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CLINICAL INVESTIGATION

Heparin suppresses mesangial cell proliferation and matrix expansion in experimental mesangioproliferative glomerulonephritis

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Heparin suppresses mesangial cell proliferation and matrix expansion in experimental mesangioproliferative glomerulonephritis. Proliferation and extracellular matrix (ECM) overproduction by glomerular mesangial cells characterizes many types of glomerulonephritis and often precedes the development of glomerulosclerosis. Heparin is a potent inhibitor of mesangial cell growth *in vitro*. We examined whether standard heparin can inhibit mesangial cell proliferation *in vivo* in the mesangioproliferative anti-Thy 1.1 nephritis. Untreated control rats were compared to rats infused with heparin either early (day −2 to 1) or late (day 2 to 5) after induction of anti-Thy 1.1 nephritis. The results show that heparin treatment significantly reduced mesangial cell proliferation regardless of when it was initiated. Heparin (either early or late treatment) also reduced mesangial basic fibroblast growth factor (bFGF) expression and platelet-derived growth factor (PDGF) receptor up-regulation as reflected by immunostaining, whereas PDGF B-chain expression was reduced only by late heparin treatment. Furthermore, heparin treatment markedly inhibited the mesangial matrix expansion for a variety of ECM proteins, including laminin, type I and IV collagen, fibronectin and entactin. Heparin did not affect the initial mesangiolysis, glomerular macrophage influx, deposition of anti-Thy 1.1 IgG or fibrinogen, or the glomerular platelet influx. These results suggest that heparin, via its antiproliferative rather than anticoagulant effect, can inhibit mesangial cell proliferation, overexpression of polypeptide growth factors, and ECM protein overproduction *in vivo*. The beneficial effect of heparin can be demonstrated even if treatment is initiated after the development of nephritis. By virtue of these properties, heparin may be an effective agent in the treatment of human mesangioproliferative disease and in the prevention of glomerulosclerosis.

One of the key features of various human glomerular diseases, including IgA nephropathy, membranoproliferative glomerulonephritis, poststreptococcal glomerulonephritis, variants of idiopathic focal sclerosis, lupus nephritis, and possibly diabetic nephropathy, is the proliferation of intrinsic glomerular mesangial cells [1, 2]. Several experimental studies have suggested that mesangial cell activation and proliferation *in vivo* not only results in glomerular hypercellularity but is also followed by overproduction of extracellular mesangial matrix (ECM) [3–7]. Both glomerular hypercellularity and expansion of the ECM are thought to be of primary importance in the development of capillary obsolescence and glomerulosclerosis [8]. The factors involved in the regulation of mesangial cell proliferation *in vitro* and *in vivo* have therefore become the focus of intense interest.

In contrast to the long list of mesangial cell mitogens *in vitro*, few substances exert anti-proliferative effects on these cells [reviewed in 9]. Among these heparin, heparan sulfate proteoglycan, and heparin-like molecules secreted by glomerular epithelial and endothelial cells have been shown to profoundly inhibit the growth of mesangial cells [10–12]. *In vivo* heparin treatment was able to suppress the formation of hypercellular mesangial nodules in the focal mesangioproliferative model in the rat, induced by injection of Habu snake venom [13]. Treatment with heparin has also been shown to reduce the mesangial hypercellularity that develops in chronic aminonucleoside nephrosis [14] and in the lupus-prone MRL-*lpr/lpr* mice [15], while it was unable to affect the endocapillary proliferation in a model of progressive Masugi nephritis [16] or the nephritis that develops in the lupus-prone [NZB × NZW]F_1 mice [15].

In the present study we have attempted to obtain direct evidence that heparin can inhibit mesangial cell proliferation *in vivo*. One model which has proven to be particularly useful for the analysis of factors involved in the initiation of mesangial cell proliferation *in vivo* and its pathological sequelae is the mesangioproliferative glomerulonephritis induced by injection of an antibody to the Thy 1.1 antigen [17–19], which is present on the surface of rat mesangial cells [20]. In this model a single injection of anti-Thy 1.1 antibody results in rapid, complement-dependent loss of mesangial cells with disruption of the mesangial matrix ("mesangiolysis"). The early mesangiolysis at day 1 is followed by marked mesangial cell proliferation and expansion of the mesangial extracellular matrix starting at day 2 [3, 19]. In this model we have analyzed whether a short course of heparin treatment instituted either prior to the initiation of mesangial cell proliferation or at a time when mesangial cell proliferation is well established, can significantly affect the glomerular cell proliferation rate. We have also investigated whether heparin treatment can affect the expression of mesangial cell growth factors such as platelet-derived growth factor

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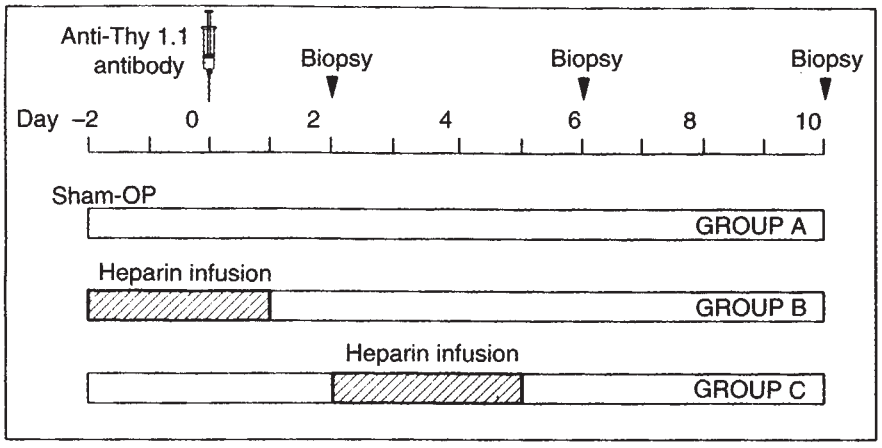

Fig. 1. *Schematic outline of the experimental design (also in* ***Methods****).*

(PDGF) and basic fibroblast growth factor (bFGF) and whether it exerts an influence on the accumulation of mesangial ECM. The results show that heparin treatment, initiated either prior to or after the onset of mesangioproliferative nephritis, led to a potent inhibition of mesangial cell proliferation in anti-Thy 1.1 nephritis, and that it also decreased the overexpression of glomerular growth factors and ECM proteins. These data support the hypothesis that cell proliferation, growth factor expression and ECM synthesis are intricately linked events in the course of glomerular disease. Heparin, by affecting all these processes, may therefore present an important therapeutic agent in the treatment of human mesangioproliferative disease and in the prevention of glomerulosclerosis.

