Kidney International, Vol. 65 (2004), pp. 2238–2248

Platelet inhibition limits TGF-β overexpression and matrix expansion after induction of anti-thy1 glomerulonephritis

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Platelet inhibition limits TGF-β overexpression and matrix expansion after induction of anti-thy1 glomerulonephritis.

Background. Although a role of platelets is well established in atherosclerosis, only little is known about their contribution to pathologic renal matrix expansion. The present study analyzes the effect of the platelet inhibitor clopidogrel on the early injury and subsequent repair phase of experimental anti-thy1 glomerulonephritis.

Methods. In male Sprague-Dawley rats, acute anti-thy1 glomerulonephritis was induced by intravenous injection of OX-7 antibody. In protocol 1 (injury), clopidogrel was given starting 5 days before antibody injection. One day after disease induction, parameters of mesangial cell injury (glomerular cell number, inducible NO synthesis, and macrophage infiltration) were analyzed. In protocol 2 (repair), clopidogrel treatment was started one day after antibody injection. On day 6, parameters of glomerular repair [glomerular matrix score, expression of transforming growth factor (TGF)-β1, fibronectin, and plasminogen activator inhibitor (PAI)-1] and thrombosis (aneurysm formation and fibrinogen deposition) were determined. In both protocols, an additional group of rats was treated with the angiotensin-converting enzyme (ACE) inhibitor enalapril.

Results. In the injury protocol, platelet inhibition did not affect mesangial cell lysis, glomerular NO production, and macrophage infiltration, while ACE inhibition was protective. In the repair protocol, clopidogrel significantly limited aneurysm formation and fibrinogen deposition, as well as glomerular matrix expansion, TGF-β1, fibronectin, and PAI-1 expression. In comparison, enalapril was less effective in preventing glomerular thrombosis, but was significantly superior to clopidogrel in limiting matrix protein expression and accumulation.

Conclusion. The present study shows that platelets play a significant role in the sequence from mesangial cell injury to renal matrix expansion in anti-thy1 glomerulonephritis. The results, directly comparing renin-angiotensin-system and platelet inhibition, suggest that platelets contribute less than angiotensin II to TGF-β overexpression and matrix accumulation in this model of acute glomerular wound repair.

Key words: platelets, ACE inhibition, TGF-β1, fibrosis.

Received for publication March 3, 2003
and in revised form October 2, 2003
Accepted for publication December 12, 2003

Overexpression of the cytokine transforming growth factor (TGF)-β and expansion of extracellular matrix are key elements of a highly orchestrated molecular and cellular program called wound repair [1, 2]. Among different organs and systems, wound repair is a relatively uniform response to various forms of tissue injury that derive from inflammation, hypoxia, diabetes, or hypertension [1, 3]. Numerous molecular factors involved in tissue injury have been shown to directly induce TGF-β overexpression [4, 5]. These factors include immune complexes, free radicals, chemokines, high glucose levels, advanced glycosylated end products, ischemia, mesangial cell stretch, protein trafficking, basic fibroblast growth factor, and TGF-β itself. TGF-β is unique in its ability to promote matrix accumulation by simultaneously increasing synthesis of matrix proteins, decreasing degradation of matrix proteins, and increasing expression of cell matrix receptors called integrins [1, 2]. The key role of TGF-β in acute and chronic tissue repair has been highlighted by experimental studies demonstrating that specific antagonism of TGF-β largely prevents matrix expansion in both acute and chronic fibrotic disease of the kidney [6, 7].

As is the case for other tissues, matrix expansion in the glomerulus is a temporally and spatially highly coordinated process, and closely directed toward the site where the initial injury has occurred. This orchestration involves a well-ordered change in the gene expression of resident glomerular cells, such as mesangial cells, podocytes, and endothelial cells, as well as a coordinated glomerular localization of blood cells (i.e., macrophages, lymphocytes, and platelets) to the site of action [1, 5, 8, 9]. While in recent years major progress has been made in the understanding of the molecular mechanism underlying glomerular matrix expansion [1, 8], and more recently, of the role of infiltrating macrophages [9], the contribution of platelets to this process is less defined by far. This is in contrast to tissue repair processes in other organs, such as dermal wounding and both acute and chronic occlusive vascular disease, in which a profibrotic role of platelets has been well established [10, 11].

In order to further characterize the role of platelets in renal matrix expansion, we tested the effect of the recently clinically introduced platelet inhibitor clopidogrel on acute anti-thy1 glomerulonephritis, a rat model of acute glomerular wound repair. Clopidogrel is structurally a thienopyridine derivate and specifically inhibits ADP-dependent platelet aggregation and adhesion via inhibition of the purinergic P2Y12 receptor [12, 13], and, unlike aspirin, has no additional effects on prostaglandin metabolism and actions [14, 15]. In two separate protocols, actions of platelet inhibition on early, anti-thy1 antibody- and nitric oxide (NO)-mediated mesangial cell injury, and subsequent TGF-β overexpression and matrix accumulation were determined (day 1 and day 6 after antibody injection, respectively). In both protocols, the effect of clopidogrel was compared side by side with the ACE inhibitor enalapril.

METHODS

Materials

Unless otherwise indicated, materials, chemicals, or culture media were purchased from Sigma Chemical-Aldrich Co. (Taufkirchen, Germany).

Animals

Male Sprague-Dawley rats (180 to 250 g) obtained from Charles River (Sulzfeld, Germany) were fed a normal protein diet (22.5% protein, Altromin, Lage, Germany) for at least three days before the start of the experiment to allow equilibration. Body weight was determined at the beginning and end of each experiment. Food and water intake were monitored. Animal care and treatment were in conformity with the guidelines of the American Physiological Society and approved by local authorities. Animals were housed in a constant temperature room with a 12-hour dark/12-hour light cycle.

Induction of acute anti-thy1 glomerulonephritis

Acute anti-thy1 glomerulonephritis was induced by tail vein injection of the monoclonal antibody OX-7 [1 mg/kg body weight in phosphate-buffered saline (PBS)] as previously described [16]. In the kidney, OX-7 binds to a thy1-like antigen on the surface of mesangial cells and causes complement- and NO-dependent cell lysis [17]. Control animals were injected with equal volumes of PBS only. OX-7 antibody was produced from a hybridoma cell line as previously described [16]. The antibodies were diluted in PBS (pH 7.4) and stored at −70°C until use.

