Fibrogenesis in progressive renal disease

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Chapter 5

Fibronectin accumulation in glomerulosclerotic lesions: self-assembly sites and the heparin II binding domain

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Abstract

Glomerulosclerosis is a severe complication of many immunologically mediated kidney diseases, eventually resulting in loss of renal function. In chronic graft-versus-host disease (GvHD) in mice, a model for human lupus nephritis, the end-stage sclerotic lesions were previously shown to contain large amounts of fibronectin (FN). This study investigated a domain-specific accumulation process of circulating plasma FN (pFN) in sclerotic lesions.

GvHD mice were injected with FITC-conjugated pFN or pFN-fragments, with or without heparin pre-incubation. pFN-fragments were generated by digestion of FN by Cathepsin D, after which the fragments were separated on a heparin affinity column. Thus, two batches of fragments were obtained with either low or high affinity for heparin.

FN accumulation was accompanied by an up-regulated expression of integrin $\alpha 5\beta 1$, the FN receptor, in the periphery of sclerotic lesions. pFN-FITC injected into GvHD mice was trapped in sclerotic glomeruli within 24 hrs. Both heparin and non-anti-coagulant heparin blocked the accumulation of pFN-FITC, indicating that the protective effect of heparin in the trapping of FN is independent of its anticoagulant properties, and probably results from preventing direct binding of FN in the sclerotic lesions.

To investigate whether FN binds in the glomerulus via the heparin-binding regions, pFN-fragments were generated and injected into GvHD mice. Whereas the fraction with high affinity for heparin did not accumulate in the sclerotic glomeruli, the fraction with low affinity for heparin did. Partial sequencing of the isolated peptides showed that in the glomerulus fibronectin does not bind via the heparin II binding region.

We hypothesize that the protective effect of heparin treatment may be the result of steric hindrance of the specific binding sites, that is, the $I_{1\text{-}5}$ and/or III_1 self-assembly sites of FN.

Introduction

Accumulation of extracellular matrix (ECM) molecules in the glomerulus leads to the development of glomerulosclerosis, which is a prominent feature of many immunologically mediated renal diseases. Glomerulosclerosis, together with tubulointerstitial fibrosis, causes irreversible end-stage renal damage with poor patient prognosis. Vleming et al. reported that of a number of matrix molecules in a variety of human renal diseases, only the amount of intraglomerular fibronectin (FN) deposition correlates with the severity of glomerular structural abnormalities, and inversely with renal function (1).

FN is a large adhesive glycoprotein, that is widely distributed as cellular FN (cFN) in the ECM of various tissues and on cell surfaces, and as soluble plasma FN (pFN) in the circulation. FN is involved in the regulation of cell adhesion, differentiation, migration, and proliferation. This multifunctional protein consists of a dimer of two disulfide-bonded subunits, each with a molecular weight of approximately 220 kD, that contain binding sites for heparin, collagen, DNA, integrins, and FN itself (2,3). In the normal kidney FN is located in the mesangium, the glomerular basement membrane (GBM), Bowman's capsule and the tubular basement membrane (TBM)(4). FN has a crucial role in the organization of ECM components, and is considered to play a key part in the pathogenesis of some glomerulonephritides, where the molecule is present in increased amounts in the expanded mesangium (5,6).

Chronic Graft-versus-Host disease (GvHD) in mice is an experimental model for human systemic lupus erythematosus (SLE), in which a transfer of donor lymphocytes into F1-hybrid recipients causes uncontrolled B-cell activation with production of autoantibodies (7). This results in an immune complex glomerulonephritis, resembling human lupus nephritis (8). Both in the GvHD model and in human renal diseases, the end-stage glomerulosclerotic lesions consist mainly of FN, which accumulates as a result of specific trapping of pFN from the circulation rather than through *de novo* synthesis (9). The mechanism by which the specific accumulation of pFN in the sclerotic lesions takes place is still unknown. FN accumulation may result from its involvement in the blood coagulation system, or via direct binding of pFN to cells or other ECM components in the damaged glomerulus.

Because the accumulation of FN in the end-stage glomerulosclerotic lesions may be the result of an activation of the coagulation cascade, we studied the effect of heparin on the glomerular accumulation of FITC-labeled pFN in the GvHD model. Heparin is a sulfated glycosaminoglycan, closely related to heparan sulfate, with a long history in the treatment of patients with thrombotic diseases. Many ECM components, such as fibronectin, laminin, thrombospondin and different types of collagen, can bind to heparin. In addition to its anti-coagulant function, heparin has an impact on interactions that involve matrix organization, cell

adhesion, proliferation, and cytokine action (reviewed by (10)). With regard to the kidney, heparin was shown to have an anti-proliferative effect on cultured vascular smooth muscle cells, epithelial cells, and mesangial cells (11-14). In several models, e.g. renal ablation, MRL/*lpr* mice, puromycine aminonucleoside-induced nephrotic syndrome, and anti-Thy 1.1 nephritis in the rat, administration of heparin diminishes proteinuria and hypertension, and decelerates the progression of renal insufficiency and glomerulosclerosis *in vivo* (15-19).