Methods

Disease model and experimental protocol

The rat anti-Thy 1.1 model [17–19] of mesangial proliferative glomerulonephritis was induced by injection of goat anti-rat thymocyte plasma, which was raised as previously described [19]. Twenty-three male Wistar rats (Bantin-Kingman, Fremont, CA), weighing 180 to 220 g, were studied. As shown in Figure 1, five rats (group A; untreated anti-Thy 1.1 nephritis) underwent a sham operation (small abdominal incision) at two days prior to the intravenous injection of anti-thymocyte plasma. Renal biopsies were obtained from each rat at days 2 and 6 and at sacrifice (day 10; Fig. 1). The remaining 12 rats were treated in an identical way, but instead of a sham operation received an intraperitoneal micro-osmotic pump (model Alzet 1003D; filling volume 100 μl; Alza Corporation, Palo Alto, California, USA). The pumps were implanted at either two days prior to the injection of anti-thymocyte plasma (group B; "early heparin"; $N = 6$) or at day 2 after the injection (group C; "late heparin"; $N = 6$; Fig. 1). The pumps were filled with heparin sodium (20,000 U/ml; from porcine intestinal mucosa; Lypho Med, Rosemont, Illinois, USA) and had a delivery time of 72 hours, that is, heparin was administered from day −2 to day 1 in group B and from day 2 to day 5 in group C (Fig. 1). Heparin delivery was calculated as 256 ± 11 U/100 g body wt/day in group B and 254 ± 9 U/100 g/day in group C. Delivery of heparin was monitored by the determination of whole blood clotting times (see below) at days −2, 0, 2, 4, 6 and 10. Six additional, non-manipulated Wistar rats were studied as normal controls.

In an additional control experiment it was assessed whether heparin treatment and/or the implantation of a micro-osmotic pump affects food or water intake. For this five rats received heparin (250 U/100 g body wt) for three days via intraperitoneal pumps and were compared to five rats which underwent a sham operation.

In each kidney biopsy the total cell number and the number of proliferating glomerular cells (as defined by immunostaining for the proliferating cell nuclear antigen (PCNA) per glomerular cross section were determined (see below). Mesangial cell proliferation and activation were assessed by double immunostaining for PCNA and Thy 1.1 as well as by immunostaining for the glomerular *de novo* expression of α-smooth muscle actin [21]. In addition, immunostaining was performed to detect glomerular expression of growth factors (PDGF, bFGF) and the PDGF receptor (β-subunit), and to assess glomerular ECM expansion as reflected by immunostaining for type I and IV collagen, laminin, fibronectin, heparan sulfate proteoglycan, and entactin. Finally, the number of glomerular monocytes/macrophages and the presence of glomerular goat IgG (anti-thymocyte antibody), fibrin/fibrinogen and platelets was determined by immunohistology.

Renal morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution [19] and embedded in paraffin. Four μm sections were stained with the periodic acid Schiff (PAS) reagent and counterstained with hematoxylin. In the PAS-stained sections the total number of nuclei per glomerular cross section was determined and mesangiolysis was graded semiquantitatively on a scale from 0 to 4+ as previously described [21]. A minimum of 20 glomeruli was examined per biopsy (range 20 to 50).

Immunoperoxidase staining

Four μm sections of methyl Carnoy's fixed biopsy tissue were processed by a direct or indirect immunoperoxidase technique as previously described [19]. Primary antibodies included:

1. 19A2 (Coulter, Hialeah, Florida, USA), a murine IgM monoclonal antibody against human PCNA, which is expressed by actively proliferating cells [22].
2. a murine monoclonal antibody to an NH_2-terminal synthetic decapeptide of α-smooth muscle actin (gift of G. Gabbiani, Geneva, Switzerland) [23].
3. ED1 (Bioproducts for Science, Indianapolis, Indiana, USA), a murine monoclonal IgG to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells.
4. PGF-007 (Mochida Pharmaceutical, Tokyo, Japan) a murine monoclonal antibody to a 25 amino acid peptide located near the COOH-terminus of the human PDGF B-chain [24].
5. a rabbit polyclonal antibody to the β-subunit of the PDGF-receptor as described elsewhere [25] (provided by R. Seifert, Seattle, Washington, USA).
6. DE6, a murine monoclonal IgG_1 antibody against rh-bFGF (provided by T. Reilly, DuPont-Merck, Wilmington, Delaware, USA) [26].
7. an IgG fraction of polyclonal rabbit anti-rat laminin (Chemicon, Temecula, California, USA).
8. an IgG fraction of polyclonal guinea pig anti-rat type I collagen [27] (provided by L. Iruela-Arispe, Seattle, Washington, USA).
9. a biotinylated [28] IgG fraction of polyclonal goat anti-mouse type IV collagen (Southern Biotech, Birmingham, Alabama, USA).
10. an affinity-purified IgG fraction of a polyclonal rabbit anti-rat fibronectin (Chemicon).
11. an IgG fraction of a polyclonal rabbit antibody to mouse core protein of heparan sulfate proteoglycan (gift of J.R. Couchman, Birmingham, Alabama, USA) [29].
12. an IgG fraction of polyclonal rabbit anti-mouse entactin/nidogen (gift of A.E. Chung, Pittsburgh, Pennsylvania, USA) [30].
13. a polyclonal goat anti-rat fibrinogen antibody (Cappel Laboratories, Cochraneville, Pennsylvania, USA).
14. PL-1, a murine monoclonal antibody against rat platelets (gift of W.W. Bakker, Groningen, The Netherlands) [31].

For all biopsies, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit IgG.

For each biopsy over 20 cross sections of consecutive cortical glomeruli containing more than 20 discrete capillary segments each were evaluated by one of the authors, who was unaware of the origin of the slides. Mean values per biopsy were calculated for the number of proliferating (PCNA+) cells and monocytes/macrophages per glomerular cross section. For the evaluation of the immunoperoxidase stains for α-smooth muscle actin, PDGF B-chain, PDGF receptor β-subunit, bFGF and the various ECM proteins, each glomerulus was graded semiquantitatively as described previously [3] and the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than intensity of mesangial matrix staining:

0 — Diffuse, very weak or absent mesangial matrix staining. No localized increases of staining.
1+ — Diffuse, weak mesangial matrix staining with up to 25% of the glomerular tuft showing focally increased staining.
2+ — 25 to 50% of the glomerular tuft demonstrating a focal, strong staining.
3+ — 50 to 75% of the glomerular tuft stained strongly in a focal manner
4+ — >75% of the glomerular tuft stained strongly.

Examples of glomerular staining scores of 1+ to 4+ are provided in Figures 2A to 2D.

For the evaluation of the glomerular deposition of goat IgG or fibrinogen, or the glomerular platelet influx, each glomerulus was graded semiquantitatively as showing no deposits or very rare platelets (grade 0), trace staining or few platelets (grade 1), moderate staining or platelet influx (grade 2), intense staining or platelet influx (grade 3), or maximal staining or platelet influx (grade 4).

Immunohistochemical double-staining

Double immunostaining for the identification of the type of proliferating cells was performed as reported previously [6] by first staining the sections for proliferating cells with 19A2, an IgM monoclonal antibody to PCNA, followed by staining with the IgG_1 monoclonal antibody OX-7 (Accurate Chemical Corporation, Westbury, New York, USA), an antibody against the Thy-1.1 antigen present on the mesangial cell membrane. Identification of mesangial cells was possible despite the presence of residual goat anti-thymocyte IgG in the glomerulus suggesting that either not all Thy 1.1 molecules on the mesangial cell surface were occupied by the goat antibody or that the monoclonal OX-7 antibody recognized a different epitope on the molecule. Cells were identified as proliferating mesangial cells if they showed positive nuclear staining for PCNA and if the nucleus was completely surrounded by cytoplasm or cell membrane positive for OX-7. Proliferating cells in which the PCNA positive nucleus did not border on the cytoplasm or cell membrane positive for OX-7 were classified as non-mesangial. Proliferating (PCNA+) cells which could not be clearly identified as OX-7 positive or negative were considered non-classifiable.