Drug administration

Both clopidogrel and enalapril were given with food. The drug-containing food was produced in our laboratory by using the flour of the standard rat chow (22.5% protein, A1311, Altromin). Clopidogrel or enalapril was mixed into the dry food flour in appropriate amounts, water was added to form pellets, and the air-dried pellets were subsequently given to the animals.

Clopidogrel, chemically the hydrogen sulfate salt of the S enantiomer of methly [2(2-chlorophenyl)-2[4,5,6,7-tretarhydrothieno(3,4-c)pyridine-5-yl]acetate, was given in a daily dose of 10 mg/kg body weight. This dose has previously been reported to maximally inhibit platelet aggregation and thrombus formation in vitro and in vivo [18]. The ACE inhibitor enalapril was used in a dose of 50 mg/kg body weight. In previous studies, this dose was maximally effective in reducing TGF-β overexpression during the repair phase of acute anti-thy1 glomerulonephritis [16].

Experimental design

In the model of acute anti-thy1 glomerulonephritis, the effects of the platelet inhibitor clopidogrel and the ACE inhibitor enalapril were determined separately on early mesangial cell lysis (injury phase, day 1, protocol 1) and on the subsequent TGF-β1 overexpression and matrix accumulation (matrix expansion phase, day 6, protocol 2). In protocol 1 (injury), treatment was started five days before and continued until 24 hours after injection of anti-thy1 antibody. Here, glomerular cell number and inducible NO production were analyzed as estimates of mesangial cell injury. In protocol 2 (repair), treatment was started one day after and continued until day 6 after antibody administration. On day 6, parameters of glomerular repair (glomerular matrix score and expression of TGF-β1, fibronectin, and PAI-1 protein) were determined.

Protocol 1

Effect on the injury phase of acute anti-thy1 glomerulonephritis (day 1 after antibody injection). Five days before antibody injection, Sprague-Dawley rats were assigned to the following groups: (*1*) PBS-injected control animals ($N = 4$, control); (*2*) anti-thy1 antibody-injected animals, no treatment (GN, $N = 8$); (*3*) anti-thy1 antibody-injected rats plus clopidogrel (GN + Clopi, $N = 7$); and (*4*) anti-thy1 antibody-injected rats plus enalapril (GN + Ena, $N = 8$).

One day after antibody injection, the histologic degree of mesangial cell lysis, the release of basal and LPS-stimulated nitrite of cultured glomeruli, and glomerular macrophage infiltration were analyzed. At this point, mesangial cell lysis is complete and inducible glomerular NO production is markedly increased [19]. Previous investigations in anti-thy1 glomerulonephritis have shown that glomerular binding of the antibody is

not affected by platelet inhibition or enalapril treatment [20, 21].

Protocol 2

Effect on the repair phase of anti-thy1 glomerulonephritis (day 6 after antibody injection). One day after antibody injection, when the mesangial cell lysis had occurred and the fibrotic response had started [19], Sprague-Dawley rats were assigned to the following groups: (*1*) PBS-injected control animals ($N = 4$, control); (*2*) anti-thy1 antibody-injected animals, no treatment (GN, $N = 8$); (*3*) anti-thy1 antibody-injected rats plus clopidogrel (GN + Clopi, $N = 8$); and (*4*) anti-thy1 antibody-injected rats plus enalapril (GN + Ena, $N = 8$).

Six days after disease induction, expression of the key fibrosis mediator and marker TGF-β served as principal therapeutic target. To document that TGF-β expression reflected actual matrix accumulation, a histologic glomerular matrix score was used. In addition, renal expression of the matrix protein fibronectin was measured as an indicator for matrix protein production. PAI-1 was used as sensitive marker of the matrix degrading system. In acute anti-thy1 glomerulonephritis, the fibrotic response peaks 6 days after antibody injection and provides a large difference between normal and disease levels [16]. In addition, signs of intraglomerular thrombosis (aneurysm formation and fibrinogen deposition) were determined histologically. In acute anti-thy1 glomerulonephritis, glomerular aneurysms develop as a consequence of mesangial cell lysis and subsequent capillary ballooning and intracapillary thrombosis. Both glomerular fibrinogen deposition and aneurysm numbers were used as indirect indices to show that potential antifibrotic effects by clopidogrel are paralleled by less glomerular platelet localization.

Bleeding time

Rat tail bleeding time was analyzed as previously reported in anesthetized animals with a standardized incision (10 mm long, 1 mm deep) on the dorsal part of the tail [22]. Bleeding time was determined exemplarily in 4 animals of each group of protocol 2 on day 5 after disease induction.

Urine collection and measurement of albuminuria

In protocol 2, a 24-hour urine was collected from each rat the day before sacrifice, using metabolic cages. Albuminuria was measured using a microplate technique and a rabbit antirat albumin peroxidase-conjugated antibody [23]. Albuminuria is expressed as mg protein/24 h.

Sacrifice

At the end of each experiment, animals were anesthetized with ether. Following a midline abdominal incision, 5 to 10 mL blood was drawn from the abdominal aorta, and the kidneys were subsequently perfused with 20 mL ice-cold PBS. For histologic examination, cortical tissue was fixed in 10% neutral buffered formalin.

Production of TGF-β1, fibronectin, and PAI-1 by glomeruli in culture

Glomeruli from individual rats were isolated by a graded sieving technique (160-, 125-, and 71-μm mesh metal sieves) as described previously [16]. Glomeruli were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 U/mL insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin. For stimulation of iNOS, 10-μg lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0127:B8) per mL was added. Glomeruli were cultured at a density of 2000 per mL for 48 hours. In previous experiments, we have shown that the TGF-β1, fibronectin, and PAI-1 production by glomeruli ex vivo is constant over 48 hours and closely correlates the actual glomerular matrix accumulation in vivo [24]. After 48 hours' incubation at 37°C/5% CO_2, supernatants were harvested and stored at −70°C until analysis of TGF-β1, fibronectin, PAI-1, or nitrite content.

Measurement of TGF-β1, fibronectin, and PAI-1

TGF-β1 content of culture supernatant was measured after acid activation using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (TGF-β1 Duoset™; R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. Fibronectin and PAI-1 levels were measured with modified inhibitory ELISA according to published methods [16]. Three samples from each rat were analyzed.