Our results show that both heparin and N-desulfated heparin can block accumulation of injected pFN-FITC and specific Cathepsin-induced FN fragments in glomerulosclerotic lesions. These results provide evidence for a domain-specific accumulation process in the accumulation of circulating FN in sclerotic lesions.

Materials and methods

Animals

DBA/2 and C57BL/10*DBA/2 F1 hybrid mice were purchased from Harlan BV (Horst, The Netherlands). Female DBA/2 mice aged 7 to 8 weeks served as donors of lymphocytes in the induction of GvHD. Eight to 10 week-old female F1 hybrids served as recipients of lymphocytes. GvHD was induced in 61 experimental F1 hybrid mice. Eleven age- and sex-matched normal F1 hybrid mice (NF1) were used as controls. The numbers of animals used for each experiment are detailed in the results section.

Induction of disease

GvHD was induced in F1 hybrid mice as described previously (20). In brief, single cell suspensions were prepared from DBA/2 spleens, lymph nodes, and thymi in Hanks' balanced salt solution (HBSS). The total number of cells and the proportion of vital cells were determined by Trypan blue staining. The suspensions were mixed, and injected intravenously in F1 recipients on days 0, 3, 7, and 10. Each dose of $25*10^6$ viable DBA/2 cells in 0.25 ml HBSS was composed of approximately 60% spleen cells, 30% thymocytes, and 10% lymph-node cells.

Accumulation experiments

All accumulation experiments were performed 10-12 weeks after the induction of GvHD, since specific trapping of pFN in the glomerulosclerotic lesions has been shown to occur at this timepoint. GvHD mice were injected intravenously with 500 μg FITC-conjugated mouse pFN with or without 250 U (non-anti-coagulant) heparin. This is approximately 15% of the normal total plasma FN content of a mouse as estimated by a quantitative sandwich-ELISA technique (unpublished results). Where appropriate, pFN and either heparin or non-anti-coagulant heparin

were incubated together for 1 h at 37°C before injection, after which the solution was spun down for 30 sec to remove FITC-crystals. pFN-heparin complexes remained soluble during the incubation and centrifugation step.

pFN-fragments were obtained as detailed below. Accumulation experiments with the pFN-fragments were performed with 250 μg of the FITC-conjugated low-affinity pFN fraction or 125 μg of the FITC-conjugated high-affinity pFN fraction, with or without 250 U heparin. The amounts of injected FITC-conjugated pFN-fragments were adjusted to equimolar amounts of molecules as compared to total pFN molecules injected in previous experiments. As controls, NF1 hybrid mice were injected intravenously with either pFN or one of the pFN fragments with or without pre-incubation with heparin.

Follow-up of F1 mice

The urine albumin content of the GvHD F1 mice was determined at two-weekly intervals, starting 2 weeks before disease induction. Animals were kept in urine-collection cages for 18 h with free access to water and food. The albumin levels were assessed by rocket electrophoresis against rabbit anti-mouse albumin, with albumin as a standard (Sigma Chemical Corporation, St.Louis, MN, USA) (20).

Groups of three experimental mice were sacrificed at week 0, 2, 4, 6, 8, 10, and 12 of GvHD. After perfusion with PBS, the kidneys were removed. For light microscopic examination with periodic acid-Schiff (PAS) reagent and phosphotungstic acid haematoxylin (PTAH), a part of the kidney tissue was fixed in 10% buffered formalin, dehydrated, and then embedded in Paraplast (Amstelstad, Amsterdam, The Netherlands). The remaining tissue was frozen in CO_2 ice-cooled isopentane and stored at –70°C until further use. Immunohistochemistry was performed with a fluorescein-isothiocyanate (FITC)-conjugated rat anti-mouse CD49e monoclonal antibody (Pharmingen, San Diego, CA, USA) to detect the integrin α5 chain. The FITC-conjugated rabbit anti-mouse CD49d monoclonal antibody (anti-integrin α4 chain) was a generous gift of Dr. E. Ruoslahti (Cancer Research Center, La Jolla, CA, USA). Fluorescence intensity was scored semi-quantitatively on a scale from 0 to 3+, in which 0 represented no staining and 3+ reflected a very strong staining. The slides were scored in a double-blinded fashion by two observers independently of each other, using a Leitz fluorescence microscope. Representative examples of the scoring are shown in figure 1.