Miscellaneous measurements

Whole blood clotting times were determined using tail vein blood in glass tubes which were inverted every 25 seconds.

Statistical analysis

All values are expressed as mean ± SD unless stated otherwise. Statistical significance (defined as $P < 0.05$) was evaluated using the Student's t-test or one way analysis of variance with modified t-tests performed using the Bonferroni correction [32].

Results

Heparin treatment reduces mesangial cell proliferation in vivo

Control rats with untreated anti-Thy 1.1 nephritis (group A) followed the typical disease course as previously reported [3, 19, 21, 25, 33]: total glomerular cell numbers decreased below normal at day 2 after disease induction, followed by a rapid increase at days 6 and 10 (Fig. 3). In contrast, glomerular cell

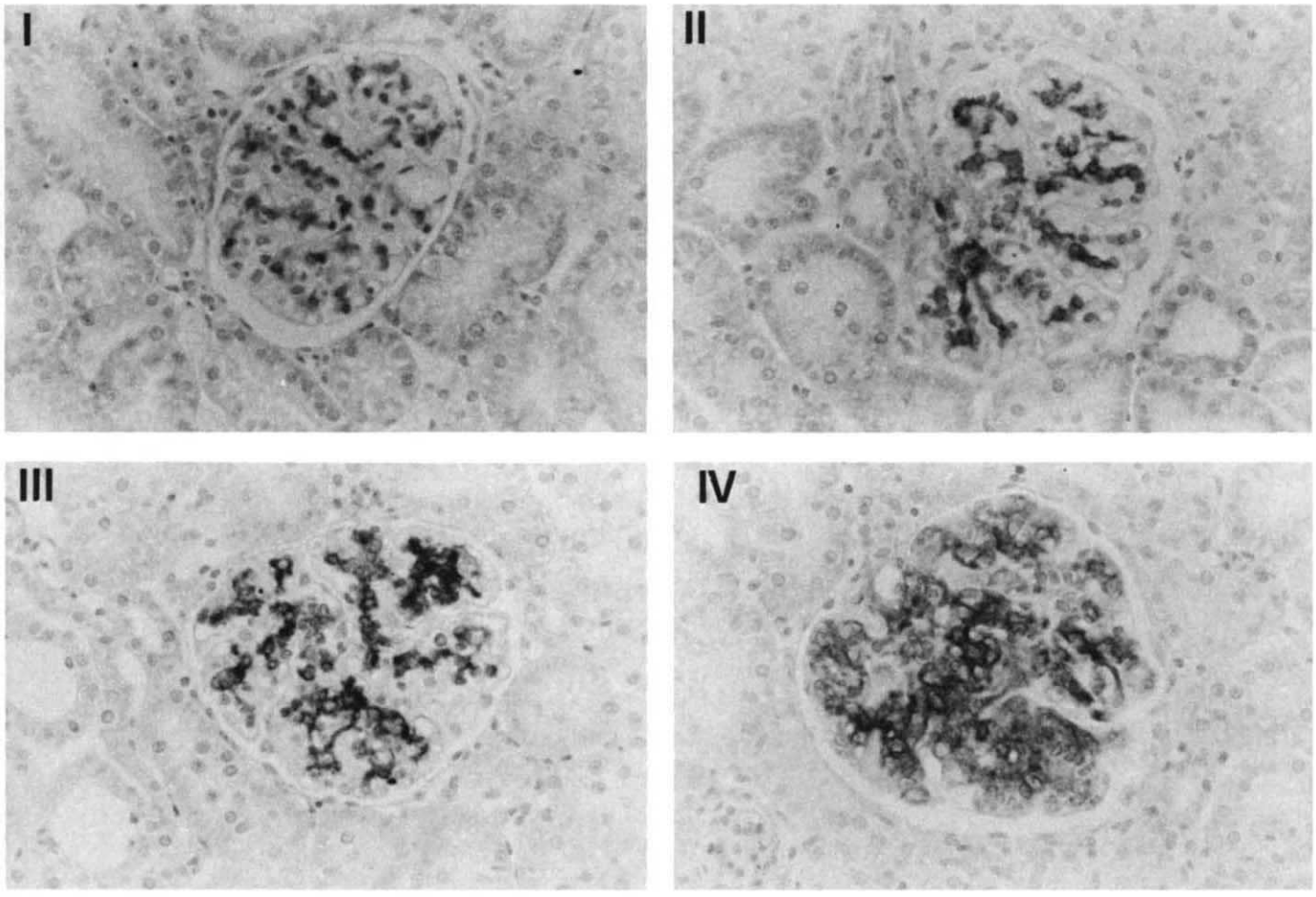

Fig. 2. *Glomerular immunostaining for PDGF receptor β-subunit at different stages of anti-Thy 1.1 nephritis, illustrating the semiquantitative scoring system (scores I to IV; see* ***Methods*** *for further details).*

proliferation was already increased above the normal range at day 2 and increased further at day 6 (Fig. 3). At day 10, glomerular cell proliferation in group A had decreased, but was still elevated above the normal range (Fig. 3). Double immunostaining for proliferating cells (PCNA+) and mesangial cells (Thy 1.1+) showed that the majority of proliferating glomerular cells at days 2, 6 and 10 were of mesangial origin (Table 1). The mesangial cell proliferation was also associated with *de novo* α-smooth muscle actin expression in mesangial regions of control rats with anti-Thy 1.1 nephritis (group A) at days 2, 6 and 10 (Fig. 4) and was consistent with previous studies [21].

In rats of group B (anti-Thy 1.1 nephritis, early heparin treatment) the total glomerular cellularity at day 2 was similar to that observed in group A (Fig. 3). However, both glomerular cell proliferation and α-smooth muscle actin expression were reduced at this time point (Figs. 3 and 4). At day 6 all three parameters were significantly lower than in group A while at day 10 only the total glomerular cellularity remained lower than that in rats of group A (Figs. 3 and 4). Double immunostaining showed that the reduction of glomerular cell proliferation at days 2 and 6 was largely due to reduced mesangial cell proliferation (Table 1). Thus, the relative contribution of proliferating mesangial cells (PCNA+, Thy 1.1+) to the total proliferating cells decreased significantly on these days in heparin treated rats of group B (Table 1).

Similar findings to those observed in group B were also obtained during a pilot experiment in which four rats with anti-Thy 1.1 nephritis were treated with subcutaneous heparin (at similar doses as used in this study) from day −1 to day 3 after disease induction and compared to four untreated rats with anti-Thy 1.1 nephritis. This experiment too demonstrated a 53 ± 8% reduction of glomerular cell proliferation at day 6 and a reduction of matrix expansion (see below).

In glomeruli of rats of group C (anti-Thy 1.1 nephritis, late heparin treatment) the total cellularity, cell proliferation and α-smooth muscle actin expression were significantly reduced at days 6 and 10 of the disease (Figs. 3 and 4). As in group B the reduction of glomerular cell proliferation at days 6 and 10 appeared to be largely due to reduced proliferation of mesangial cells (Table 1).