Measurement of nitrite

Nitrite served as indicator of NO production, and was determined by the Griess reaction in glomerular culture supernatants [25]. Briefly, 100-μL of sample was mixed with 100-μL Griess reagent (0.05% N-[1-naphthyl] ethylene diamine dihydrochloride, 0.5% sulfanilamide in 45% glacial acetic acid) in 96-well plates. After 10 minutes' incubation in the dark, absorbance was read at 546 nm in an automated plate reader (MRX II; Dynex Technologies, Frankfurt am Main, Germany). Standard samples were prepared with sodium nitrite.

Glomerular iNOS mRNA expression

Glomerular total RNA was extracted by a guanidinium isothiocyanate method using Trizol™ reagent (Gibco BRL, Berlin, Germany) according to the

manufacturer's instructions. iNOS mRNA expression was determined by a standard relative reverse transcription (RT)-polymerase chain reaction (PCR) method as previously described [26]. The following primer pairs were used: GAPDH: sense CCATCTTCCAGGAGC GAGAT, antisense GATGACCTTGCCCACAGCCT (24 cycles, 59°C); iNOS: sense GCAGAATGTGACCAT CATGG, antisense ACAACCTTGGTGTTGAAGGC (34 cycles, 64°C). PCR products were separated on a 2% agarose gel and analyzed by an imaging densitometer (Typhoon 8600; Amersham Pharmacia Biotech, Buckinghamshire, UK). The density of the bands for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as housekeeping gene. For quantitation purposes, values of iNOS bands were divided by the band density for GAPDH of the same sample.

Light and immunohistochemistry microscopy

All microscopic examinations were performed in a blinded fashion. Three-μm sections of paraffin-embedded tissue were stained with periodic-acid Schiff (PAS). In protocol 1, the number of cell nuclei was counted in 20 glomeruli of 80 to 100 μm diameter from each animal for calculation of mesangial cell lysis [19]. The number of infiltrating macrophages was analyzed in 20 glomeruli of each animal using a primary mouse antibody (Serotec, Hamburg, Germany) and a secondary goat antimouse antibody coupled with the Envision™ staining system (DakoCytomation, Hamburg, Germany). In protocol 2, glomerular matrix expansion was evaluated on PAS-stained slides by rating the percentage of mesangial matrix occupying area in 20 glomeruli from each rat (0% to 100%) [16]. Glomerular cell proliferation was analyzed using a primary mouse anti-proliferating cell nuclear antigen (PCNA) antibody (DakoCytomation) and a secondary goat antimouse antibody coupled with the Envision™ staining system (DakoCytomation). PCNA-positive cells were counted in at least 15 glomeruli of each animal. Furthermore, the percentage of aneurysmatic glomeruli was calculated in at least 20 glomeruli of each rat. Fibrinogen deposition was determined with a primary rabbit antifibrinogen antibody and a secondary goat antirabbit antibody coupled with the Envision™ staining system (DakoCytomation). Glomerular fibrinogen deposition is expressed as the percentage of fibrinogen-positive area in 20 glomeruli from each rat (0% to 100%).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis between the groups was performed by one-way analysis of variance (ANOVA) and subsequent *t* testing with Bonferroni correction for multiple comparison. Data on mRNA expression were analyzed by Mann-Whitney *U* test. A P value < 0.05 was considered significant.

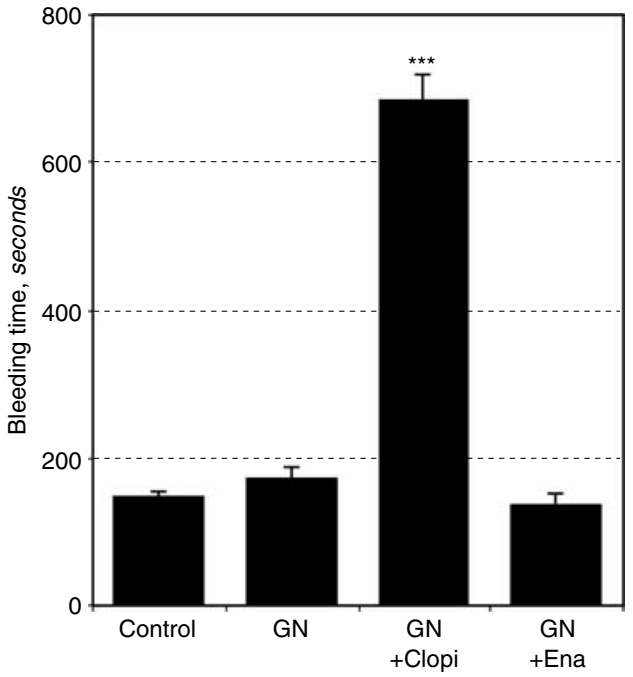

Fig. 1. Tail bleeding time in rats with induced anti-thy1 glomerulonephritis (GN). Shown are the effects of the platelet inhibitor clopidogrel (+Clopi) and the angiotensin-converting enzyme (ACE) inhibitor enalapril (+Ena). Normal control animals (Control) were injected with phosphate-buffered saline (PBS). Bleeding was induced by a standard insertion (10 mm long and 1 mm deep) in anesthetized animals. ***$P < 0.001$ vs. aGN.

RESULTS

Body weight and bleeding time

In protocols 1 and 2, there were no significant differences in body weight at the beginning and at the end of the experiment. Rat tail bleeding time was comparable in normal and disease control rats (146 ± 9 sec and 173 ± 14 sec, P = NS) (Fig. 1). Treatment with clopidogrel prolonged bleeding time as expected (683 ± 37 sec, $P < 0.001$), while enalapril was without a significant effect (136 ± 15 sec, NS vs. control or GN). The increased bleeding time caused by clopidogrel documents the in vivo bioavailability of the drug.

