence of TGF-β early in the development of CSS. Border et al. (19) have shown that TGF-β plays a role in the development of glomerulosclerosis in αThy1 nephritis. The same is true for the αGBM model, in which TGF-β is involved in the development of the disease (42). Inclusion of EDA or EDB within the FN molecule was not detected in GVHD mice, a model in which TFG-β positive cells in the glomeruli or an increased glomerular mRNA level of TGF-β could not be found at any stage of the disease. In summary, results from various experimental models show that levels of TGF-β are associated with the extent of EDA and EDB inclusion.

The effect of IL-4 and TGF-β was investigated *in vitro* to achieve a better understanding of the effects of cytokines on FN transcription and splicing at the EDA region. Evidence for the involvement of IL-4 in the pathogenesis of glomerulonephritis has been provided by several investigators who have shown that intrinsic human glomerular cells express mRNA for both IL-4 and IL-4 receptor (22), and that IL-4 expression is upregulated in various types of GN. In crescentic GN, early IL-4 treatment was shown to reduce proteinuria and inflammation (43). Our results demonstrated that overexpression of IL-4 in cultured mesangial cells led to a reduction of the amount of EDA+ FN mRNA. Also, a reduced overall production of FN by decreased FN

transcription was observed in IL-4 transfected MC. The decreased EDA inclusion might be explained by the significant decrease in TGF-β mRNA production found in IL-4 overexpressing cells. Decreased TGF-β1 serum levels (44) were observed in mice with constitutive transgenic expression of IL-4. These results suggest that IL-4 may play a role in the induction of quantitative and qualitative alterations in matrix composition, during the development of glomerulosclerosis.

Chronic GHVD was the only model tested in which we could not find an indication for a role for TGF-β and EDA inclusion. We wanted to investigate whether these findings could be confirmed in human glomerulosclerosis. Therefore, we measured the mRNA levels of fibronectin and the ratio between EDA+ and EDA- mRNA in controls and diseased renal tissue. We also analyzed TGF-β mRNA levels in these tissues. The renal biopsies with glomerulosclerosis were divided into two subgroups. One group consisted of patients suffering from lupus nephritis, which corresponded to the GHVD model in the mice, while the other group consisted of various other chronic kidney diseases. TGF-β, fibronectin mRNA, and the EDA+/EDA- mRNA ratio were significantly increased in patients with renal disease in comparison to the controls. In contrast, patients with lupus nephritis did not show a significant increase in TGF-β, FN, and EDA+/EDA- in comparison to controls, which corroborates the experimental models. The steady-state level of fibronectin mRNA correlates with the EDA+/EDA- mRNA ratio, indicating that the increased fibronectin mRNA observed in chronic disease is mainly EDA positive.

The question remains what triggers the development of GS in mice with GVHD-induced lupus nephritis and in patients with lupus nephritis. In an earlier study we have shown that in these mice the accumulation of FN protein during the sclerotic phase of the disease is not a result of an increased FN production but a result of specific trapping of plasma fibronectin from the circulation (45). Circulating FN was shown to bind through its heparin binding site to integrin α5β1, which was upregulated in glomeruli in the later stage of the disease (6). IL-4 has been found to induce upregulation of ß1-integrins in lung fibroblasts (46). Therefore, EDA negative FN from the circulation might be trapped in the kidney through binding to integrins, of which the expression is induced by IL-4. In addition, mice with constitutive transgenic expression of IL-4 show progressive glomerulosclerosis with mesangial accumulation of collagens type I, IV, and V. Seven-day-old IL-4-transgenic animals showed early renal fibrotic changes in the absence of immune deposits or TGF-β1 upregulation (44). Treatment of these mice with IL-4 neutralizing antibody prevented renal disease (47). It has also been found that IL-4 is capable of activating the human type I collagen promoter in lung fibroblasts and may thus contribute to lung fibrosis (48). In addition, NZM.2410 mice rendered deficient in STAT6, a transcription factor involved in the production and function of type 2 cytokines, are completely protected from the development of glomerulosclerosis(49). The renoprotective effects of ACE inhibitors in lupus nephritis may be explained in part by their negative action on IL-4 and IL-10

production (50). From our study we conclude that alternative splicing of the EDA domain of FN mRNA is correlated with the presence of TGF-β. The results suggest that TGF-β is a key player in the development of GS by inducing FN production and alternative FN mRNA splicing. On the other hand, we showed that *in vitro* TGF-β and IL-4 have opposing effects with respect to spicing at the EDA region of FN. In lupus glomerulonephritis, in which the role of TGF-β in GS is less prominent, type 2 cytokines such as IL-4 probably predominate in the induction of collagen synthesis and FN accumulation from the circulation, which leads to increased matrix accumulation and glomerulosclerosis. Therefore, neither alternative FN splicing, nor a high transcription level of TFG-β appears to be a general prerequisite for the development of GS.

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## Refereces


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