Heparin treatment decreases glomerular immunostaining for PDGF, PDGF receptor, and bFGF during mesangioproliferative glomerulonephritis

Immunostaining patterns for PDGF B-chain, PDGF receptor β-subunit, and bFGF in glomeruli of normal rats and rats with

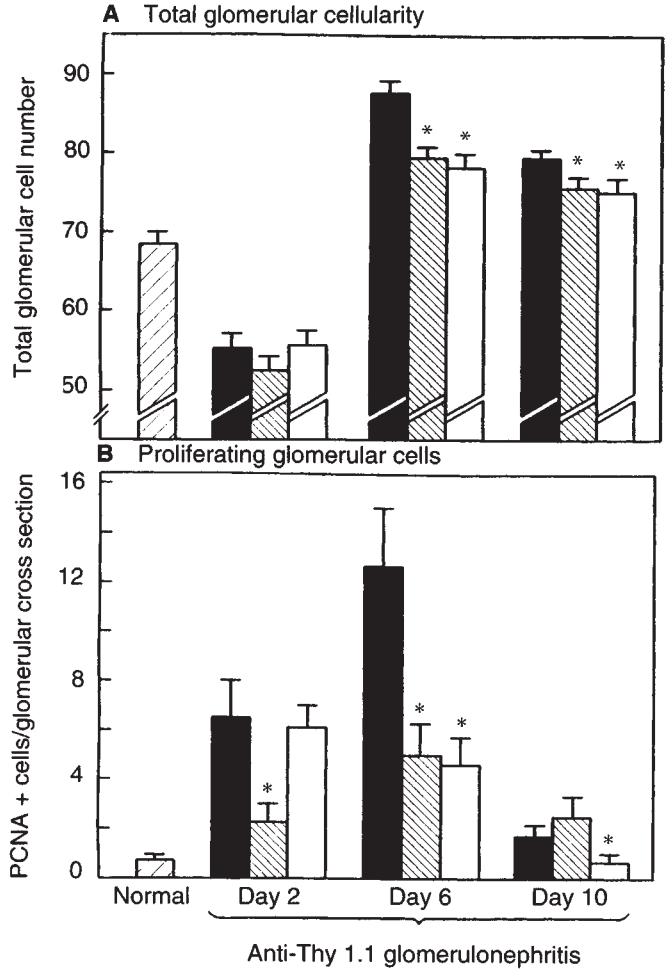

Fig. 3. *Total cell counts and proliferating cells (as defined by immunostaining for the PCNA antigen) per glomerular cross section in normal rats, untreated rats with anti-Thy 1.1 nephritis (■, group A), and rats with anti-Thy 1.1 nephritis which received either early (▨, group B) or late (□, group C) heparin treatment.* *$P < 0.05$ versus group A.

Table 1. Double immunostaining for PCNA and the Thy 1.1 antigen on the mesangial cell surface in normal Wistar rats ("day 0"), rats with anti-Thy 1.1 nephritis (control), in rats with anti-Thy 1.1 nephritis and heparin treatment from day −2 to day 1 (early heparin), and in rats with anti-Thy 1.1 nephritis and heparin treatment from days 2 to 5 (late heparin)

	Days after disease induction	Control (group A)	Early heparin (group B)	Late heparin (group C)
Proliferating, mesangial (PCNA+, Thy 1.1+)	0	0.2 ± 0.1	—	—
	2	2.7 ± 0.3	0.7 ± 0.1[a]	2.2 ± 0.4
	6	7.4 ± 0.4	1.9 ± 0.3[a]	2.2 ± 0.3[a]
	10	1.0 ± 0.1	1.3 ± 0.2	0.3 ± 0.2[a]
Proliferating, non-mesangial (PCNA+, Thy 1.1−)	0	0.4 ± 0.1	—	—
	2	1.2 ± 0.3	0.9 ± 0.1	1.3 ± 0.1
	6	0.7 ± 0.2	1.0 ± 0.2	0.8 ± 0.1
	10	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
Not classifiable	0	0.1 ± 0.0	—	—
	2	0.7 ± 0.2	0.2 ± 0.0	0.5 ± 0.1
	6	1.0 ± 0.2	0.5 ± 0.2	0.4 ± 0.2[a]
	10	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.0[a]

Data are mean ± SD. Results are expressed as cells per glomerular cross section. Due to the different staining technique total PCNA-positive cells per glomerulus are lower than those detected during immunoperoxidase staining for PCNA only (Fig. 3).

[a] $P < 0.05$ versus group A (Bonferroni *t*-tests)

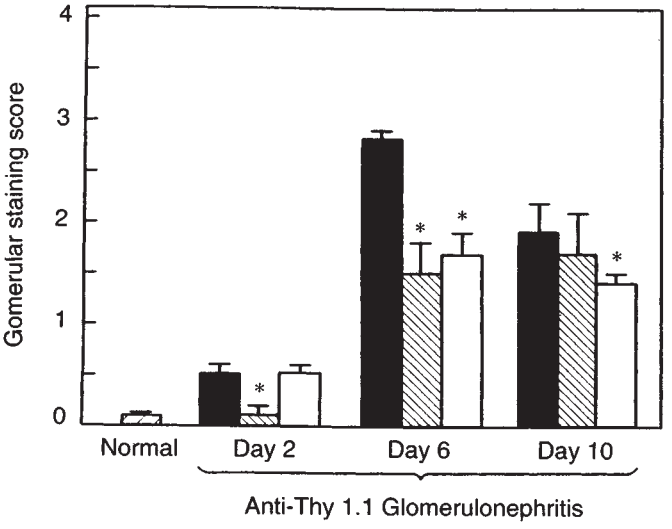

Fig. 4. *Glomerular immunostaining scores (see **Methods**) for the mesangial cell activation marker α-smooth muscle actin in normal rats, untreated rats with anti-Thy 1.1 nephritis (■, group A), and rats with anti-Thy 1.1 nephritis which received either early (▨, group B) or late (□, group C) heparin treatment.* *$P < 0.05$ versus group A.

anti-Thy 1.1 nephritis using the same antibodies as employed in this study have been published previously [6, 25, 34] and were not different in the present study (data not shown).

During anti-Thy 1.1 nephritis the glomerular immunostaining scores for PDGF B-chain, PDGF receptor β-subunit and bFGF in rats of group A (control) remained either unchanged or decreased below normal (bFGF) at day 2 of the disease (Fig. 5). At days 6 and, to a lesser degree, day 10 all three immunostaining scores increased above the normal range (Fig. 5).

In glomeruli of rats of group B (early heparin) immunostaining scores for PDGF B-chain were not different from controls at any time point (Fig. 5). In contrast, the glomerular staining scores for the PDGF receptor β-subunit were reduced at days 2 and 6 of the disease (Fig. 5). Immunostaining for bFGF was significantly reduced at days 6 and 10 in group B (Fig. 5).

In rats of group C (late heparin) the glomerular staining scores for PDGF B-chain, PDGF receptor β-subunit and bFGF were significantly reduced at day 6 (Fig. 5). A significantly lower staining score for PDGF receptor β-subunit and bFGF persisted at day 10 in this group (Fig. 5).