Eighteen hours after the injection of pFN or pFN-fragments, groups of mice were sacrificed. The numbers of animals used for each experiment are detailed in the results section. After perfusion with PBS, the kidneys were removed and stored for light and fluorescence microscopy as described above. The amount accumulation of pFN or pFN-fragments was scored semi-quantitatively on a scale from 0 to 3+, in which 0 represented no accumulation and 3+ reflected

a very strong accumulation. The slides were scored in a double-blinded fashion by two observers independently of each other, using a Leitz fluorescence microscope. Representative examples of the scoring are shown in figure .

pFN isolation and purification of pFN fragments

pFN was isolated from normal mouse plasma, in the presence of 30 mM citrate as an anti-coagulant. The plasma was dialyzed overnight against PBS/10 mM EDTA at 4°C. The plasma was spun down at 3,000g for 20 min. to remove protein aggregates, after which the supernatant was circulated over a gelatin-Sepharose 4B affinity column for 2 h. The column was rinsed with PBS/10 mM EDTA. pFN was eluted from the column with 6 M urea in 0.1 M citric acid/50 mM Tris pH 4.7. The eluate was dialyzed overnight against PBS/10 mM EDTA at 4°C before labeling with FITC (21). For preparation of pFN fragments 10 mg of affinity-purified pFN was dialyzed overnight against 50 mM sodium acetate/50 mM NaCl/10 mM EDTA pH 6.4. Addition of a 10% solution of glacial acetic acid was used to adjust the pH of the dialysate to 3.5. Digestion with Cathepsin D (Sigma-Aldrich N.V./S.A., Bornem, The Netherlands) was performed for 18 h at 37°C in a weight ratio of FN:Cathepsin D = 200:1. Adjusting the pH to 7 with 2.5 M Tris (pH 9) terminated the reaction. The digested pFN solution was tested by SDS-PAGE, under reducing conditions, before purification of the fragments.

The heparin-Sepharose 4B affinity column was equilibrated with 20 mM Tris/50 mM NaCl/ 10 mM EDTA/5 mM Caproic acid/0.2 mM PMSF pH 7.4. The digested pFN was allowed to bind to the column by recirculation of the effluent, followed by the washing with three column volumes of equilibration buffer. The pFN fragments were eluted by a discontinuous gradient subsequently consisting of 0.25 M, 0.6 M and 1 M NaCl in equilibration buffer. The separate fractions were analyzed by SDS-PAGE under reducing conditions, dialyzed overnight against PBS/10 mM EDTA at 4°C, and labeled with FITC (21). Fractions were concentrated and washed by high-pressure diafiltration in a Centricon (Amicon), and stored at –20°C until further use.

Preparation of non-anti-coagulant heparin

Heparin (Organon Teknika Nederland BV, Boxtel, The Netherlands) was converted into N-desulfated non-anti-coagulant heparin according to the procedure of Inoue and Nagasawa (22). In brief, sodium-heparin salt dissolved in 0.1 M HCl was converted into a pyridin-heparin salt on a DOWEX column (Pharmacia, Uppsala, Sweden). Desulfation was performed by using dimethyl sulfoxide/5% methanol. Activated partial thromboplastin time (aPTT) was performed according to the manufacturers protocol (Boehringer, Mannheim, Germany). N-desulfated non-anti-coagulant heparin had no effect on the clotting time. In an ELISA under isotonic conditions, non-anti-coagulant heparin and anti-coagulant heparin were found to have comparable binding

capacities to pFN (unpublished results).

Statistical analysis

Results are presented as means ± SD where indicated, and were analyzed using the Chi-square test. $P \leq 0.05$ was considered statistically significant.

Results

Clinical course of GvHD

Four weeks after the induction of GvHD, mice developed abnormal proteinuria. Albuminuria reached the highest levels at week 12 (results not shown). At this timepoint the majority of the animals developed ascites and edema. Light-microscopy of kidney sections of GvHD mice showed a lupus type of nephritis, complicated by focal and segmental glomerulosclerosis starting 10 weeks after the induction of the disease (Fig. 1), confirming earlier results (20). Furthermore, PTAH staining showed fibrin deposited in sclerotic regions, suggesting that the coagulation pathway may be involved in the development of glomerulosclerosis in GvHD (results not shown).

