Kidney International, Vol. 60 (2001), pp. 2222–2234

Isotretinoin alleviates renal damage in rat chronic glomerulonephritis

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Background. Retinoids, derivatives of vitamin A, have strong anti-inflammatory and antiproliferative properties. We previously demonstrated that the pan-agonists all-*trans*retinoic acid (RA) and isotretinoin (13-cis RA) alleviate renal damage in rat acute glomerulonephritis (GN) induced by anti-Thy-1.1 mAb OX-7.

Methods. The present study examined the effects of low dose and high dose treatment with isotretinoin in the chronic glomerulonephritis model, Thy-GN. Thy-GN was induced by a single intravenous injection of monoclonal antibody (mAb) 1-22-3 in uninephrectomized Wistar rats ($N = 7$ to 10 per group). Control and nephritic groups were treated with vehicle (veh), low dose isotretinoin (2 mg/kg body wt), or high dose isotretinoin (10 mg/kg body wt). The experiment was terminated 60 days after induction of Thy-GN.

Results. In animals with Thy-GN, isotretinoin abrogated the increase in blood pressure and significantly reduced albuminuria. Glomerulosclerosis index, glomerular and interstitial cell counts, as well as the area of the interstitial space were significantly lower in nephritic rats treated with low and high dose isotretinoin compared to vehicle-treated nephritic controls. Treatment with isotretinoin also significantly reduced the number of glomerular and interstitial macrophages. The increase of transforming growth factor (TGF)-β1, TGF receptor II and preproendothelin-1 gene expression in vehicle-treated nephritic rats was significantly attenuated by isotretinoin.

Conclusions. Treatment with isotretinoin significantly reduces glomerular and interstitial damage in rats with chronic glomerulonephritis as indicated by different functional and histological markers. Retinoids may provide a novel therapeutic option for the treatment of glomerulonephritis.

In the treatment of progressive renal disease, the focus of research has shifted to some extent from blood pressure control toward therapeutic interference with the network of factors that are involved in glomerular cell proliferation, inflammation and extracellular matrix formation [1]. In that respect, retinoids, which are derivatives of vitamin A, exhibit a number of potentially beneficial effects such as antiproliferative, anti-inflammatory, antimigratory and antifibrogenic actions [2–4]. Retinoic acids (RA), unlike retinol (vitamin A), act via specific receptors, that is, retinoid A (RAR) and retinoid X receptor (RXR) with the subtypes α, β, γ. These receptors are expressed in the rat and human kidney [5, 6]. Retinoid receptors belong to the superfamily of ligand-activated nuclear receptors that include vitamin D, thyroid receptor and peroxisome-proliferator-activated receptor (PPAR) [7]. These receptors have a role in kidney development, since double knockout mice with functionally inactive retinoid receptors are characterized by renal aplasia or loss of the renal bud [8–10]. In the adult animal retinoids are involved in cellular differentiation and control of proliferative and inflammatory processes [11, 12]. These receptors regulate expression of target genes directly via binding to retinoic acid response elements [13], which are often located on the promoters of genes involved in extracellular matrix production and degradation. Alternatively, retinoids may act indirectly via inhibition of transcription factors such as activator protein-1 (AP-1) [14, 15], nuclear factor-κB (NF-κB) [16, 17] or cAMP-responsive binding element binding protein (CREB) [18], and others whose roles in renal disease are currently evolving.

We have previously demonstrated that in the early repair phase of experimental mesangioproliferative glomerulonephritis of the rat, all-*trans* RA and isotretinoin limit glomerular cell proliferation, reduce immigration of monocytes, macrophages and reduce the expression of growth factors such as platelet-derived growth factor-B (PDGF B) or transforming growth factor-β1 (TGF-β1) [19, 20]. Retinoids were shown to inhibit the proliferative response of vascular smooth muscle cells to angiotensin II via an activator protein-1 (AP-1) related mecha-

Key words: retinoids, isotretinoin, experimental glomerulonephritis, renal damage, renoprotection, interstitial damage, progressive renal disease.

Received for publication February 7, 2001
and in revised form June 13, 2001
Accepted for publication July 17, 2001

nism [21]. The operation of a similar mechanism in vivo is suggested by the observation that retinoids reduce glomerular c-fos expression in the nephritic kidney. Retinoids significantly reduced renal damage as indicated by a reduced capillary occlusion score and a marked reduction in albuminuria.

Such beneficial effects in an acute model of glomerulonephritis do not provide information, if retinoids are effective when given over a prolonged period of time. To address this issue, we examined the effects of two different doses of isotretinoin in the chronic model of rat mesangioproliferative glomerulonephritis using the monoclonal antibody (mAb) 1-22-3 in uninephrectomized rats [22–25], an established model of chronic mesangioproliferative glomerulonephritis [26, 27].

METHODS

Induction of chronic glomerulonephritis

Male Wistar rats weighing 170 to 190 g (Charles River, Sulzfeld, Germany) were used in this study. The rat anti-Thy 1.1 model of chronic mesangioproliferative glomerulonephritis (Thy-GN) was induced by a single intravenous injection of 0.5 mg mAb 1-22-3 into rats one hour after unilateral nephrectomy [22, 23] . Monoclonal antibody 1-22-3 was dissolved in phosphate-buffered saline (PBS) as previously described [25]. Unilateral nephrectomy was performed under anesthesia by injection of 100 mg/kg body weight (BW) intramuscularly (IM) Ketamine (Ketanest, Parke-Davis, Freiburg, Germany) and 5 mg/kg BW IM Xylazine (Rompun, Bayer, Leverkusen, Germany). A small flank incision was made, the right adrenal gland and renal capsule were separated from right kidney, the right renal artery, vein and ureter were ligated at the renal pedicle and the kidney was removed. All rats were uninephrectomized. Animal experimentation was performed in accordance with the German animal protection laws. The experiment was terminated 60 days after induction of glomerulonephritis. Treatment was started one day after injection of the antibody.

Experimental groups

Animals were randomly allocated to six experimental groups: Group 1, uninephrectomized + vehicle (Control/Vehicle), $N = 7$; Group 2, uninephrectomized + low dose isotretinoin (Control/Iso-low), $N = 6$; Group 3, uninephrectomized + high dose isotretinoin (Control/Iso-high), $N = 7$; Group 4, uninephrectomized + 0.5 mg mAb 1-22-3 + vehicle (Thy-GN/Vehicle), $N = 7$; Group 5, uninephrectomized + 0.5 mg mAb 1-22-3 + low dose isotretinoin (Thy-GN/Iso-low), $N = 9$; Group 6, uninephrectomized + 0.5 mg mAb 1-22-3 + high dose isotretinoin (Thy-GN/Iso-high), $N = 10$.

The low dose of isotretinoin was 2 mg/kg BW per os daily and the high dose was 10 mg/kg BW per day. Treatment was started one day after injection of the antibody.