Heparin treatment reduces glomerular immunostaining for ECM proteins during mesangioproliferative glomerulonephritis

In agreement with previously published results employing the same antibodies as in the present study [3], the immunostaining of glomeruli for laminin, type I and IV collagen, fibronectin, heparan sulfate proteoglycan, and entactin/nidogen remained unchanged as compared to normal rats at day 2 of anti-Thy 1.1

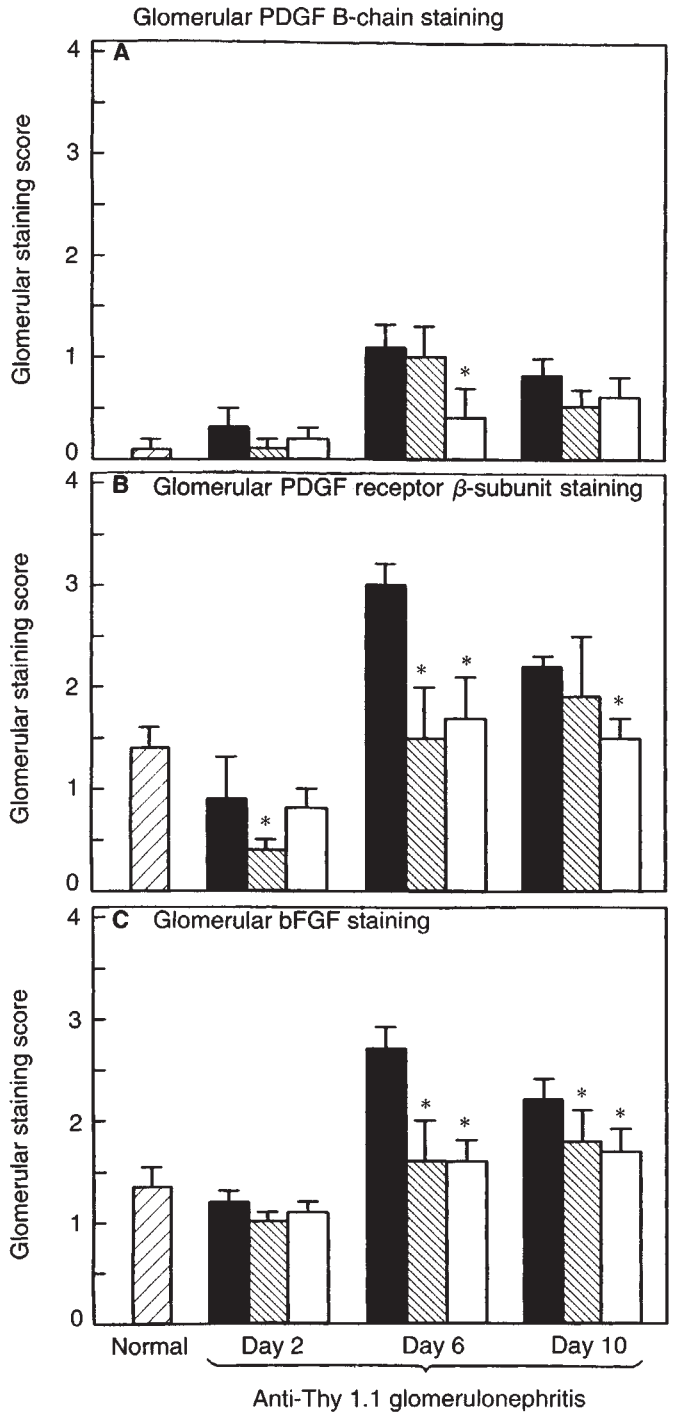

Fig. 5. *Glomerular immunostaining scores (see **Methods**) for PDGF B-chain, PDGF receptor β-subunit, and bFGF in normal rats, untreated rats with anti-Thy 1.1 nephritis (■, group A), and rats with anti-Thy 1.1 nephritis which received either early (▨, group B) or late (□, group C) heparin treatment.* $^*P < 0.05$ versus group A.

nephritis, increased markedly at day 6, and decreased again at day 10 in rats of group A (Fig. 6).

In group B (early heparin) there was a minor, but significant decrease of the glomerular immunostaining for laminin at day 2 as compared to group A (Fig. 6). None of the other ECM protein staining scores was altered at this time point (Fig. 6). At day 6 all ECM staining scores, with the exception of heparan sulfate proteoglycan, were significantly lower in glomeruli of group B as compared to group A (Fig. 6). For all ECM proteins the immunostaining in glomeruli showed high intra-biopsy variability at day 6, and glomeruli with marked ECM expansion could be detected adjacent to glomeruli with reduced immunostaining. At day 10 of anti-Thy 1.1 nephritis, the immunostaining for the various ECM proteins, with the exception of entactin, was no longer different between groups B and A (Fig. 6).

In group C (late heparin) the immunostaining scores of glomeruli for the various ECM proteins, with the exception of heparan sulfate proteoglycan, were significantly reduced at day 6 (Fig. 6). As in group B, glomerular immunostaining for ECM proteins was highly variable at this time point. At day 10 only the glomerular staining scores for heparan sulfate proteoglycan and entactin were lower in group C in comparison to group A (Fig. 6).

Effects of heparin treatment on mesangiolysis, glomerular monocyte/macrophage influx, goat IgG deposition, fibrin deposition and platelet influx

As shown in Figure 7, marked mesangiolysis was present in rats of group A at day 2. Mesangiolysis decreased at day 6 and was no longer present at day 10 (Fig. 7). In contrast, both early (group B) and late (group C) heparin treatment resulted in greater, persistent mesangiolysis at days 6 (group B) and 10 (groups B and C; Fig. 7). Microaneurysms were noted at day 10 in 5 to 10% of the glomeruli of group B, and 1 to 5% of the glomeruli of group C as opposed to <1% of the glomeruli of group A.

In agreement with previous observations [25, 35], induction of anti-Thy 1.1 nephritis resulted in an early glomerular influx of monocytes/macrophages which then slowly decreased during the course of the disease. This monocyte/macrophage influx was not affected by early (group B) or late (group C) heparin treatment (data not shown) and the glomerular monocyte/macrophage counts were not different from those observed in untreated rats (group A) at days 2 (9.6 ± 0.5 macrophages per glomerular cross section), 6 (7.0 ± 0.7) and 10 (6.3 ± 1.4).

Similar to the glomerular macrophage influx, the deposition of goat IgG in glomeruli after the injection of goat anti-rat thymocyte plasma was not affected by heparin treatment. At day 2 glomeruli of all groups exhibited moderate staining (score in group A: 2.1 ± 0.2), which decreased to weak or absent staining (score in group A: 0.6 ± 0.1) at day 6, and absent staining at day 10.