Altered expression of integrins that specifically bind to FN may well lead to enhanced binding of pFN in the glomerulosclerotic lesions. Immunohistochemistry showed that both the integrin chains α4 and α5 were expressed in minimal amounts in the glomeruli of NF1 mice (fluorescence intensity +/-, diffuse pattern). The expression of the integrin α4 did not alter during the development of GvHD. However, from week 6 on we observed increased amounts of the α5 integrin chain in the periphery of sclerotic lesions (segmental staining, fluorescence intensity 3+, Fig. 1). Control staining with an irrelevant non-immune IgG showed no fluorescence pattern in NF1 or GvHD mice.

pFN isolation and purification of pFN fragments

Figure 2 shows the results of SDS-PAGE analysis of isolated pFN and pFN fragments under reducing conditions. Total pFN was isolated by affinity chromatography and has a molecular weight of 220 kD (lane B). Incubation of pFN with Cathepsin D for 18 hr led to complete digestion of pFN (lane C). Digestion for a longer period of time or at a higher protein/proteinase ratio did not provide any additional FN fragmentbands. Incubation for a shorter period showed incomplete digestion of FN, leaving remains of the 220 kD total FN band in the SDS-PAGE gel (results not shown). Purification of the pFN fragments on a heparin affinity column revealed two separate fractions that could be eluted from the column with 0.25 M NaCl in an equilibration

buffer (lane D) and with 1 M NaCl (lane E) respectively. No fragments could be eluted with 0.6 M NaCl. The fraction with low affinity for heparin contains a specific pFN fragment with an apparent MW of 120 kD (lane D) and a mixture of additional bands with an apparent MW around 100 kD. The fraction with high affinity for heparin contains a specific pFN fragment with an apparent MW of 60 kD (lane E) and also a mixture of additional bands with an apparent MW around 100 kD. No pFN protein bands were visible in the gel below the 60 kD band.

Although the mouse FN protein sequence (Swissprot: FINC_mouse, 2477aa) shows more than 80% homology with the human FN protein sequence (Swissprot: FINC_human, 2386aa), the digestion of mouse FN with Cathepsin D generated several different fragments (120 kD, 60 kD, and 100 kD) than digestion of human FN (70 kD gelatin bindin domain, 27 kD N-terminal heparin binding domain, 40 kD collagen binding domain, 120 kD cell binding domain, and 55-65 kD C-terminal heparin binding domain (23,24)). Results of N-terminal sequencing of the fractions revealed that the protein-sequence of the 60 kD high-affinity fragment started at residue 1704 (AQNRNGESQP). This fragment thus includes the high-affinity heparin II binding domain. Repeated N-terminal sequencing of the low-affinity fraction suggests that the N-terminus of the 120 kD fragment was located near the low-affinity heparin-binding domain, but we were not able to find one unequivocal starting point for the sequence. The 100 kD proteins in both samples were not identical to each other, but the 100 kD protein fragment in the low-affinity fraction shared sequence homology with the 120 kD pFN fragment, while the 60 kD pFN fragment shared sequence homology with the 100 kD protein fragment in the high-affinity fraction. Figure 3 shows a proposed schematic drawing of the FN protein sequence indicating the proposed locations of the FN fragments.

Both heparin and N-desulfated non-anti-coagulant heparin prevent binding of pFN to glomerulosclerotic lesions

FITC-conjugated pFN injected intravenously into mice 10-12 weeks after induction of GvHD accumulated in the glomerulosclerotic lesions (n=9, fluorescence intensity 3+, Fig. 4A). The segmental fluorescent staining pattern was comparable to the occurrence of PAS-positive glomerulosclerotic lesions. This accumulation was not observed in NF1 mice (n=2), confirming earlier results by Bergijk et al. (9). Ex vivo pre-incubation of pFN-FITC with heparin resulted in a significantly reduced accumulation of pFN-FITC upon injection ($p < 0.05$). Hardly any fluorescence signal was detectable in 4 out of 5 animals with GvHD (Fig. 4B, fluorescence intensity +/-). Injection of pFN pre-incubated with non-anti-coagulant, N-desulfated heparin also prevented the accumulation of pFN-FITC in the glomerular lesions ($p < 0.05$). Five GvHD mice showed no accumulation, while only 2 animals had a weak fluorescent staining of 1+. There was no statistically significant difference between the accumulation of pFN-FITC pre-