Galenic preparation of isotretinoin

To improve homogeneity and oxidative stability, isotretinoin (F. Hoffmann-La Roche Ltd, Basel, Switzerland) was first incorporated into a lactose-gelatin granular carrier substance including 5% ascorbic acid (Sigma-Aldrich Chemie, Deisenhofen, Germany) using the wet-granulation method [28]. For preparation of the carrier substance an oscillating damp-granulating machine was applied (Frewitt). Subsequently, the isotretinoin carrier was pressed into standard rat chow (Altr. 1324; Altromin, Lage, Germany). The chow of vehicle rats consisted of a carrier substance including 5% ascorbic acid incorporated into standard rat chow without isotretinoin. The isotretinoin carrier substance was produced with the friendly support of Dr. M. Bultmann of the Institute of Pharmaceutical Technology, University of Heidelberg.

Rat chow was stored in portions, packed in vacuumized light-tight sealed plastic bags at −20°C. Rats were fed after 6 p.m. when lights were turned off in the animal facility.

Animals were pair-fed to ascertain comparable calorie and isotretinoin intakes in nephritic animals and non-nephritic controls and adjusted per os by offering the amount of pellets calculated to deliver the respective dose. The rats had free access to tap water.

Blood pressure measurement

Systolic blood pressure (SBP) was determined on day 0, week 3, week 6, week 7 and week 8 after the induction of nephritis by tail cuff plethysmography under light ether anesthesia. The SBP for each rat was calculated as the average of three separate measurements at each session.

Measurement of urinary albumin and creatinine clearance

For determination of albumin in urine, rats were placed in metabolic cages and urine was collected for 24 hours. Urine was frozen at −20°C until measurement. Albuminuria in rats was determined essentially as in the study of Magnotti et al [29] on a 96-well ELISA-plate using a peroxidase-conjugated anti-rat-albumin-antibody (ICN-Biomedical, Eschwege, Germany). Measurements were performed in quadruplicate.

Creatinine clearance was calculated after enzymatic determination of serum and urinary creatinine (from a 24-hour urine collection 24 hours before the animals were sacrificed; Creatinine Kit, Hoffmann La Roche, Basel, Switzerland) on a Hitachi autoanalyzer.

Processing of renal tissue

For sacrifice, animals were injected with Xylazine (5 mg/kg BW IM; BayerVital, Leverkusen, Germany) and Ket-

amine 10% (100 mg/kg BW IM; WDT, Garbsen, Germany). Rats were saline-perfused containing 0.5 g/L procaine hydrochloride at a defined pressure of 110 mm Hg by retrograde insertion of a cannula into the abdominal aorta [30]. The kidneys were removed immediately, and further processed for histological studies and RNA extraction.

Renal morphology

Tissue for light microscopy was fixed in 10% buffered formalin and embedded in paraffin. Sections of 4 μm thickness were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. The investigator was unaware of the treatment protocol in all morphological determinations.

Glomerular sclerosis index (GSI). A semiquantitative score was used to evaluate the degree of glomerular sclerosis according to the method of Raij, Azar and Keane [31]. The severity of the lesions was examined in 30 glomeruli selected at random, graded from 0 to 4 points according to the percentage of morphological changes on each glomerulus and assigned a score beginning with 0 = 0%, 1+ = 1-25%, 2+ = 26 to 50%, 3+ = 51 to 75%, and 4+ = 76 to 100%. The number of glomeruli showing a lesion of 0 was n_0, of 1+ n_1, of 2+ n_2, of 3+ n_3, of 4+n_4, respectively. Thirty glomeruli were examined independently, and the GSI was obtained by the following formula: $(0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4)/30$.

The tubulointerstitial lesions score was evaluated according to the method of Veniant et al [32]. At least 20 non-overlapping cortical areas were examined using a grid containing 121 fields (Leica, Wetzlar, Germany).

Total glomerular cell count. The total glomerular count was determined in PAS-stained sections in 30 cortical glomeruli per kidney with a diameter of at least 100 μm and the mean number of cells per glomerulus was calculated [33].

Interstitial space and cells. For evaluation of interstitial space and number of interstitial cells PAS staining was performed. Cross-sections were analyzed using a grid containing 121 fields [34, 35]. In each kidney, at least 20 non-overlapping cortical areas of two different sections were evaluated. The proportion of interstitial versus tubular area was quantified and the number of interstitial cells per grid was counted.

Immunohistochemistry

Renal tissue was fixed in 10% buffered formalin (KI-67, fibronectin) or methyl Carnoy's solution (ED-1, collagen I), paraffin-embedded and cut into 4 μm slices.

The primary antibodies were a mouse anti-rat KI-67 antibody (Dianova, Hamburg, Germany), a rabbit-anti-rat fibronectin antibody (Chemicon, Temecula, CA, USA), a mouse-anti-rat ED-1 antibody (Serotech, Oxford, UK) and a goat-anti-rat collagen I antibody (Dianova). Before incubating with primary antibodies against KI-67 microwave pre-treatment was performed in citrate buffer (pH 6.0) for 3 × 3 minutes (750 W). For staining, the labeled avidin-biotin method and 3-amino-9-ethylcarbazole (AEC) as substrate were applied using the Histostain-SP kit (Zymed, San Francisco, CA, USA) according to the manufacturer's recommendations. Sections were counterstained with Mayer's hemalum (Merck, Darmstadt, Germany) and mounted under glass coverslips.

For each biopsy 20 cross sections of consecutive cortical glomeruli with a diameter of at least 100 μm were evaluated. Mean values per glomerular cross sections were calculated for the number of proliferating (KI-67+) cells and monocytes/macrophages (ED-1+). For evaluation of monocytes/macrophages interstitial cross-sections were analyzed using a grid containing 121 fields (Leica, Wetzlar, Germany). In each kidney, at least 20 non-overlapping cortical areas of two different sections were evaluated. In immunoperoxidase stains for fibronectin and collagen I, 20 glomeruli and 20 non-overlapping cortical areas were graded semiquantitatively according to the intensity of immunostaining: no staining (grade 0), faint (grade 1), moderate (grade 2), intense (grade 3) or maximal (grade 4) staining.

RNA isolation and reverse transcription

The Trizol (Life Technologies, Grand Island, NY, USA) method was used for RNA isolation according to the manufacturer's recommendations. RNA was checked for degradation of total RNA on 1% agarose gel. RNA concentrations were determined by spectrophotometric measurements at wavelengths of 260/280 nm. Reverse transcription was performed as described elsewhere [36]. For each biopsy reverse transcription was carried out three times and the resulting cDNA was pooled.

Quantitative polymerase chain reaction assay

Quantification of specific mRNAs was performed essentially as described by Paul, Wagner and Dzau [37] and Wagner et al [36]. For each gene, a DNA deletion mutant was cloned exhibiting the same sequences as the endogenous genes with identical primer binding sites, but a deletion of maximally 20% resulting in a shorter amplification product [38]. Reverse-transcribed (RT) RNA (0.1 μg) was used for amplification in the presence of defined concentrations of DNA deletion mutants as internal standards. The concentration of standard DNA was selected to allow comparable degrees of amplification of wild type and mutant genes. Primers were used for TGF-β1, 5′-CAC CAT CCA TGA CAT GAA CC-3′ (sense primer) and 5′-TCA TGT TGG ACA ACT GCT CC-3′ (antisense), for TGF receptor II, 5′-CTA CAA GGC CAA GCT GAA GC-3′ (sense) and 5′-AGC CAT GGA GTA GAC ATC CG-3′ (antisense) [39], for pre-

pro-endothelin-1 5′-TGG CTT TCC AAG GAG CTC C-3′ (sense) and 5′-GCT TGG CAG AAA TTC CAG C-3′, and for fibronectin 5′-AGG ATT CCG AGT GGA GTA CG-3′ (sense) and 5′-AGG AGG TGT CCA CAT GAT GG-3′ (antisense).