Protocol 1

Effect on the injury phase of acute anti-thy1 glomerulonephritis. Compared with the normal control animals, injection of anti-thy1 antibody resulted in a significantly reduced glomerular cell number (56.5 ± 1.4 vs. 43.0 ± 1.2, $P < 0.001$) (Fig. 2) basal (0.8 ± 0.1 vs. 4.1 ± 0.7 nmol/mL, $P < 0.001$) (Fig. 3A) and LPS-stimulated glomerular NO production (2.9 ± 0.7 vs. 37.7 ± 5.0 nmol/mL, $P < 0.001$)

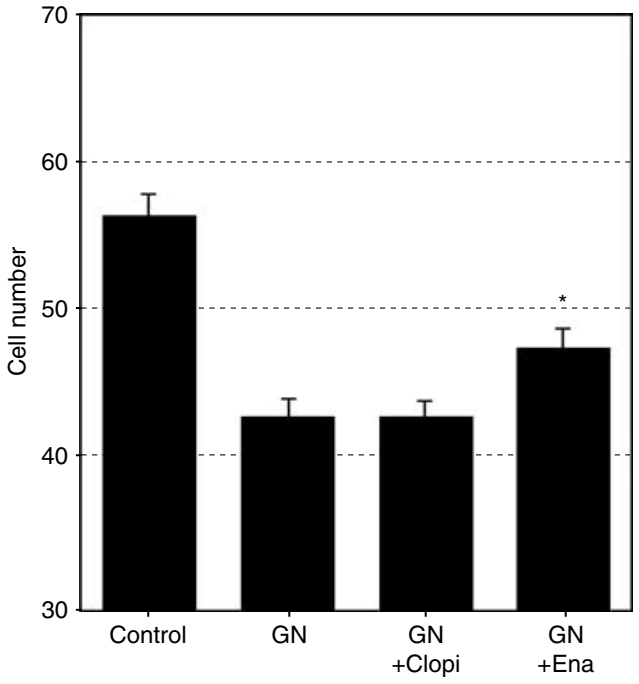

Fig. 2. Glomerular cell number indicating mesangial cell lysis in rats 1 day after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 1, injury). Depicted are the effects of clopidogrel (+Clopi) and enalapril (+Ena). Normal control animals (Control) were injected with phosphate-buffered saline (PBS). $^*P < 0.05$ vs. aGN.

(Fig. 3B), and iNOS mRNA expression (14 ± 4% vs. 100%, $P < 0.001$) (Fig. 3C and D). Treatment with clopidogrel had no significant influence on these parameters (glomerular cell number: 42.9 ± 1.2; basal NO production: 4.7 ± 1.1 nmol/mL; LPS-stimulated NO production 37.4 ± 5.8 nmol/mL; and iNOS mRNA expression 104 ± 15%, P = NS for all parameters). In contrast, administration of the ACE inhibitor enalapril significantly reduced mesangial cell lysis (glomerular cell number: 47.6 ± 1.3, $P < 0.05$ vs. GN), basal NO production (1.1 ± 0.4 nmol/mL, $P < 0.001$ vs. GN), LPS-stimulated NO production (23.7 ± 3.1 nmol/mL, $P < 0.05$ vs. aGN), and iNOS mRNA expression (63 ± 5%, $P < 0.001$ vs. GN). Because macrophages have been described as an important source of glomerular iNOS expression in anti-thy1 glomerulonephritis, the number of infiltrating macrophages was determined 24 hours after disease induction (Fig. 4). Compared with the normal control animals (0.21 ± 0.03), injection of anti-thy1 antibody resulted in a marked increase in the glomerular macrophage number (3.1 ± 0.2). While clopidogrel did not affect glomerular macrophage infiltration (3.0 ± 0.2), the number of positive cells was significantly reduced in the animals treated with enalapril (2.4 ± 0.1, $P < 0.01$).

Together, the results from protocol 1 consistently show that platelet inhibition does not affect early mesangial cell injury in acute anti-thy1 glomerulonephritis, while ACE inhibition significantly limits macrophage infiltration, induction of inducible NO production, and mesangial cell lysis.

Protocol 2

Effect on the repair phase of acute anti-thy1 glomerulonephritis. Six days after injection of anti-thy1 antibody, disease was characterized by a significant increase in albuminuria (95.2 ± 24.2 mg/24 h) (Fig. 5A), glomerular cell proliferation (9.6 ± 0.5 PCNA-positive cells/glomerular cross-section) (Fig. 5B), histologic matrix accumulation (matrix score 78 ± 1.5%), and glomerular production of TGF-β1 (1136 ± 102 pg/mL), fibronectin (16915 ± 1869 ng/mL), and PAI-1 (435 ± 19 ng/mL, $P < 0.001$ vs. control for all parameters) (Fig. 6A-D). In addition, a marked increase in the percentage of aneurysmatic glomeruli (20.0 ± 3.8%) and in the relative glomerular fibrinogen deposition (13.5 ± 2.4%) was observed on day 6 (Fig. 7).

Treatment with clopidogrel decreased albuminuria (51.3 ± 15.9 mg/24 h) (Fig. 5A) and significantly reduced glomerular cell proliferation (6.9 ± 0.8 PCNA-positive cells/glomerular cross-section). Furthermore, clopidogrel administration significantly decreased histology matrix accumulation (matrix score 68.7 ± 3.0%) and glomerular production of TGF-β1 (797 ± 71 pg/mL), fibronectin (12285 ± 1419 ng/mL), and PAI-1 (239 ± 36 ng/mL, $P < 0.05$ vs. GN for all parameters) (Fig. 6A-D). Compared to clopidogrel, enalapril therapy was more effective in reducing albuminuria (26.0 ± 14.2 mg/24 h) (Fig. 5), histologic matrix accumulation (matrix score 58.2 ± 2.7%), and glomerular production of TGF-β1 (504 ± 83 pg/mL) and fibronectin (7500 ± 989 ng/mL, all $P < 0.01$ vs. GN and $P < 0.05$ vs. GN + Clopi) (Fig. 6A-D). Enalapril limited glomerular cell proliferation more effectively than clopidogrel, but this difference did not reach statistical significance (6.0 ± 0.9 PCNA-positive cells/glomerular cross-section) (Fig. 5B). PAI-1 protein expression was slightly but not significantly more reduced by clopidogrel than by enalapril (298 ± 30 ng/mL, both $P < 0.01$ vs. GN) (Fig. 6D). The animals treated with clopidogrel showed a significant reduction in the relative number of aneurysmatic glomeruli (5.8 ± 2.2%, $P < 0.01$ vs. GN) and the score indicating glomerular fibrinogen deposition (2.5 ± 0.9%, $P < 0.01$ vs. GN). Enalapril reduced these parameters as well but in a far less pronounced way and not reaching statistical significance levels (9.8 ± 3.8% aneurysmatic glomeruli, $P = 0.07$ vs. GN, fibrinogen deposition 8.2 ± 3.1%, $P = 0.18$ vs. GN) (Fig. 7). Exemplary pictures of glomerular fibrinogen deposition in each group are shown in Figure 8.