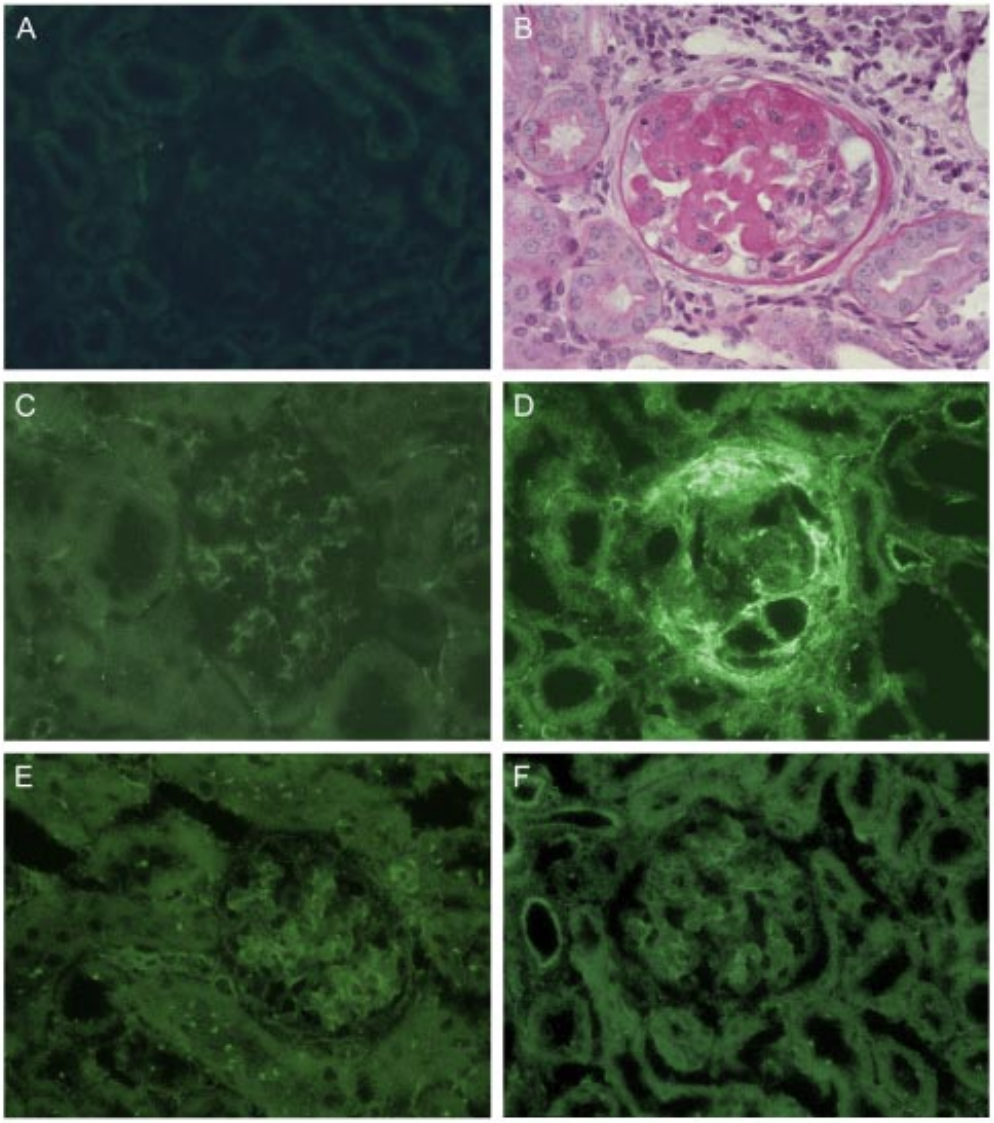

Figure 1. (Immuno-)histochemical staining of renal tissue. Figure A shows a control staining with a non-immune IgG antibody. Figure B is a PAS staining of a mouse glomerulus 10 weeks after the induction of GvHD, showing extensive glomerulosclerosis. The integrin α5 chain is expressed in minimal amounts in a glomerulus of a NF1 mouse (C). Increased expression of the integrin α5 chain is visible in the periphery of the glomerulosclerotic lesion 8 weeks after the induction of GvHD (D). The integrin α4 chain was expressed in trace amounts in glomeruli of NF1 mice (E). The expression of the integrin α4 chain did not alter during GvHD (Fig. F, GvHD week10).

treated with heparin or non-anti-coagulant heparin. Results of the accumulation experiments are summarized in Table 1.

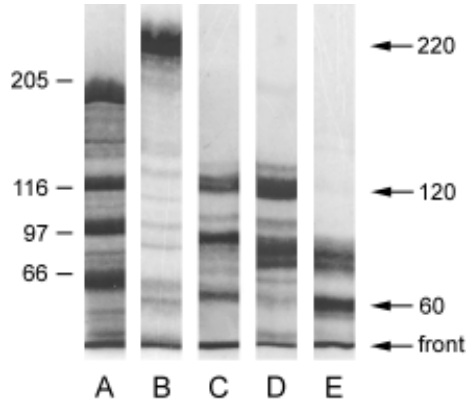

Figure 2. Polyacrylamide gel electrophoresis under reducing conditions of plasma-fibronectin isolated from normal mouse plasma using a gelatin-Sepharose 4B affinity column. Lane A represents molecular weight markers. Lane B contains total FN with an apparent MW of 220 kD. Lane C shows Cathepsin D induced fragments of total FN. There is no 220 kD FN band left after enzymatic digestion. Affinity purification of the fragment-mixture, on a heparin-Sepharose 4B column, resulted in a low-affinity fraction containing a specific 120 kD FN fragment (Lane D) and a high-affinity fraction containing a specific 60 kD FN fragment (Lane E). The low-affinity fraction (120kD) was completely depleted of the 60 kD band, while the high-affinity fraction (60 kD) did not contain any 120 kD FN fragments. Additional pFN fragments with an apparent MW of about 100 kD are present in both fractions.