The polymerase chain reaction (PCR) reaction mix contained 0.25 mmol/L deoxynucleoside triphosphate (dNTP; Promega, Madison, WI, USA), 2.5 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 80 nmol/L levels sense and antisense primers (Life Technologies,) and 1 U of Taq DNA Polymerase (GIBCO Life Technologies, Paisley, UK). The thermal profile used consisted of denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one minute carried out 31 times for TGF-β1, 26 times for TGF receptor II, 31 times for prepro-ET-1 and 27 times for fibronectin. In all experiments possible contamination with genomic DNA was excluded by PCR-amplification in absence of reverse transcriptase. Amplification products were separated by agarose gel electrophoresis and then digitized using a gel documentation system (Intas, Göttingen, Germany) and Scion Image (NIH, Bethesda, MD, USA). The ratio between the optical density of the endogenous cDNA and the optical density of the mutant DNA (ODR) was determined. Each sample was measured in triplicate of individual PCR assays for each gene [40].

Statistical analysis

All results are presented as mean values ± SEM. The statistical significance was evaluated by analysis of variance (ANOVA) and Bonferroni's Multiple Comparison Test, and significance was accepted at $P < 0.05$.

RESULTS

Effects of isotretinoin treatment on blood pressure, body weight, creatinine clearance and albuminuria

Throughout the experimental period systolic blood pressure was significantly higher in vehicle-treated glomerulonephritic rats than in non-nephritic control rats (Fig. 1A). Treatment with isotretinoin had no significant effect on blood pressure in non-nephritic controls. Nephritic rats treated with either low or high dose isotretinoin, however, had significantly lower blood pressure levels than vehicle-treated nephritic rats. In isotretinoin-treated nephritic rats blood pressure was not significantly different from control (Fig. 1A).

Body weights of the animals increased throughout the experiment, but were similar in animals treated with low or high dose isotretinoin or vehicle (Fig. 1B).

Creatinine clearance was not significantly changed in vehicle-treated nephritic rats (1.4 ± 0.14 mL/min/kg BW) compared to non-nephritic control rats (1.3 ± 0.2 mL/min/kg BW). Creatinine clearance was not altered in the presence of either low dose (1.2 ± 0.1 mL/min/kg BW) or high dose (1.5 ± 0.2 mL/min/kg BW) isotretinoin treatment in nephritic rats compared to the vehicle-treated group.

After 60 days, 24 hour albuminuria was 5 mg/day in vehicle-treated nephritic rats compared to 0.3 mg/day in non-nephritic controls (Fig. 1C). Similar albuminuria values were obtained at weeks 3 and 5 in vehicle-treated nephritic rats ($P < 0.01$, data not shown). In nephritic animals treated with low or high dose isotretinoin, albuminuria was significantly less (Fig. 1C).

Effects of isotretinoin on glomerular damage

Figure 2 depicts typical PAS-stains of glomeruli of vehicle-treated non-nephritic (Fig. 2A) and nephritic rats that were treated with vehicle (Fig. 2B) or low dose isotretinoin (Fig. 2C). Note the reduction in mesangial cell proliferation and mesangial matrix expansion in isotretinoin-treated rats.

The glomerulosclerosis index was significantly higher in vehicle-treated nephritic rats than in non-nephritic controls. Isotretinoin had no significant effect in non-nephritic controls, but almost normalized the glomerulosclerosis index in nephritic rats treated with either low or high dose isotretinoin (Fig. 3A). The total glomerular cell number was not affected by isotretinoin-treatment in non-nephritic controls as compared to the vehicle group. In vehicle-treated nephritic rats the glomerular cell number was markedly higher and was normalized in nephritic rats treated with either low or high dose isotretinoin (Fig. 3B). Glomerular cell proliferation as indicated by the number of Ki67(+) glomerular cells was slightly, but significantly higher in vehicle-treated nephritic rats, but was not significantly different from controls in nephritic rats treated with either low or high dose isotretinoin (Fig. 3C). Glomerular staining for ED-1 positive cells showed significantly more glomerular ED-1(+) cells in vehicle-treated nephritic rats than in non-nephritic controls. ED-1 (+) cells were significantly less in nephritic rats treated with either low or high dose isotretinoin compared to the vehicle-treated group (Fig. 3D).

Effects of isotretinoin on tubulointerstitial damage

The tubulointerstitial lesions score was slightly, but significantly higher in vehicle-treated nephritic rats than in non-nephritic controls. Treatment either with low or high dose isotretinoin significantly reduced the tubulointerstitial lesions in nephritic rats (Fig. 4A).

Figure 4B indicates that the number of interstitial cells was significantly higher in vehicle-treated nephritic rats compared to non-nephritic controls. Neither dose of isotretinoin had an effect on interstitial cell number in non-nephritic rats, but in nephritic rats treated with low or high dose isotretinoin the interstitial cell numbers were significantly lower compared to those in the vehicle group.