Following the induction of anti-Thy 1.1 nephritis the coagulation cascade was activated in the glomeruli as evidenced by intense glomerular fibrin/fibrinogen deposition at day 2 in control rats (group A; glomerular staining score: 2.8 ± 0.1; Fig. 8). Glomerular fibrin/fibrinogen deposition then decreased during the course of the disease in rats of group A (score at day 6: 1.4 ± 0.1; score at day 10: 0.4 ± 0.1). In rats of group B and C this staining pattern was not altered to a significant degree despite the significant prolongation of the whole clotting time

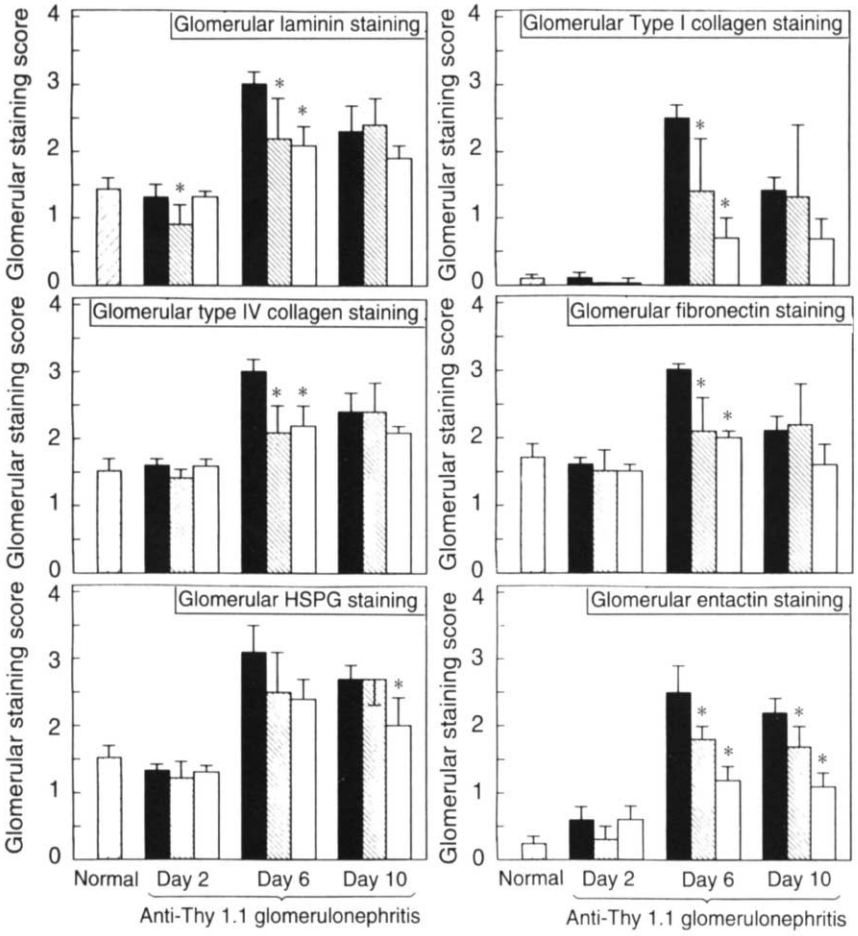

Fig. 6. *Glomerular immunostaining scores (see **Methods**) for various extracellular matrix proteins in normal rats, untreated rats with anti-Thy 1.1 nephritis (■, group A), and rats with anti-Thy 1.1 nephritis which received either early (▨, group B) or late (□, group C) heparin treatment.* $*P < 0.05$ versus group A. HSPG = heparan sulfate proteoglycan.

during heparin treatment (see below). In addition to the activation of the coagulation cascade, the induction of anti-Thy 1.1 nephritis was also associated with a marked glomerular influx of platelets (Fig. 9). Early heparin treatment (group B) did not affect the initial platelet influx, but rather led to a longer persistence of glomerular platelet deposits (Figs. 9, 10). Late heparin treatment (group C) had no significant influence on the glomerular platelet influx (Fig. 9).

Basic data

As shown in Table 2, whole blood clotting times in untreated rats with anti-Thy 1.1 nephritis (group A) did not differ from those observed in normal rats. Heparin treatment from day −2 until day 1 (group B) resulted in a significant prolongation of the clotting time at days 0 and 2 (Table 2). Similarly, heparin treatment from days 2 to 5 (group C) led to a significant prolongation of the clotting time at days 4 and 6 (Table 2).

Neither the induction of anti-Thy 1.1 nephritis nor late heparin treatment (group C) had an influence on hematocrits (Table 2). In contrast, in rats of group B there was a small but significant drop in hematocrits at days 0, 2, and 4 (Table 2).

Heparin treatment and the intraperitoneal implantation of a micro-osmotic pump did not significantly affect daily food or water intake (data not shown).

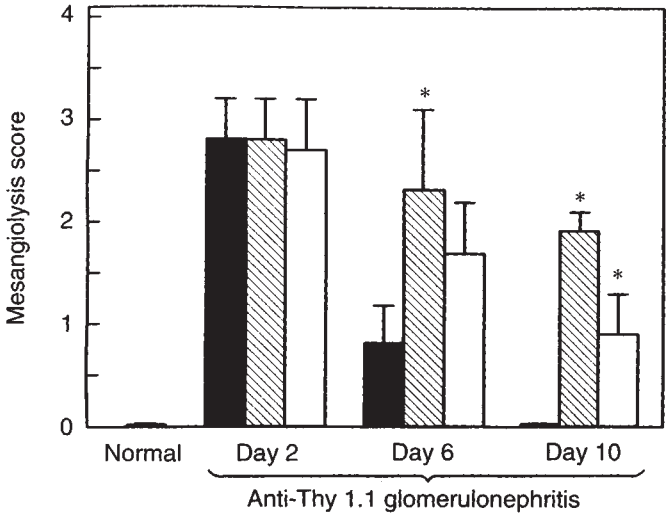

Fig. 7. *Mesangiolysis score (see **Methods**) in normal rats, untreated rats with anti-Thy 1.1 nephritis (■, group A), and rats with anti-Thy 1.1 nephritis which received either early (▨, group B) or late (□, group C) heparin treatment.* $*P < 0.05$ versus group A.

Discussion

In the present study we have obtained direct evidence that the administration of standard heparin either prior to or during

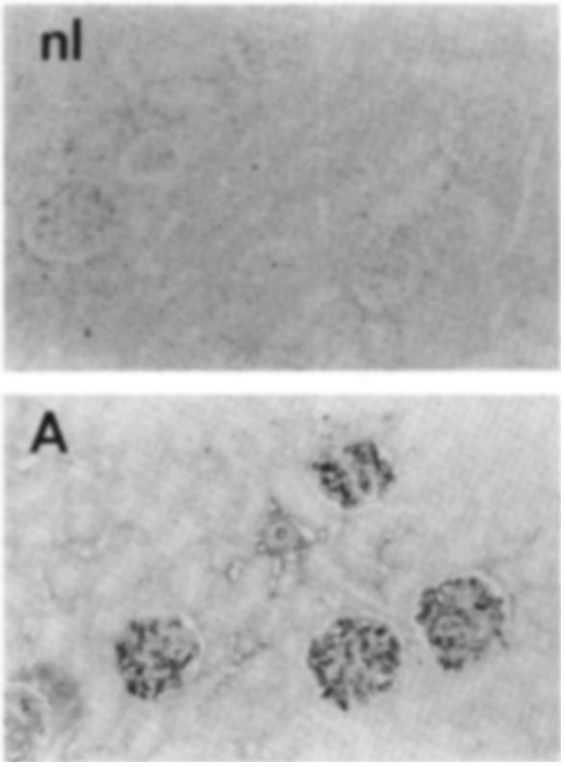

Fig. 8. *Glomerular immunostaining for fibrinogen in a renal biopsy of an untreated rat with anti-Thy 1.1 nephritis (group A) at day 2 ("A") and a normal Wistar rat ("nl").* Compared to the normal rat, marked glomerular fibrin/fibrinogen deposition is present in the rat with anti-Thy 1.1 nephritis. Immunoperoxidase stain with methyl green counterstain. ×400.