Taken together, the results from protocol 2 show that platelet inhibition significantly limits the fibrotic response

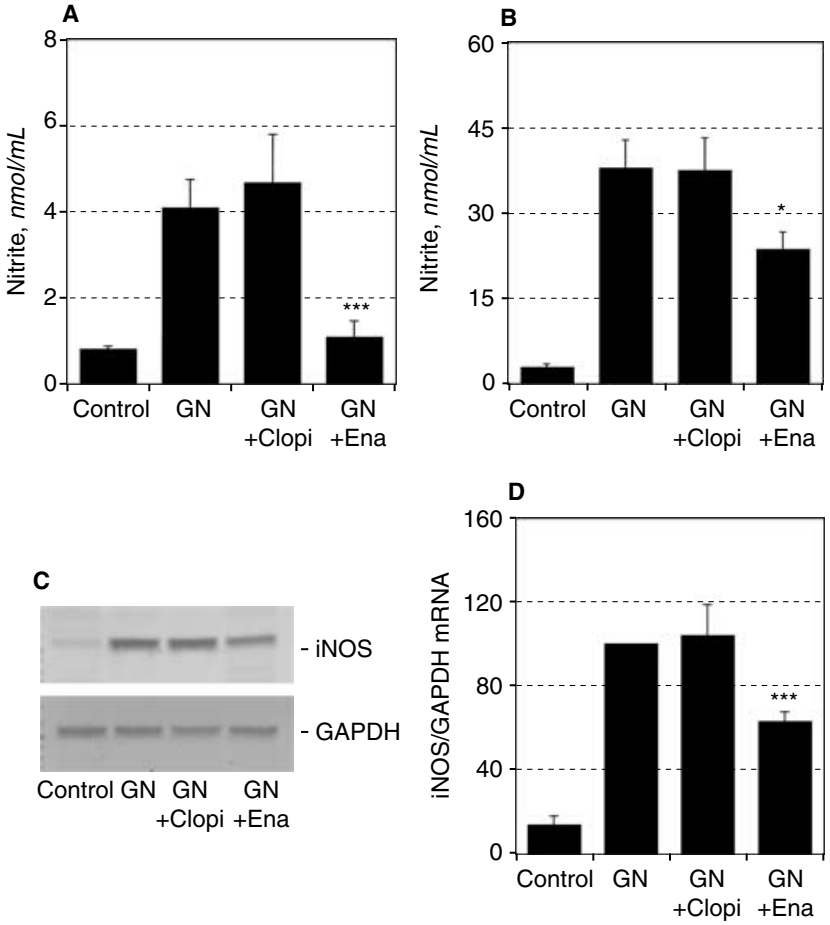

Fig. 3. Indices of glomerular inducible nitric oxide synthase (iNOS) expression (protocol 1, injury). Shown are basal (*A*) and lipopolysaccharide (LPS)-stimulated nitrite production (*B*) of cultured glomeruli (2000 glomeruli per mL and 48 hours) of animals treated with clopidogrel (+Clopi) or enalapril (+Ena). Glomeruli were harvested 1 day after injection of anti-thy1 antibody (GN). Normal control animals (Control) received an injection with phosphate-buffered saline (PBS). A representative iNOS polymerase chain reaction (PCR) gel (*C*) and the densitometric analysis of iNOS mRNA expression corrected for GAPDH (*D*) are shown, respectively. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. aGN.

after induction of anti-thy1 glomerulonephritis. However, the antifibrotic efficacy of clopidogrel appears to be lower than that of the inhibition of RAS, as shown by direct side-by-side comparison to the ACE inhibitor enalapril.

DISCUSSION

Localization of platelets to the site of tissue damage is a key mechanism in the initiation and advance of subsequent wound repair in many tissues and organs, including skin, brain, and heart [2, 10, 11]. The evidence for a profibrotic role of platelets in diseases affecting the glomerular capillaries (i.e., glomerulonephritis and glomerulosclerosis) has mainly been indirect (for reviews see [15, 27]): (*1*) platelets and their degranulation products have been documented in both experimental and human glomerulopathies, such as mesangioproliferative glomerulonephritis, membranous glomerulonephritis, and lupus nephritis; (*2*) shortened biological half-life of platelets, indicating increased platelet consumption, has been demonstrated in patients with various glomerular diseases; and (*3*) platelets contain a number of vasoactive, chemotactic, and profibrotic substances, which, when released within the glomerulus, are likely to amplify the tissue repair process. These substances include platelet-activating factor, platelet secretary products, polycationic macromolecules, platelet factor-4, β-thromboglobulin, various growth factors, and, important in the context of this study, high concentrations the profibrotic cytokine TGF-β.

Using the pharmacologic platelet inhibitor clopidogrel, the present study provides causal evidence that platelets in part mediate overexpression of TGF-β and subsequent matrix accumulation after the induction of experimental anti-thy1 glomerulonephritis. Using cytotoxic antibodies specific to platelets, a previous investigation has shown that platelet depletion decreases early mesangial cell proliferation on day 3 in a similar model of anti-thy1 glomerulonephritis [20]. This finding is now expanded in a number of important ways. Platelet inhibition (*1*) limits subsequent glomerular matrix accumulation in the later course of the disease; (*2*) involves a reduction in the overproduction of the key fibrosis mediator TGF-β; and (*3*) temporally localizes the beneficial effect of platelet inhibition downstream from the initial mesangial cell injury. In addition, this study directly compares the relative contribution of platelets to glomerular matrix expansion to that of