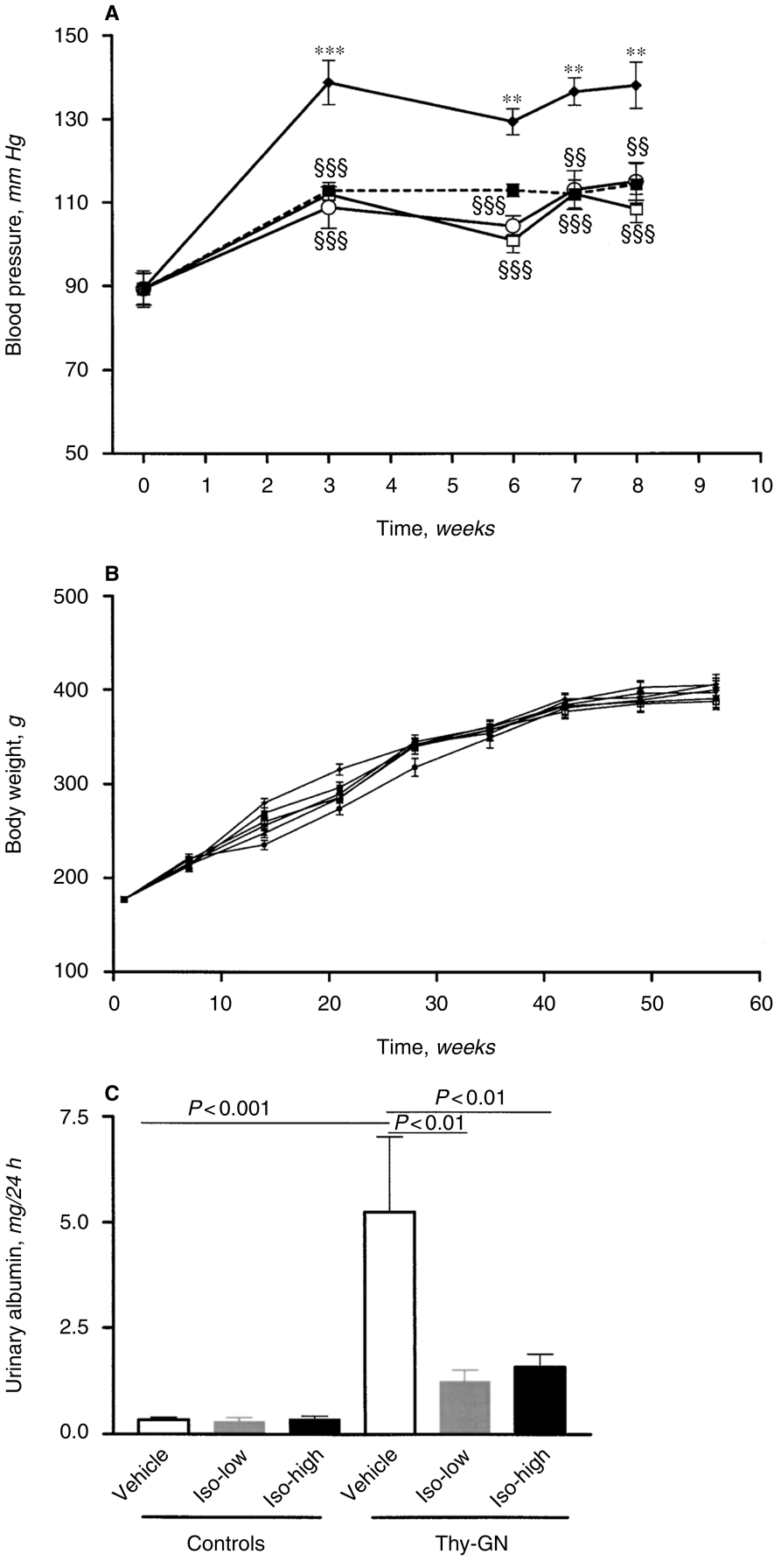

Fig. 1. Effects of isotretinoin treatment on blood pressure, body weight and albuminuria. (*A*) Time course of systolic blood pressure (BP). Isotretinoin treatment prevented the blood pressure increase in anti-Thy1.1-nephritis (Thy-GN). Data are presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Con/Vehicle and §§$P < 0.01$, §§§$P < 0.001$ vs. Thy-GN/Vehicle. Symbols are (◆) Thy-GN/Vehicle; (■) Control/Vehicle; (○) Thy-GN/Iso-low; (□) Thy-GN/Iso-high. (*B*) Time course of body weight. Body weights were measured weekly. No significant differences were found between the groups. Symbols are: (■) Control/Vehicle; (▲) Control/Iso-low; (▼) Control/Iso-high; (◆) Thy-GN/Control; (●) Thy-GN/Iso-low; (□) Thy-GN/Iso-high. Data are presented as mean ± SEM. (*C*) Twenty-four–hour albumin excretion (mg/24 h) at day 60. Treatment of nephritic rats with low and high dose isotretinoin reduces albumin excretion by 77 or 70%, respectively. Similar findings were found at days 21 and 35. Data are presented as mean ± SEM.