pFN binding in glomerulosclerotic lesions does not occur via the high-affinity binding site for heparin

Intravenous injection of the FITC-conjugated pFN fraction with low affinity for heparin resulted in intense segmental staining of glomeruli from 8 out of 11 mice with GvHD at week 10 (fluorescence intensity 2+, Fig. 4C). This accumulation did not occur when the low-affinity fraction was injected in NF1 mice (n=3). Ex vivo incubation of the low-affinity pFN-FITC fraction with heparin inhibited its accumulation in the glomerulus of GvHD mice (n=2). The FITC-conjugated pFN fraction with high affinity for heparin did not accumulate in the glomerulus of GvHD mice at week 10 (n=10, Fig. 4D), nor of NF1 mice (n=3). There was no statistically significant difference between the accumulation of the low-affinity fraction and the accumulation of total pFN-FITC, but there was a strong difference between the accumulation of total pFN-FITC and the accumulation of the high-affinity fraction ($p < 0.02$)

Discussion

Circulating pFN, which is produced by the liver, accumulates in glomerulosclerotic lesions at a late stage in GvHD (9). This dimeric glycoprotein is known to play a role in a variety of cellular processes, immune complex clearance, and wound healing. Several domains within the FN molecule may be involved in the specific binding to the sclerotic lesion. The actual accumulation of pFN may result from 1) direct binding to other ECM components in the damaged glomerulus, 2) direct binding to beta-1 integrins on cell surfaces, 3) involvement of the coagulation system, or 4) binding to itself by polymerization via self-assembly sites into insoluble fibrils, or from a combination of these mechanisms.

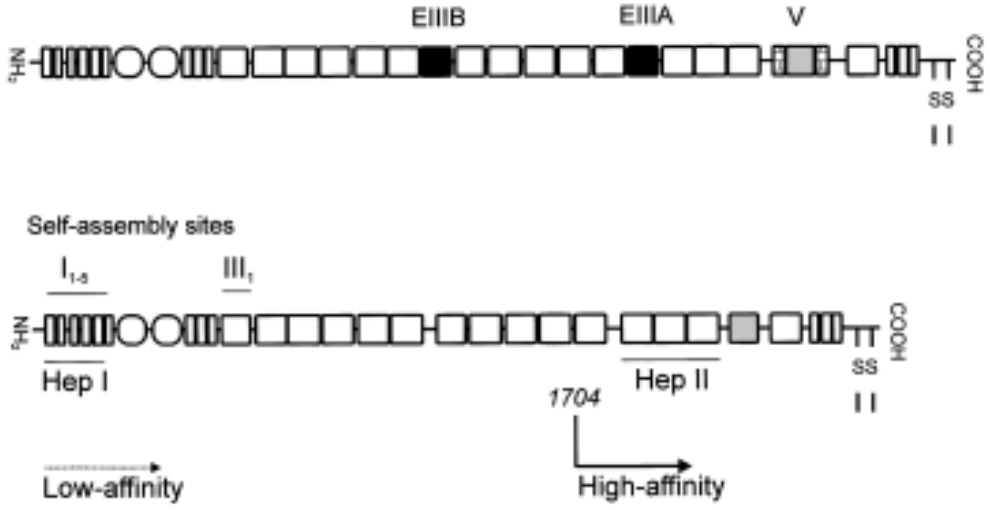

Figure 3. Schematic drawing of the total FN protein sequence (top) and the pFN protein sequece (bottom), with the proposed locations of the FN fragments involved in the accumulation of FN in glomerulosclerotic lesions. Indicated are the EIIIA, EIIIB and V regions, which can be included in or excluded from the protein by alternative splicing of the FN pre-mRNA. Rectangles represent homologous type I repeats, circles represent homologout type II repeats, and squares represent homologous type III repeats. The protein sequence of the high-affinity fraction starts at residue 1704 (homology repeat III_{11}), just before the high-affinity heparin-binding domain (Hep II). The N-terminus of the low-affinity fraction is suggested to be located near the low-affinity heparin-binding domain (Hep I, homology repeat I_{1-5}).

Firstly, FN can bind to other ECM molecules, such as collagen and laminin, that are produced and deposited in the kidney to maintain a stable matrix. Alterations in the ECM turnover that may contribute to the accumulation of FN have been described to precede the development of glomerulosclerosis in the GvHD model (25). Secondly, stabilization of the tissue is also obtained

Table 1. Summary of FN accumulation experiments 10-12 weeks after induction of GvHD.*

	Pre-incubation with		
Administration of	Buffer	Heparin	N-desulfated heparin
Total pFN	+++ (n = 9)	+/- (n = 5)	+/- (n = 5)
High-affinity pFN fraction	0 (n = 10)	0 (n = 2)	nd
Low-affinity pFN fraction	++ (n = 11)	0 (n = 2)	nd

* Results are scored semi-quantitatively on a scale from 0 to +++, in which 0 represents no accumulation of FITC-conjugated pFN or pFN fragments, and +++ reflects very strong accumulation in the glomerulosclerotic lesions, as determined by direct immunofluorescence. Abbreviations are: ND, not determined; N, number of animals.