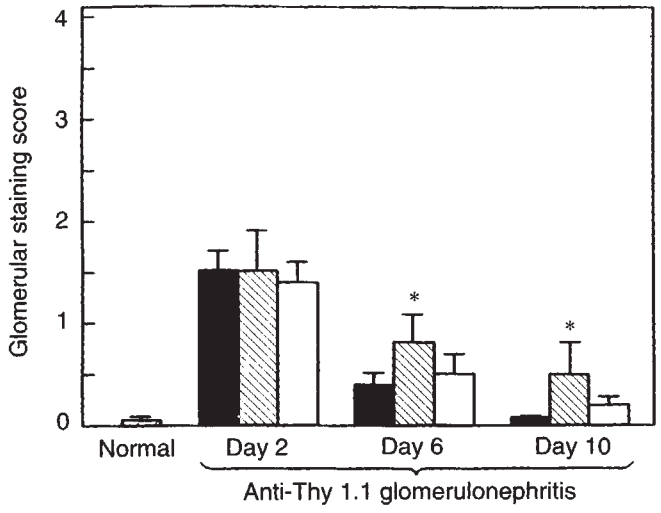

Fig. 9. *Glomerular immunostaining score (see **Methods**) for platelets in normal rats, untreated rats with anti-Thy 1.1 nephritis (■, group A), and rats with anti-Thy 1.1 nephritis which received either early (▨, group B) or late (□, group C) heparin treatment.* $*P < 0.05$ versus group A.

the mesangioproliferative phase of anti-Thy 1.1 nephritis results in a 65% decrease of glomerular cell proliferation. This observation is in agreement with preliminary data of other authors, showing that heparin treatment of rats with anti-Thy 1.1 nephritis can reduce both mesangial hypercellularity [36, 37] and the glomerular incorporation rate of a thymidine analogue, BrdU [37]. The decrease in glomerular cell proliferation observed in our study appeared to be largely due to the suppression of mesangial cell proliferation as indicated by the findings obtained with double immunostaining for the PCNA antigen and the Thy 1.1 antigen as well as by the observation of reduced glomerular α-smooth muscle actin expression. We have previously shown that the glomerular *de novo* expression of this actin isotype specifically marks activated and/or proliferating mesangial cells [21, 38]. Both early and late administration of heparin led to a long-lasting effect on mesangial cell proliferation, which extended well beyond the period of heparin administration but was ultimately reversible (as in the case of day 10 of the early heparin treatment group). This finding is in contrast to data obtained in the Habu snake venom model of mesangioproliferative nephritis, in which heparin had to be present during the period of mesangial cell proliferation in order to reduce mesangial hypercellularity [13]. Heparin treatment in our study also resulted in a reduction of the total glomerular cellularity. These beneficial actions of heparin did not appear to be due to an effect of heparin on the initial mesangiolysis, since the mesangiolysis score, the initial drop of glomerular cell numbers, the deposition of anti-mesangial cell antibody (goat IgG) as well as the resultant influx of monocytes/macrophages were not affected to a detectable degree by heparin.

One potential mechanism by which heparin could affect mesangial cell proliferation *in vivo* is via its anticoagulant activity. Thus, factors of the coagulation cascade such as thrombin as well as factors released from activated platelets such as PDGF are potent mitogens for mesangial cells in culture [reviewed in 9]. Previous data [18, 33, 39] as well as the data of the present study show that glomerular activation of the coagulation pathway and platelet localization indeed occur in anti-Thy 1.1 nephritis. In platelet-depleted rats we have also obtained direct evidence that platelets are involved in the initiation of mesangial cell proliferation in this model [19]. However, various observations of the present study argue against a significant role of the anticoagulant activity of heparin as being responsible for the reduction of mesangial cell proliferation. Thus, heparin treatment was equally effective no matter whether it was given early or late during the course of anti-Thy 1.1 nephritis, while both glomerular fibrin deposition and platelet influx were maximal during the early phases of the disease [this study and 34]. Furthermore, at the heparin dosage chosen, no significant suppression of either glomerular fibrin deposition or platelet influx could be demonstrated. In fact, early heparin treatment led to persistence of glomerular platelet accumulation in group B as compared to group A, which may relate to the persistent mesangiolysis in group B.

Alternatively, the therapeutic effectiveness of heparin in the anti-Thy 1.1 nephritis could reside in its antiproliferative activity. Thus, heparin has been shown to inhibit the proliferation of cultured glomerular mesangial, visceral epithelial, and endothelial cells [10, 40, 41]. Strong evidence that heparin may exhibit similar activity *in vivo* is proposed by studies in which heparin species, which were devoid of anticoagulant but not of antiproliferative activity, were able to ameliorate the progression of glomerular disease in rats with subtotal renal ablation [42] or

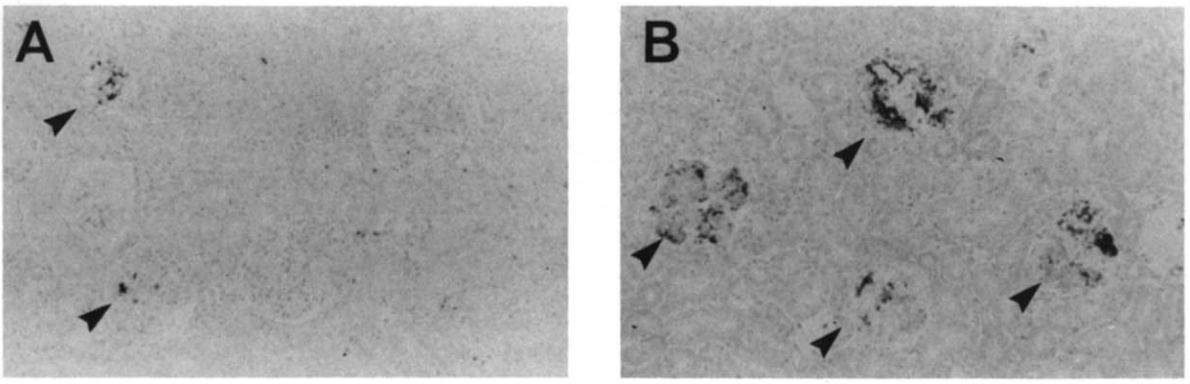

Fig. 10. *Glomerular immunostaining for platelets in a renal biopsy of rats with anti-Thy 1.1 nephritis that were either untreated (A) or had received heparin treatment from day −2 to 1 after disease induction (B).* Compared to the rat of group A, there is persistence of platelet accumulation in the glomerulus of the rat of group B (arrows). Immunoperoxidase stain with methyl green counterstain. ×400.