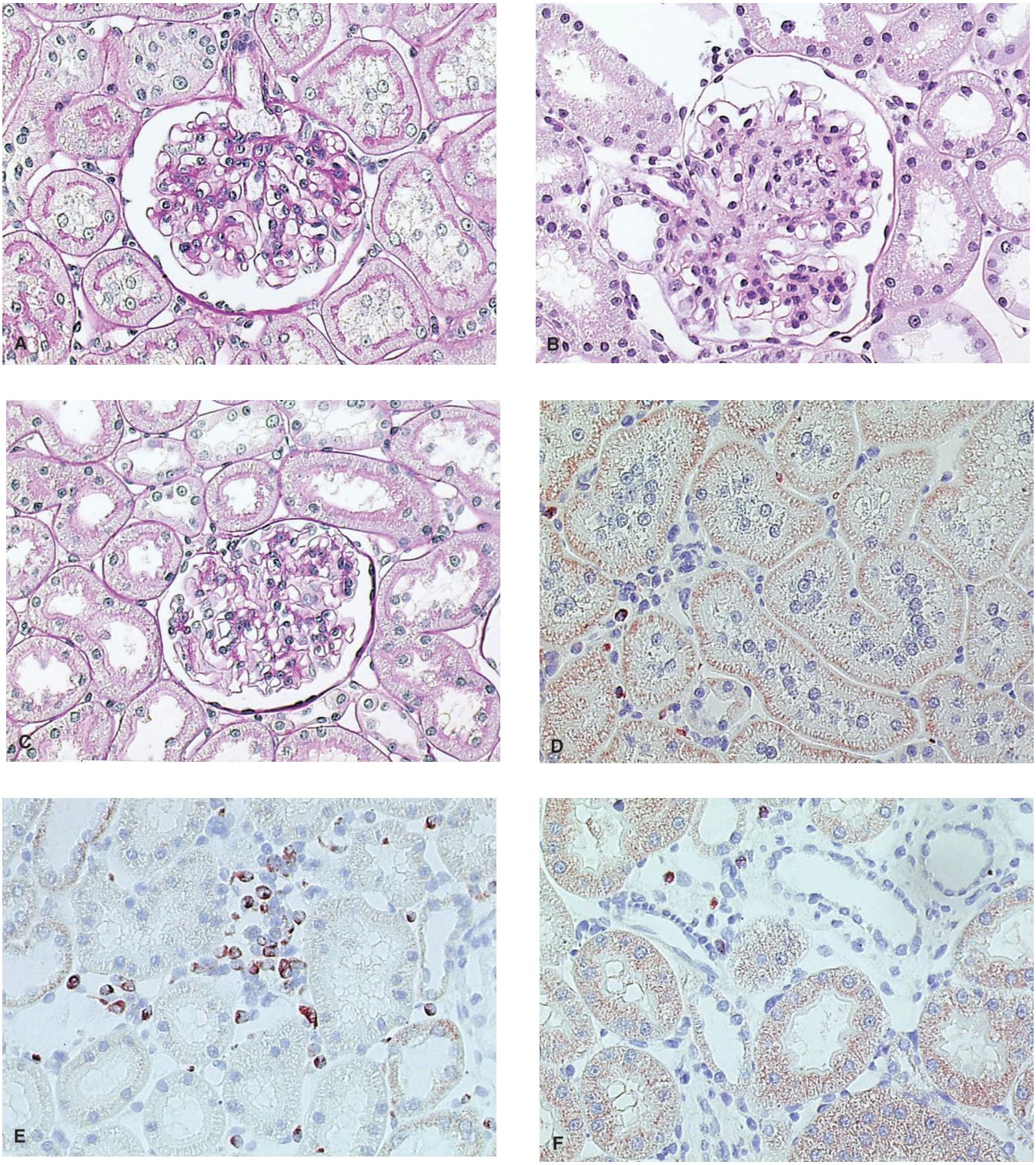

Fig. 2. Representative examples of PAS-stains of glomeruli and immunostaining for monocytes/macrophages (ED-1 positive) in tubulointerstitial areas. Panels A to C show typical PAS-stains of glomeruli of vehicle-treated non-nephritic (*A*) or nephritic rats (*B*) or low-dose isotretinoin-treated nephritic rats (*C*). Note the reduction of mesangial cell proliferation and mesangial matrix expansion in low-dose isotretinoin-treated animals. Panels D to F depict monocytes/macrophages (positive staining for ED-1) in interstitial areas of vehicle-treated non-nephritic rats (*D*) and nephritic rats, which were treated with vehicle (*E*) or low-dose isotretinoin (*F*); ED-positive staining was reduced in nephritic rats with low-dose isotretinoin treatment.

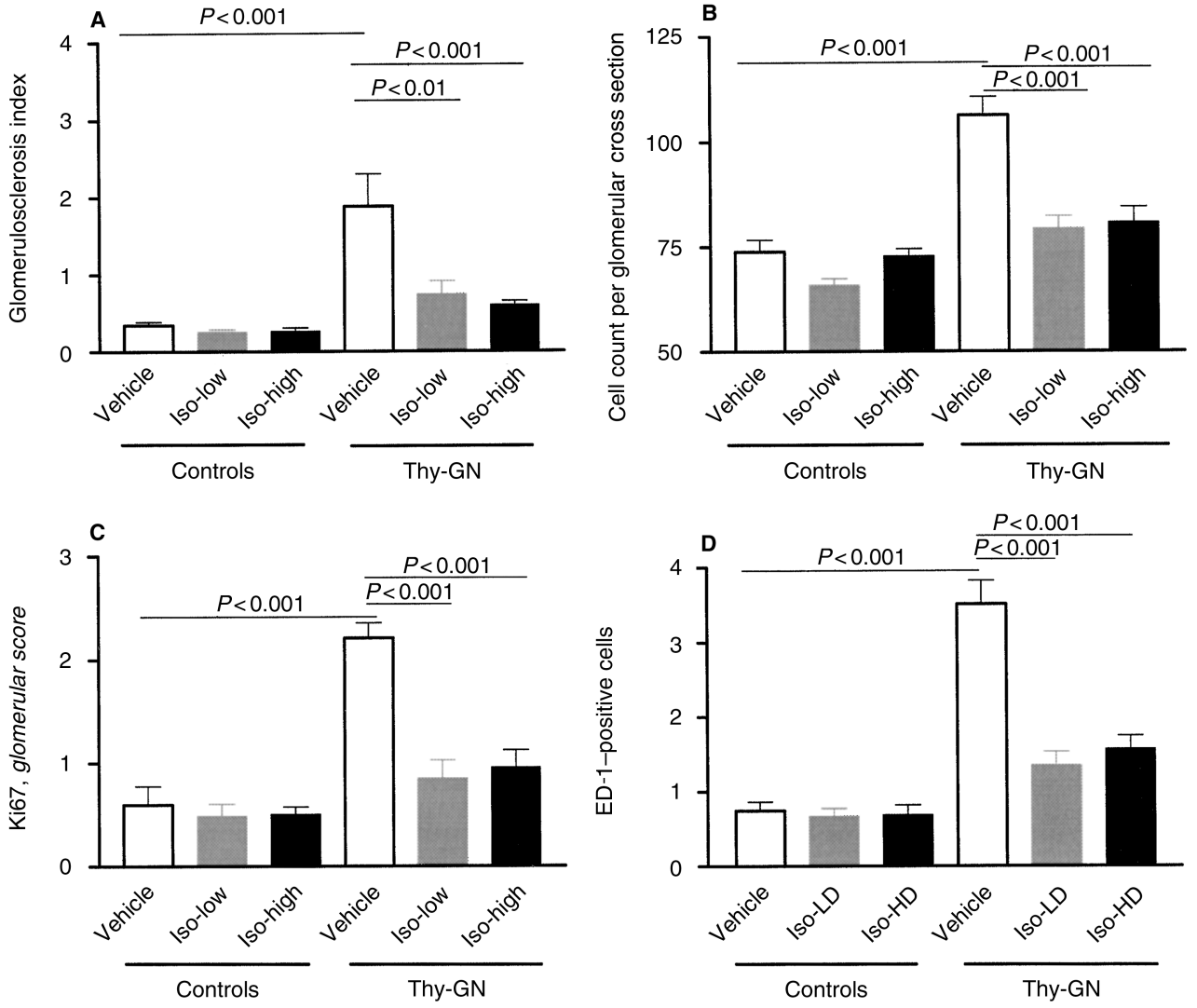

Fig. 3. Effects of isotretinoin on glomerular damage. (*A*) Glomerulosclerosis index (GSI). There was a significant reduction of GSI by treatment with both high and low dose isotretinoin in rats with Thy-GN. The score was not affected in control rats. Data are presented as mean ± SEM. (*B*) Glomerular cell count. Isotretinoin reduced total glomerular cell numbers in rats with anti-Thy1.1-nephritis. Cell numbers were significantly elevated in vehicle-treated nephritic rats as compared to controls. (*C*) Proliferating glomerular cells (Ki67 positive). The number of Ki67 positive glomerular cells was significantly lower in nephritic rats treated with low or high dose isotretinoin than in vehicle-treated rats. (*D*) Glomerular monocytes/macrophages (positive staining for ED-1). In vehicle-treated nephritic rats increased glomerular infiltration by monocytes/macrophages was detected as compared to controls. Infiltration of monocytes/macrophages was significantly lower in nephritic rats with low or high dose isotretinoin.

The area of the renal interstitium (given as percent of grid area) was significantly higher in nephritic rats in comparison to non-nephritic animals. In nephritic rats treated with either low or high dose isotretinoin, interstitial area was significantly smaller (Fig. 4C).

The number of interstitial ED-1 positive cells was significantly higher in nephritic animals as compared to non-nephritic controls. Either dose of isotretinoin almost normalized interstitial ED-1 positive cell counts in nephritic rats (Fig. 4D).

Figure 2 D through F show representative examples of ED-1 immunostains of tubulointerstitial areas in vehicle-treated non-nephritic (Fig. 2D) or nephritic rats (Fig. 2E) or low dose isotretinoin-treated nephritic rats (Fig. 2F).