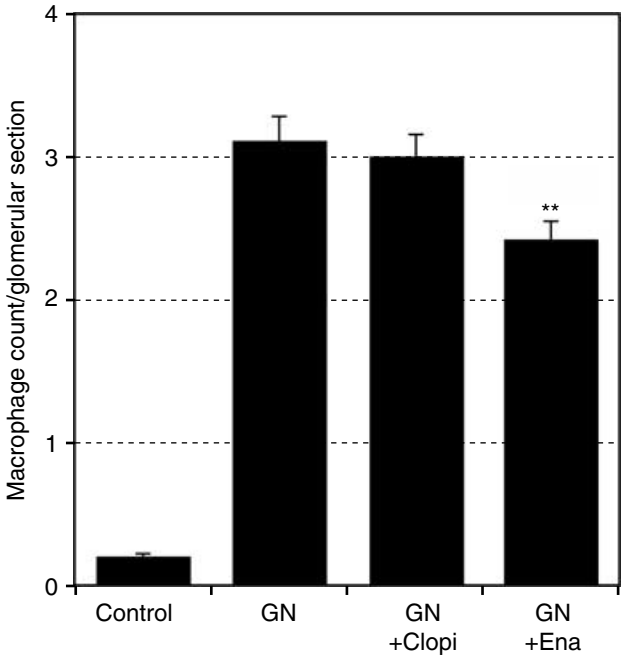

Fig. 4. Macrophage number per glomerular cross-section 1 day after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 1, injury). An ED1 antibody was used for immunohistochemical detection of macrophages. Depicted are the effects of clopidogrel (+Clopi) and enalapril (+Ena). Normal control animals (Control) were injected with phosphate-buffered saline (PBS). $^{**}P < 0.01$ vs. aGN.

the RAS, and expands the beneficial effect of platelet inhibition on glomerular disease into a clinically applicable approach.

In acute anti-thy1 glomerulonephritis, the initial tissue damage is primarily directed toward mesangial cells. However, the finding of increased glomerular thrombus formation and fibrin deposition in day 6 animals strongly indicates that the surrounding glomerular endothelial cells are significantly injured as well. Damage and disruption of the endothelial cell lining and exposure to subendothelial matrix components is a prerequisite for local vascular platelet adhesion and activation [10, 11]. Activation of platelets involves a change of shape, formation of filopedia, and release of alpha and dense granules. Some of the released substances, especially ADP, act directly on other platelets and cause secondary platelet aggregation, leading to fibrin and thrombus formation. In acute anti-thy1 glomerulonephritis, the endothelial cell injury may be caused by direct actions of inflammatory mediators, but the structural consequences of mesangial cell lysis and loss of their normal structural support of the glomerular architecture may play a role as well. The finding of endothelial damage itself is consistent with several recent studies directly documenting a significant loss of endothelial cells and rarefaction of the glomerular capillaries in early anti-thy1 nephritis [28, 29]. Here, the endothelial cell damage was most prominent in glomeruli with large capillary aneurysmal balloonings.

At present, platelet function in human disease may be inhibited by a number of pharmacologic approaches [11, 15, 27]. We chose clopidogrel instead of the clinically more widely used aspirin because its mode of action is more specifically directed toward platelet inhibition. Aspirin acts mainly via cyclooxygenase on the thromboxane pathway, but may have potentially deleterious effects on the production of prostaglandins. In the kidney, especially in chronic renal disease, prostaglandins can be important for maintaining GFR and renal plasma flow, thereby counterbalancing the vasoconstrictive action of angiotensin II [15]. In addition, clinical trials in vascular occlusive disease have suggested that clopidogrel is more effective than aspirin [30, 31]. Inhibitors of the glycoprotein IIb/IIIa receptor were considered as well [11]. The glycoprotein IIb/IIIa receptor is essential for the binding of fibrinogen. However, because clinically effective inhibition of this pathway requires continuous intravenous administration, which restricts its applicability in renal disease, this mode was not used in this study [11]. It may be of interest that another way of anticoagulation therapy, heparin, has shown beneficial effects in fibrotic renal disease as well [32]. Because heparin, which inhibits thrombin via activating antithrombin, ultimately decreases the formation of fibrinogen-platelet aggregates, parts of its beneficial action may be explained by its indirect antiplatelet action.

The antifibrotic action of platelet inhibition documented here in acute glomerular wound repair may be relevant for chronic fibrotic renal disease as well, which in a simplistic perspective may be seen as ongoing pathologic wound repair [1]. Using another clinically introduced ADP-inhibitor, ticlopidine, Zoja et al [33] previously demonstrated a significantly slower progression in rats with surgically reduced renal mass, as shown by proteinuria, GFR, and renal matrix expansion. Given the key role of TGF-β in tissue fibrosis in both acute and chronic renal disease, including renal mass reduction, the findings by Zoja et al imply that a reduction in renal TGF-β expression may be a mechanism of the antifibrotic effect of ticlopidine. In addition, corresponding to the glomerular endothelial damage in anti-thy1 glomerulonephritis, the benefit of ticlopidine may be indicative of a relevant injury of the peritubular endothelial cell layer in this model of progressive interstitial fibrosis.

In the repair protocol, expression of TGF-β served as the main measure of therapeutic efficacy for clopidogrel and enalapril. Thereby, this study contributes to a recent study series comparing and contrasting antifibrotic therapies in anti-thy1 glomerulonephritis, using the same disease model, rat strain, anti-thy1 antibody, harvest times, and measures of fibrosis. This series so far covers ACE inhibition, angiotensin type-1 receptor

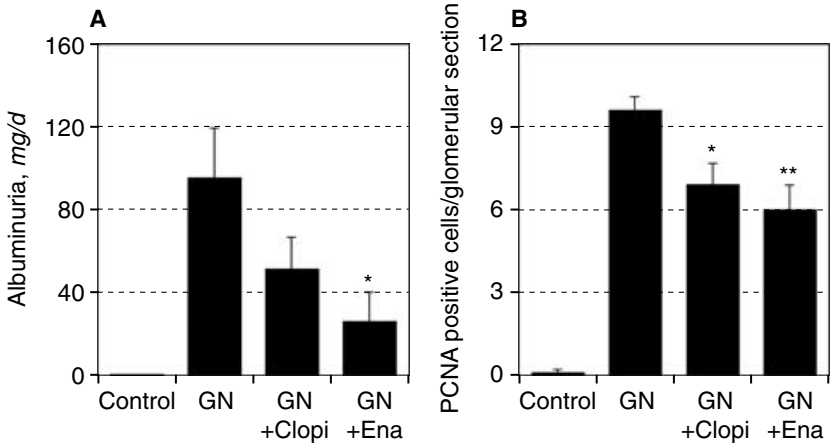

Fig. 5. Urinary albumin excretion (*A*) and proliferating cell nuclear antigen (PCNA)-positive cells per glomerular section (*B*) 6 days after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 2, repair). Treatment with clopidogrel (+Clopi) and enalapril (+Ena) was started 24 hours after disease induction. Urine was collected for 24 hours using metabolic cages. PCNA staining was used as a marker for proliferating cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. aGN.