Table 2. Whole blood clotting times and hematocrits in rats with untreated anti-Thy 1.1 glomerulonephritis (group A), in rats with anti-Thy 1.1 nephritis and heparin treatment from day −2 to day 1 (group B), and in rats with anti-Thy 1.1 nephritis and heparin treatment from days 2 to 5 (group C)

Day prior to or after disease induction	Whole blood clotting time (*minutes*) of group			Hematocrit (%) of group		
	A	B	C	A	B	C
−2	ND	3.4 ± 0.3	ND	ND	42 ± 1	ND
0	3.2 ± 0.5	7.0 ± 2.4[a]	3.2 ± 0.4	44 ± 1	39 ± 2[a]	44 ± 1
2	3.6 ± 0.6	5.3 ± 1.2[a]	4.0 ± 1.0	44 ± 2	40 ± 3[a]	45 ± 2
4	4.0 ± 0.7	3.7 ± 0.5	7.2 ± 1.5[a]	44 ± 2	40 ± 1[a]	42 ± 3
6	3.9 ± 0.6	3.9 ± 0.6	5.6 ± 1.8[a]	43 ± 2	40 ± 3	41 ± 4
10	4.0 ± 1.2	3.8 ± 0.3	3.8 ± 0.6	45 ± 1	41 ± 3	44 ± 2

Data are mean ± SD ($N = 5$ in group A, $N = 6$ in groups B and C). Abbreviation ND is not determined.
[a] $P < 0.05$ versus group A

aminonucleoside nephrosis [14]. In conjunction with our finding that increased mesangial cell proliferation occurs early after subtotal renal ablation [6], this suggests that heparin may indeed exert a beneficial effect *in vivo* by inhibiting the proliferation of intrinsic glomerular mesangial cells.

Multiple mechanisms have been proposed by which heparin could inhibit cell proliferation. Studies in vascular smooth muscle cells, which exhibit various features reminiscent of mesangial cells [reviewed in 43, 44], have shown that heparin can synchronize and arrest the cells in the G_0 phase of the cell cycle [45]. Furthermore, heparin decreases the transcription of protooncogenes expressed in the mid but not early G_1 phase [46] and may thereby interrupt the completion of the cell cycle.

Although only assessed by a semiquantitative scoring system, the data of the present study also suggest that heparin treatment resulted in a reduction of the mesangial expression of PDGF β-receptor subunit and bFGF as well as in a minor decrease of the mesangial expression of PDGF B-chain. In previous studies we have presented evidence that all three proteins are overexpressed in anti-Thy 1.1 nephritis and that bFGF and PDGF may be involved in the initiation and maintenance of mesangial cell proliferation in this model [25, 34, 47]. Heparin can interfere with the PDGF synthesis and actin in multiple ways. Thus, *in vitro* heparin decreases PDGF mRNA transcription [48], binds to PDGF [49], interferes with PDGF signaling [45], and heparin treatment *in vivo* can remove platelet cationic proteins, such as PDGF, from glomeruli [50]. In contrast to PDGF, the effects of heparin on the biological actions of bFGF are less consistent. Thus, heparin can cooperate with bFGF to stimulate growth in some cultured cell lines [51] and it can protect bFGF from denaturation and degradation [52]. However, heparin infusion can also displace matrix-bound bFGF and release it into the circulation [53, 54]. The data of the present study, showing that early heparin treatment effectively suppressed mesangial cell proliferation, argue against the first possibility, in which heparin should have amplified the mitogenic effect of bFGF released from injured mesangial cells [35]. Thus, multiple mechanisms exist by which heparin could interfere with the action of mesangial cell growth factors such as PDGF and bFGF. However, although heparin may have these various direct effects on PDGF and bFGF, we cannot exclude the possibility that decreased expression of these cytokines may be a secondary phenomenon related to the decrease in mesangial cell numbers and consequently decreased staining for intracellular bFGF or PDGF.

Apart from its effects on the cell cycle and on polypeptide growth factors, another *in vitro* activity of heparin, which is likely to be of relevance for the course of anti-Thy 1.1 nephritis, is the inhibition of mesangial cell migration by heparin and heparin-like glycosaminoglycans [55]. It is likely that mesangial cell migration is involved in association with the mesangial cell proliferation in the remodeling of the mesangium that follows the mesangiolysis in the Thy 1.1 model. Although it has not been possible to demonstrate mesangial cell migration in our

model, the demonstration that there was persistence of microaneurysms in the heparin-treated rats suggests that heparin may also be mediating effects on this mesangial cell function. A similarity in this regard relates to studies in rats with carotid balloon injury. In these rats heparin treatment not only reduced the proliferation rate of vascular smooth muscle cells, which in many ways resemble mesangial cells [reviewed in 43, 44], but it also abrogated the migration of the smooth muscle cells into the intima [56].

The third main finding of this study was that heparin also suppressed the accumulation of a variety of ECM proteins that accompanies the mesangial cell proliferation in the anti-Thy 1.1 model [3]. This observation is in agreement with findings in other progressive models of experimental and human glomerular disease in which heparin treatment resulted in a reduction of glomerulosclerosis [14, 42, 57–59, reviewed in 60]. Our data thereby lend further support to the hypothesis that mesangial cell activation and proliferation may be linked to subsequent overproduction of ECM proteins and to the development of glomerulosclerosis [9]. Alternatively, heparin could also exert direct effects on the cellular production of ECM proteins. Data derived from cell culture experiments, however, are conflicting in this respect. While preliminary findings in rat mesangial cells suggested that heparin decreases the synthesis of collagenous proteins [61], in vascular smooth muscle cells and endothelial cells heparin led to an augmentation of fibronectin, thrombospondin and heparan sulfate proteoglycan synthesis [62–64]. In the present study the glomerular accumulation of heparan sulfate proteoglycan in heparin treated rats appeared to be reduced to a lesser degree than that of the other ECM proteins. This observation argues against the hypothesis of Coffey and Karnovsky [13] that heparin replaces the heparan sulfate proteoglycan lost from the glomerular basement membrane during glomerulonephritis and thereby substitutes its anti-proliferative effect on mesangial cells. Rather, our findings would suggest that the persistence of heparan sulfate proteoglycan induced by heparin treatment provides an additional mechanism by which heparin could inhibit mesangial cell proliferation [10].

So far clinical studies of heparin treatment have mostly focused on glomerular diseases characterized by acute onset and evidence of intraglomerular coagulation, such as rapidly progressive glomerulonephritis, pre-eclampsia or acute allograft rejection [reviewed in 60]. In these studies heparin was usually given in combination with other agents such as steroids or cytostatic drugs and no definite conclusions on the effectiveness of anticoagulant therapy have been reached [60]. Very few studies have investigated the efficacy of heparin in chronic glomerulopathies. In a controlled clinical trial in 18 patients with biopsy proven chronic proliferative glomerulonephritis, Cade et al [59] were able to show that a monotherapy of daily subcutaneous heparin injections for one year could improve glomerular filtration rates. More importantly, in the eight patients, who underwent pre- and post-treatment renal biopsies, glomerular hypercellularity had regressed after one year [59].

In summary, the present study shows that a brief course of heparin treatment, instituted either prior to the initiation of mesangial cell proliferation or after it has been established, can exert a marked growth inhibitory effect on mesangial cells *in vivo* and can effectively suppress ECM overproduction by these cells. This observation suggests that cell proliferation and ECM overproduction are intricately linked events *in vivo* and also provides a further rationale for the use of heparin as an (adjunctive) therapy in mesangioproliferative and progressive glomerular diseases. In contrast to promising laboratory studies with neutralizing antibodies against PDGF or transforming growth factor-β (TGF-β), which also show beneficial effects on the mesangial cell proliferation and/or matrix expansion in experimental mesangioproliferative disease [47, 65], heparin is clinically available. Despite the potential problem of bleeding, experimental data such as provided in the present study support continued investigation of a possible role for heparin in the therapy of proliferative glomerular diseases, particularly if non-anticoagulant heparin with antimitogenic properties becomes available.

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