Effects of isotretinoin on expression of genes involved in renal damage

Renal cortical expression of TGF-β1 was significantly enhanced in vehicle-treated nephritic rats compared to non-nephritic controls. In nephritic animals treated either with low or high dose isotretinoin renal expression of TGF-β1 was almost completely normalized. No effect of isotretinoin treatment on renal TGF-β1 gene expression was observed in non-nephritic controls (Fig. 5A). Simi-

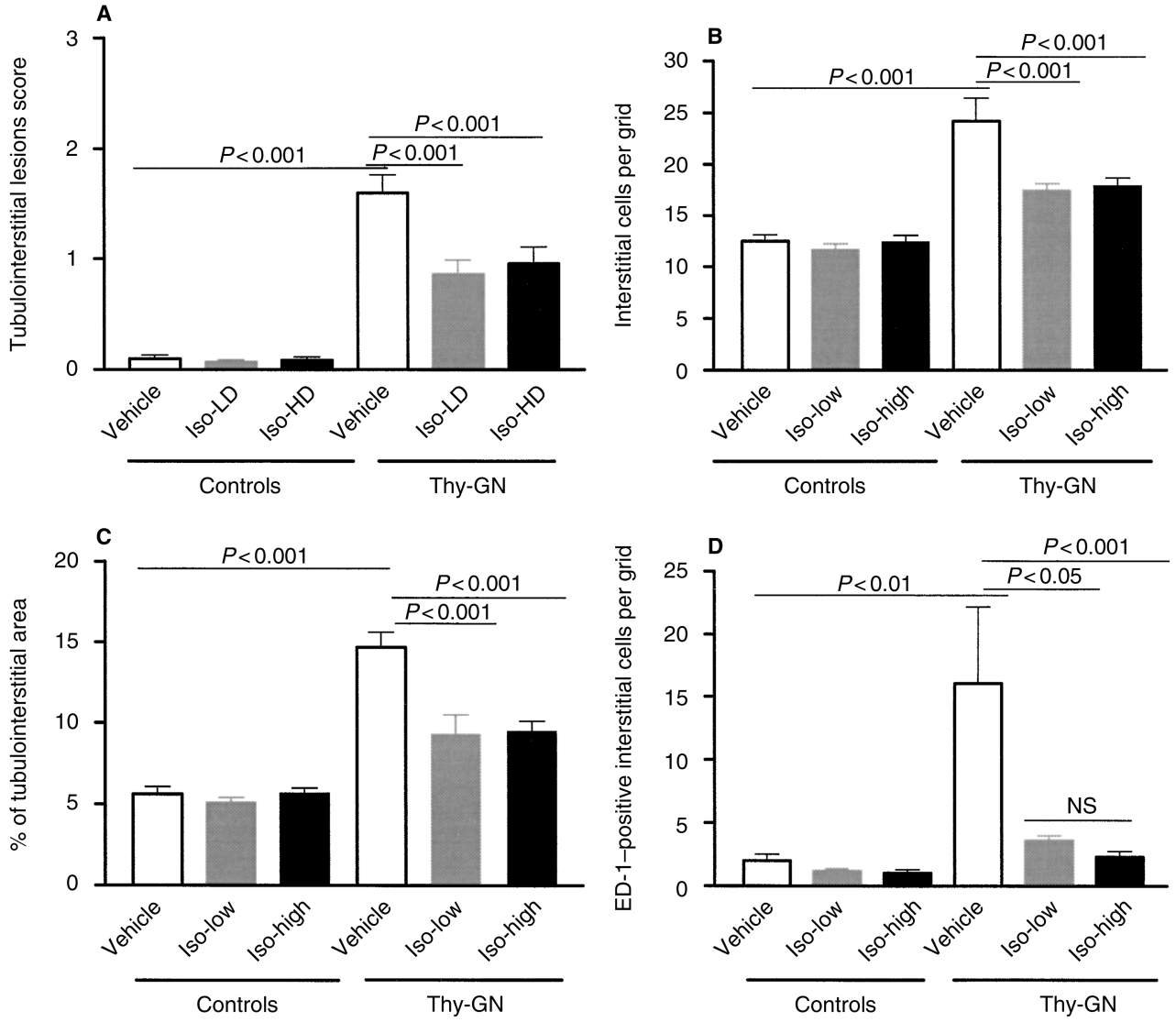

Fig. 4. Effects of isotretinoin on tubulointerstitial damage. (*A*) There was a significant reduction of tubulointerstitial lesions score by treatment with both high and low dose isotretinoin in rats with Thy-GN. The score was not affected in control rats. Data are presented as mean ± SEM. (*B*) Interstitial cell counts were twofold higher in nephritic rats compared to non-nephritic controls. The number of interstitial cells was significantly less after treatment with isotretinoin. (*C*) In the vehicle-treated nephritic group, the interstitial area was significantly expanded compared to non-nephritic controls. In contrast, the interstitial area was significantly smaller in nephritic rats treated with low or high dose isotretinoin. (*D*) Monocytes/macrophages in the interstitium (positive ED-1 staining). The number of interstitial ED-1 positive cells was markedly higher in nephritic rats as compared to non-nephritic controls. Isotretinoin limited the increase in interstitial ED-1 positive cells in nephritic rats.

larly renal cortical TGF receptor II mRNA was higher in the renal cortex of nephritic rats treated with vehicle, than in non-nephritic controls. TGF receptor II gene expression was lower in nephritic rats treated with either low or high dose isotretinoin, but this difference failed to reach statistical significance in the high dose group. No isotretinoin effects were found in non-nephritic controls (Fig. 5B).

Renal cortical prepro-endothelin-1 gene expression also was significantly higher in vehicle-treated nephritic rats compared to non-nephritic controls ($P < 0.001$). Renal prepro-endothelin-1 gene expression was significantly lower ($P < 0.001$) in nephritic rats treated either with low or high dose isotretinoin. The values were comparable to those in non-nephritic vehicle-treated controls (Fig. 5C).

Fibronectin and collagen I immunostaining

Glomerular fibronectin immunostaining was very low in vehicle- and isotretinoin-treated kidneys of non-nephritic rats. Immunostaining was markedly higher in glomeruli of vehicle-treated nephritic rats than in non-nephritic controls. It was less in nephritic rats treated either with low or high dose isotretinoin (Fig. 6A).