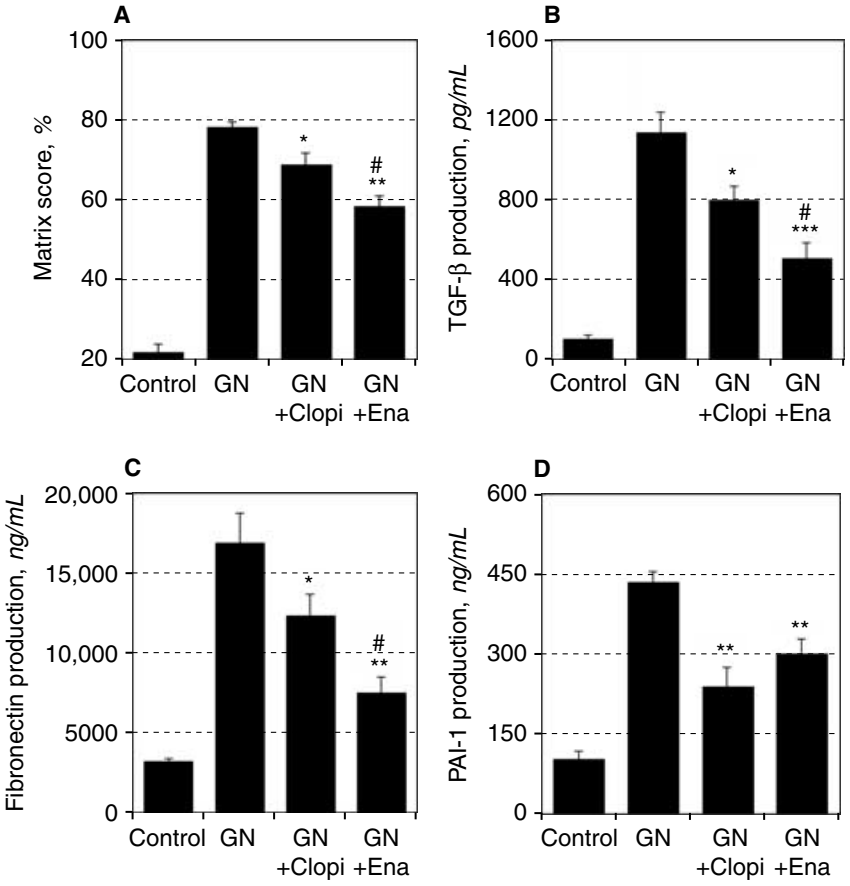

Fig. 6. Markers of glomerular matrix expansion 6 days after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 2, repair). Shown are the glomerular matrix accumulation (*A*) and glomerular production of transforming growth factor (TGF)-β1 (*B*), fibronectin (*C*), and plasminogen activator inhibitor (PAI)-1 (*D*). Control animals received phosphate-buffered saline (PBS) (Control). Treatment with clopidogrel (+Clopi) and enalapril (+Ena) was started 24 hours after disease induction. Glomeruli were harvested from individual animals and cultured at a density of 2000 per mL for 48 hours. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. aGN, $^{\#}P < 0.05$ vs. aGN+Clopi.

antagonisms, adrenergic β-receptor blockade, low-protein diet, and L-arginine supplementation [16, 23, 24, 34]. In the present study, direct side-by-side comparison showed that ACE inhibition with enalapril reduced TGF-β1 overexpression significantly more effectively than clopidogrel. Similar results were observed with regard to proteinuria, actual glomerular matrix expansion, and fibronectin expression. Because both drugs were given in maximally effective high doses [16, 18], and as far as pharmacologically feasible, our results in anti-thy1 glomerulonephritis imply that the relative impact of platelets on TGF-β1 overexpression is not as great as that of the RAS.

In human renal disease, the situation on platelet inhibition is characterized by a general lack of controlled clinical trials with a sufficient number of patients. As early as 1972, benefits from antiplatelet drugs were reported by Kincaid-Smith in a small group of patients with membranoproliferative glomerulonephritis treated with cyclophosphamide, the anticoagulant warfarin, and

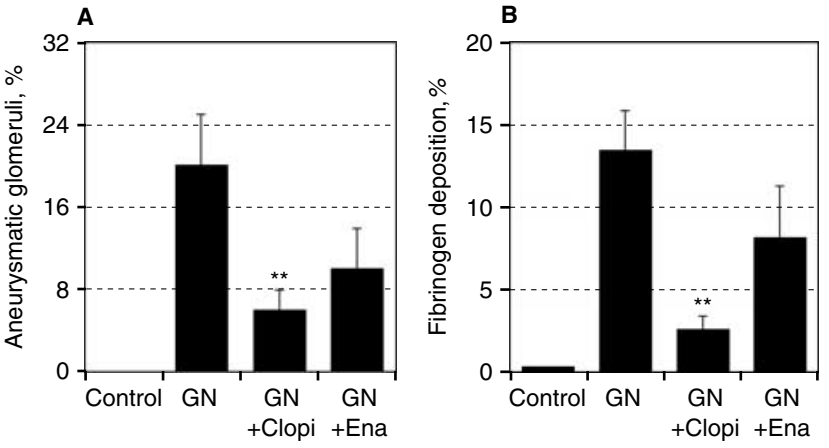

Fig. 7. Markers of glomerular thrombosis 6 days after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 2, repair). Shown are the histologic scores for glomerular aneurysm formation (*A*) and fibrinogen depositions (*B*). Treatment with clopidogrel (+Clopi) and enalapril (+Ena) was started 24 hours after disease induction. $^{**}P < 0.01$ vs. aGN.