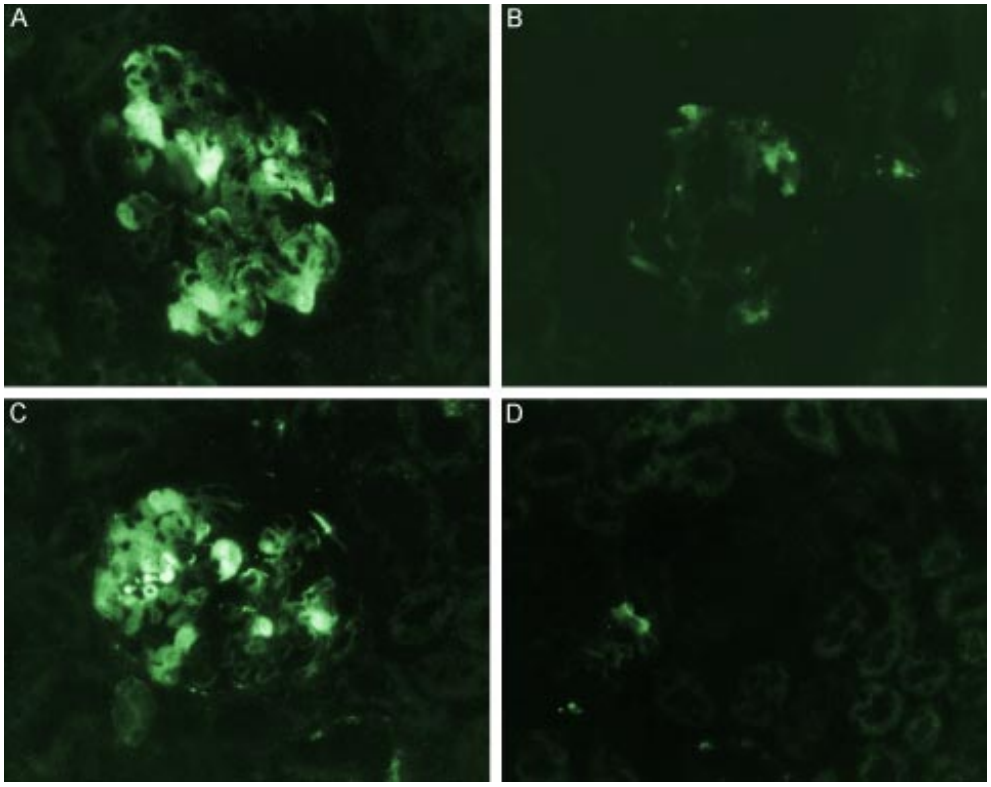

Figure 4. Immunofluorescence micrographs of mouse glomeruli 10 weeks after the induction of GvHD, and 18 h after injection with pFN-FITC (A), pFN-FITC pre-incubated with heparin (B), low-affinity pFN-FITC fraction (C), or high-affinity pFN-FITC fraction (D). Injection of the low-affinity pFN fraction pre-incubated with heparin shows a significantly reduced pattern of accumulation, comparable to the pattern shown in figure B.

by the cell-to-cell and cell-to-substrate attachments via integrin receptors. FN has two major integrin binding sites. The first binding site is an RGD (Arg-Gly-Asp) sequence in the central cell binding domain which is recognized by $\alpha 5\beta 1$ integrins. The second binding site, recognized by $\alpha 4\beta 1$ integrins, is an LDV (Leu-Asp-Val) sequence located in the alternatively spliced V-region. This study shows an increase in the expression of the $\alpha 5$ integrin chain in areas surrounding the glomerulosclerotic lesions (Fig. 1). This altered integrin expression in the glomerulus during the course of the disease may lead to enhanced binding of pFN.