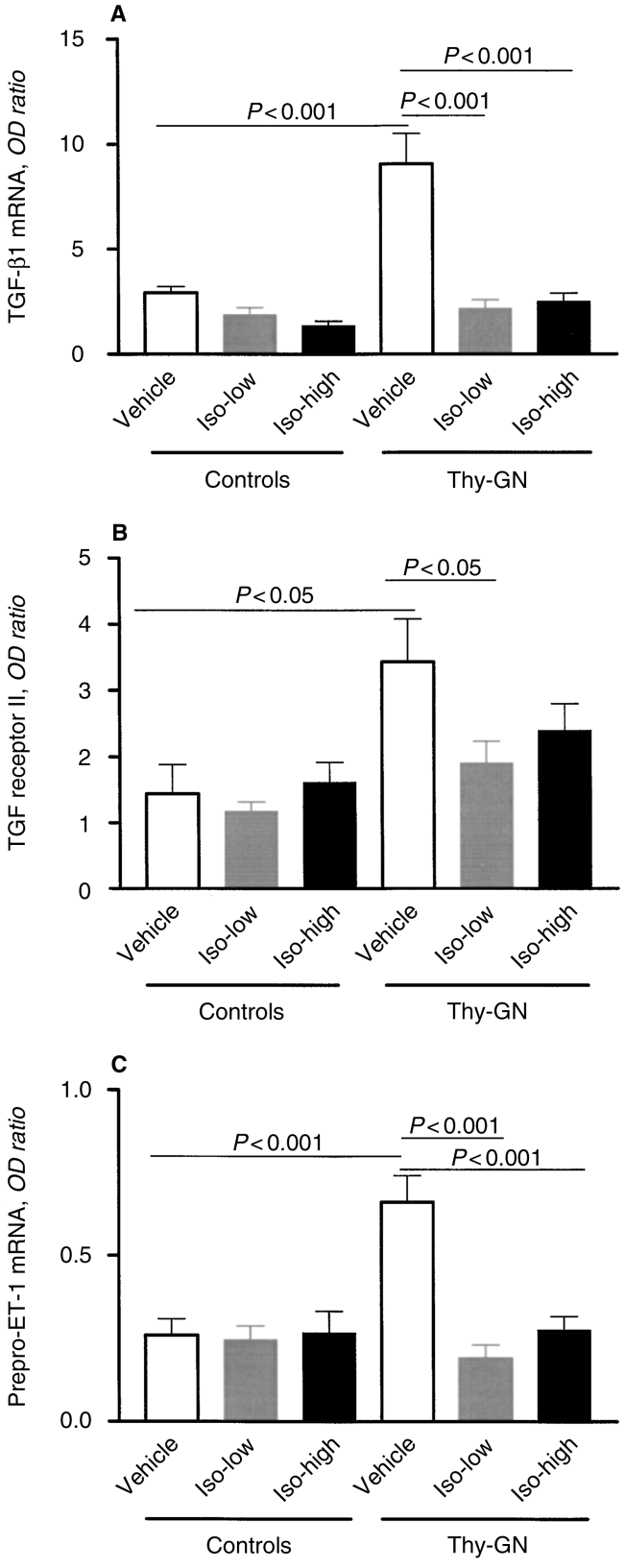

Fig. 5. Renal gene expression of TGFβ-1, TGF receptor II, and prepro-endothelin-1. (*A*) TGF-β1 mRNA in the renal cortex was significantly elevated in nephritic rats compared to non-nephritic controls. Isotretinoin suppressed cortical TGFβ-1 over-expression in nephritic rats. No change of TGFβ-1 expression was observed in control rats. Data are presented as mean ± SEM. (*B*). TGF receptor II mRNA expression in the renal cortex was significantly less in the low dose, but not in the high dose isotretinoin-treated group. TGF receptor II gene expression was significantly higher in nephritic rats in presence of vehicle than that in the respective non-nephritic group. (*C*) Renal cortical expression of prepro-endothelin-1 was significantly higher in vehicle-treated nephritic rats compared to non-nephritic controls. Both high and low dose isotretinoin completely normalized renal expression of prepro-endothelin-1 in nephritic groups.

The immunostaining for fibronectin in the tubulointerstitial space was significantly enhanced in vehicle-treated nephritic rats compared to non-nephritic controls. It was significantly reduced and essentially normalized by treatment with either low or high dose isotretinoin in nephritic rats (Fig. 6B).

Glomerular immunostaining of collagen I protein was markedly higher in vehicle-treated nephritic rats when compared to non-nephritic controls. Collagen I immunostaining was significantly less in glomeruli of nephritic rats treated with either dose of isotretinoin (Fig. 6C).

The tubulointerstitial staining for collagen I was markedly higher in nephritic rats than in non-nephritic controls. Significantly less staining was found in the tubulointerstitial space of nephritic rats after treatment with either dose of isotretinoin (Fig. 6D).

DISCUSSION

The data document that isotretinoin lowers albuminuria and glomerulosclerosis in a rat model of chronic mesangioproliferative glomerulonephritis. These data indicate that retinoids are not only effective in short term, but also in long-term models of inflammatory renal damage, which are more akin to glomerulonephritis in humans.

The model of chronic mesangioproliferative glomerulonephritis in the rat is well established. Cheng et al observed that uninephrectomized rats treated with a single injection of mAb 1-22-3 developed hypertension, massive proteinuria and severe glomerular injury [23]. We confirmed these findings with respect to hypertension and glomerulosclerosis. With regard to albuminuria, we saw no progressive increase and it remained stably elevated over the entire experimental period. In contrast, in the acute nephritic model, urinary albumin excretion rates of up to 30 mg/day were reported [19]. The reasons for the more benign course in the present study are not entirely clear, but may reflect different genetic backgrounds of the experimental animals or different efficiencies of the antibody preparations. The milder course of chronic glomerulonephritis also was reflected by the lack of a reduction in creatinine clearance in vehicle-treated nephritis rats compared to non-nephritic controls, which also has been described for serum creatinine