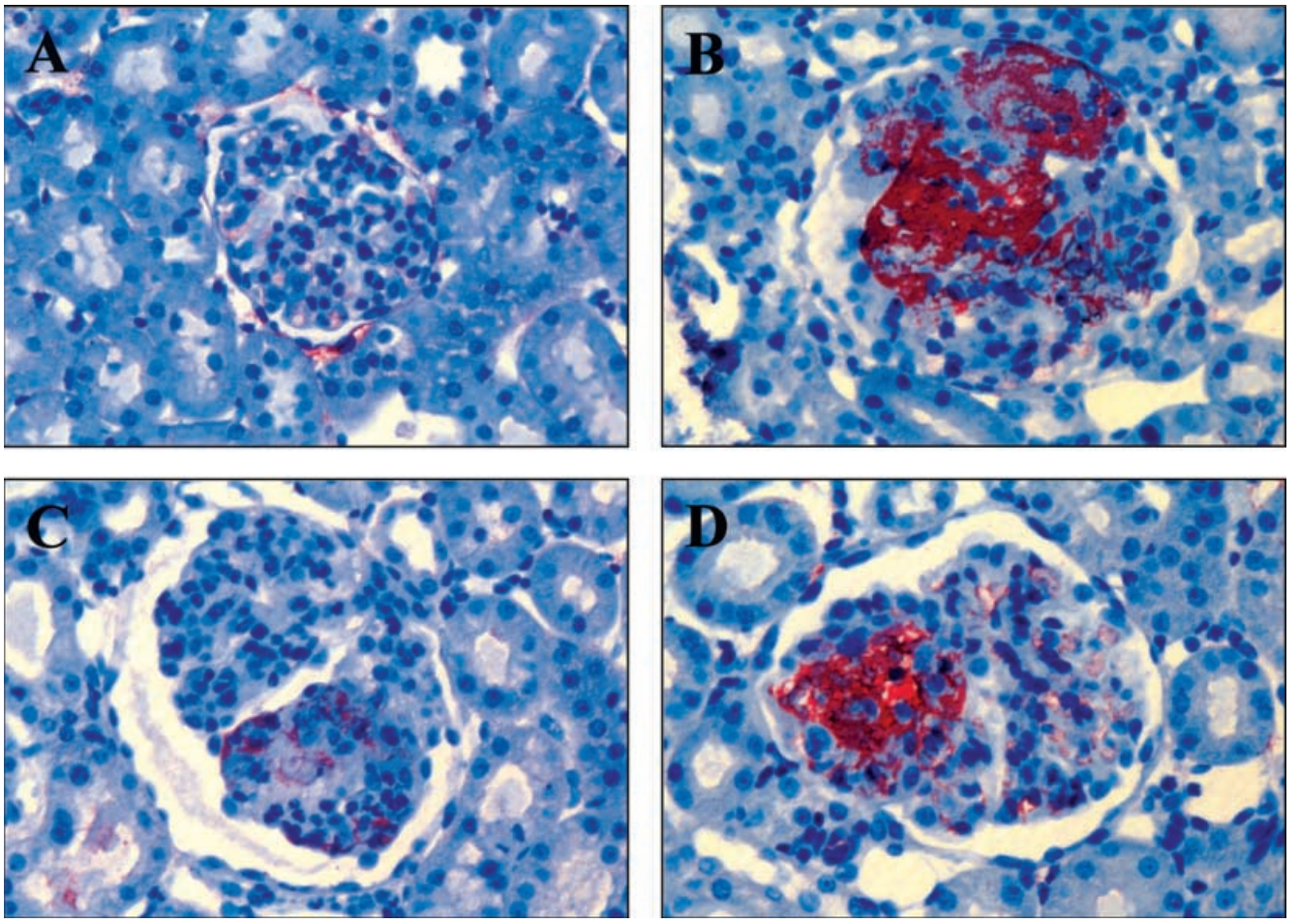

Fig. 8. Glomerular fibrinogen deposition 6 days after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 2, repair). Characteristic photographs show tissue from normal control animals (*A*), anti-thy1 animals without (*B*) or with clopidogrel (*C*) or enalapril (*D*) treatment. Immunohistochemistry was performed using a primary rabbit anti-fibrinogen and a secondary goat anti-rabbit antibody coupled with the Envision™ staining system.

dipyridamonle [35]. Although a few small studies using different antiplatelet and anticoagulant strategies were able to confirm the improvement, others showed no significant benefit [15]. With regard to ADP antagonism, a small study in 11 patients with chronic glomerulopathies may be of interest, in which a two-year course of ticlopidine reduced proteinuria significantly [15]. The antifibrotic benefit of clopidogrel, now demonstrated in acute anti-thy1 glomerulonephritis, underscores the need for control trials. In addition, as a result of the relatively

smaller antifibrotic potential of clopidogrel, our data suggest that larger patient numbers will be needed than in the ACE inhibitor trials.

In this study, actions on mesangial cell injury in anti-thy1 glomerulonephritis were analyzed separately because it has been shown that positive or negative changes in the initial mesangial cell lysis are reflected in opposing changes in the later matrix expansion phase [5, 19]. In the injury protocol, clopidogrel did not affect the initial injury phase; however, enalapril reduced the number of lysed mesangial cells significantly. This benefit went in tandem with reduced iNOS mRNA expression and NO production. Given the critical role of NO for anti-thy1 antibody-induced mesangial cell lysis [17], the data suggest that a reduction in inducible NO production was the mechanism. Part of the decreased glomerular iNOS expression can be explained by the reduced macrophage infiltration, which is an important source of iNOS enzyme in this model. Similar to this study, an anti-inflammatory action of ACE inhibition has previously been demonstrated in chronic, iNOS-mediated lupus nephritis of MRL/lpr mouse [36] [abstract; Perez de Lema et al, *J Am Soc Nephrol* 13:174A, 2002]. Together, these studies are consistent with the concept that angiotensin II, independent of blood pressure, acts as a proinflammatory molecule capable of directly regulating the expressions of nuclear transcription factor-kappaβ, chemokines (MCP-1 and RANTES), and iNOS [37, 38].

CONCLUSION

The present study shows that platelets play a significant role in the sequence from mesangial cell damage to TGF-β overexpression and matrix expansion in acute anti-thy1 glomerulonephritis. Furthermore, the comparison of platelet and ACE inhibition directly side-by-side suggests that the contribution of platelets to TGF-β overexpression is not as great as that of the RAS in this glomerular model of acute wound repair.

ACKNOWLEDGMENTS

This study was supported by a grant from Bristol-Myers Squibb GmbH, Germany, and in part by a grant from the Deutsche Forschungsgemeinschaft (PE 558/2–1). The excellent technical assistance of Ms. Tanja Loof and Ms. Adelina Stössel is highly appreciated.

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