A third mechanism that may participate in the accumulation of pFN in the glomerulus, is activation of the coagulation system. PTAH staining showed deposition of fibrin in the glomerulosclerotic lesions during the development of GvHD, indicating an activation of the coagulation cascade during the induction of the disease. Therapeutical intervention in the coagulation cascade can be achieved by treating the mice with heparin. We earlier performed an

experiment that investigated whether heparin treatment in a late stage of GvHD could significantly affect the development of glomerulosclerosis in vivo (Abstract; Bergijk EC, *et al*. *J Am Soc Nephrol* 6:892, 1995). Seven weeks after induction of GvHD the glomeruli showed mesangial matrix expansion and thickening of the GBM. From this moment on, mice (N=5/group) were treated with different doses of heparin (20 or 40 U/24h). High dose treatment significantly diminished the development of fibronectin-containing end-stage sclerotic lesions in 50-80% of the animals at week 11 of the disease. This points toward the involvement of the coagulation cascade in the process of FN-accumulation leading to the development of end-stage sclerotic lesions. However, to our surprise, treatment of mice with N-desulfated non-anti-coagulant heparin showed a similar effect. This indicates that the protective effect of heparin in the trapping of FN in the glomerulosclerotic lesions is independent of the anti-coagulant properties of heparin. We then hypothesized that heparin treatment directly interferes with FN accumulation by binding to FN, i.e., without participation of the coagulation system.

FN has been shown to contain two heparin-binding sites. The high-affinity heparin II binding domain located at the carboxyterminal site of FN (III_{12-14}) has a dual function. The heparin II binding domain is thought to interact with cell surface glycosaminoglycans to facilitate cell adhesion and spreading (26,27), but it also plays an important role in matrix assembly (28), probably via interactions with I_{1-5}. In addition to the high-affinity heparin II binding domain, FN also has a low-affinity binding domain for heparin (heparin I binding domain) located on $I_{1-5.}$ The heparin I domain is also involved in matrix assembly and, in particular, FN self-assembly (29,30).

To test the possible involvement of either of the heparin-binding sites of FN in its glomerular accumulation, we performed an experiment in which we injected GvHD mice with pFN-FITC pre-incubated with heparin or N-desulfated heparin. Our results show that pre-incubation with heparin prevents FN accumulation in the sclerotic lesions. Similar results were obtained using N-desulfated non-anti-coagulant heparin (Table 1). This suggests the involvement of either one of the heparin binding sites of FN in its glomerular accumulation. To investigate whether FN binds to the glomerulus via its high- or low-affinity heparin-binding domain, FN was digested with Cathepsin D, after which the fragments were separated on a heparin affinity column. Thus, two batches of FN fragments were obtained with either low or high affinity for heparin, respectively. The 100 kD fragments present in both fractions appeared to have no functional effect during the accumulation experiments. Further molecular dissection of these fragments will be required to obtain conclusive evidence for their non-involvement.

In contrast to our expectation, the fraction with FN fragments containing the high- affinity heparin II binding domain did not accumulate in the sclerotic glomeruli, whereas the FN fraction containing the low-affinity binding site for heparin (heparin I binding domain) did. The

accumulation of the latter fraction could be inhibited by pre-incubation with heparin (Table I), suggesting that FN does not bind to the glomerulus via its high-affinity heparin-binding region, but probably via its low-affinity heparin-binding domain, or via a site nearby. In the latter case, we hypothesize that the protective effect of heparin treatment could result from steric hindrance of the specific binding site, or heparin might act via other unknown mechanisms. Therefore, we conclude that i) the protective effect of heparin is independent of its anti-coagulant properties, suggesting that the coagulation cascade is not involved in the trapping of FN in the sclerotic lesion, ii) the process of glomerulosclerosis is not mediated via the heparin II binding site of FN, and iii) the site involved in the accumulation of FN in the glomerulus is located on or near the heparin I binding site.

A final remark concerns the possible functional involvement of the heparin I binding site in the glomerular accumulation and stabilization of FN in the diseased glomeruli. As mentioned above, it is known that the heparin I binding domain contains a self-assembly site which is instrumental in polymerization of FN into insoluble FN fibrils (29,30). Self-assembly of FN dimers into insoluble fibrils is thought to involve primarily interactions between the first five type I (I_{1-5}) repeats located on the low-affinity heparin I binding domain, and the first type III (III_1) repeat located nearby (31-34). Treatment of purified FN in solution with an III_1-C fragment resulted in the appearance of FN multimers, called superfibronectin, which resemble matrix fibrils (35). It has been suggested that this self-assembly site of FN is protected from spontaneous polymerization by keeping it cryptic, and that it is only unmasked in response to either physiological stimuli, or to tissue repair reactions. Based on these observations and ours, we hypothesize that self-assembly of soluble pFN after binding to FN molecules already present in the glomerular ECM contributes to the observed accumulation of FN during the development of glomerulosclerosis in a late stage of GvHD.

In conclusion, our results provide evidence for progressive accumulation of FN in glomerulosclerotic lesions in our model via its low-affinity heparin binding site or a site nearby. This may involve a self-assembly process involving the first five type I (I_{1-5}) repeats, or the first type III (III_1) repeat, or both. However, the extent to which this self-assembly process is preceded by initial binding of FN to either ECM or beta 1 integrins requires further investigation.

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