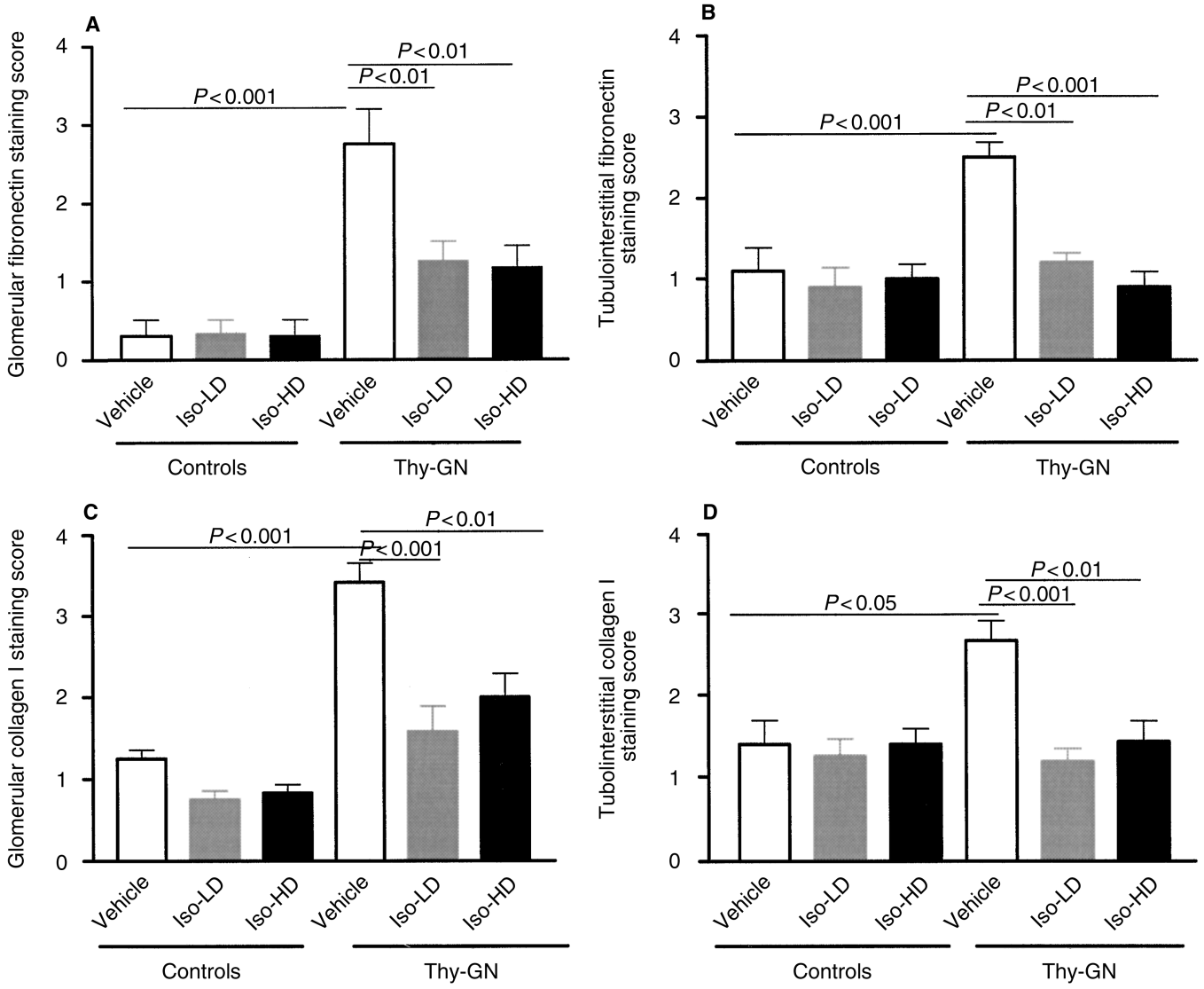

Fig. 6. Staining score for extracellular matrix proteins. (*A*) Glomerular fibronectin immunostaining. Significantly less staining was found in glomeruli of nephritic rats after treatment with either dose of isotretinoin. The staining for fibronectin was markedly higher in nephritic rats than in non-nephritic controls. (*B*) Tubulointerstitial fibronectin immunostaining. Immunostaining was markedly higher in tubulointerstitial areas of vehicle-treated nephritic rats than in non-nephritic controls. It was significantly less in nephritic rats treated either with low or high dose isotretinoin. (*C*) Glomerular collagen I immunostaining. The immunostaining for collagen I in glomeruli was significantly reduced by treatment with either low or high dose isotretinoin in nephritic rats. In vehicle-treated nephritic rats strong staining was found in contrast to the faint staining of vehicle-treated control rats. (*D*) Tubulointerstitial collagen I immunostaining. Tubulointerstitial immunostaining of collagen I protein was markedly higher in vehicle-treated nephritic rats when compared to that in non-nephritic controls. Collagen I immunostaining was significantly less in the tubulointerstitial area of nephritic rats treated with either dose of isotretinoin compared to vehicle-treated nephritic rats.

by Kawachi et al in the chronic model [24]. The decrease in albuminuria by treatment with isotretinoin is not due to a decrease in glomerular filtration rate, since creatinine clearance is not diminished in presence of either dose of isotretinoin both in non-nephritic controls and in nephritic rats. The beneficial effects of isotretinoin are not due to interference with the early immunologic injury: In acute-anti-Thy1.1-nephritis, institution of retinoid treatment 3 days before (pre-treatment) or after injection (post-treatment) of the antibody did not influence the retinoid effects [19].

As in acute anti-Thy1.1-nephritis, isotretinoin reversed the blood pressure increase in vehicle-treated nephritic rats. So far the effects of retinoids on blood pressure have not been reported to our knowledge. The antihypertensive effect is, however, not specific for isotretinoin, since in the anti-Thy1.1-nephritis model all-trans RA or Ro257386, a retinoid X receptor-specific compound, lowered blood pressure as well. The exact mechanism of a retinoid-mediated decrease of blood pressure has not been clarified. The effect may be non-specific and reflect alleviation of renal damage by retinoids. It is of

note however, that even in the absence of renal injury, retinoids lower blood pressure after chronic angiotensin II (Ang II) infusion. We have shown that retinoids lower Ang II type 1 (AT1) receptor binding in vascular smooth muscle and abrogate the increase of glomerular AT1 receptor expression in the acute anti-Thy1.1-nephritis model [21]. Angiotensin II contributes to renal damage in chronic glomerulonephritis induced by mAb 1-22-3 as shown by the beneficial effects of AT1 receptor blockade by candesartan [26]. These findings do not prove, but suggest, that retinoids influence blood pressure not only indirectly by reducing renal damage, but also directly by interfering with the activity of the renin-angiotensin system.

The glomerulosclerosis index was markedly elevated in vehicle-treated nephritic rats [26]. Isotretinoin reduced, but failed to completely normalize, the glomerulosclerosis index. There were no significant differences between high and low doses of isotretinoin indicating that the low dose is as effective as the high dose in treating renal disease. This is of interest, since a dose of 2 mg/kg/day of isotretinoin is in the range of doses that also are used in humans for the treatment of skin diseases [41]. It is not excluded that even lower doses would be effective, potentially improving the benefit/side effect ratio.

It is unlikely that in isotretinoin-treated rats the reduction of renal damage is only due to blood pressure lowering, since in the acute model of anti-Thy1.1-nephritis renal damage was markedly less in retinoid-treated rats, where an effect of blood pressure on renal damage is supposed to be of less importance.

The lower glomerulosclerosis index probably reflects the direct effects of retinoids on glomerular cell proliferation and immigration of inflammatory cells. Isotretinoin significantly reduced the total glomerular cell number. An antiproliferative action of retinoids has been demonstrated in many cell types, which provided the rationale for the use of retinoids in oncology [2, 42]. Apart from malignant cells, retinoids inhibit proliferation of mesangial, vascular smooth muscle and endothelial as well as tubular cells [14, 21, 43]. Inhibition of vascular smooth muscle cell growth by retinoids was reported in restenosis injury after balloon arterioplasty. In acute anti-Thy 1.1 nephritis and in non-inflammatory kidney models such as unilateral ureteral obstruction, antiproliferative actions were seen as well [19, 44]. The lower number of glomerular cells in isotretinoin-treated kidneys is due, at least in part, to inhibition of cell proliferation, since isotretinoin treatment reduces the number of Ki67(+) cells. It is notable, however, that retinoids do not influence the basal cell number in glomeruli as shown by the results in the control groups.

The mechanism of the antiproliferative action of retinoids has not been completely elucidated but probably involves different pathways, such as interference with activator protein-1, or cyclins, or possibly suppression of pro-proliferative growth factors [15]. Retinoids inhibited Ang II or endothelin-1, and α-adrenergic-induced cell growth in cardiac myocytes or vascular smooth muscle cells [21, 45].

The reduction of the number of glomerular monocytes and macrophages may be a further important aspect to explain the beneficial effect of retinoids. Monocytes and macrophages contribute to glomerular damage. They are sources of pro-inflammatory cytokines and growth factors such as TGF-β1 that contribute to the sclerosis in this model [26]. Retinoids have anti-migratory properties [3] and are involved in the monocyte/macrophage differentiation process [46]. A reduction of glomerular ED-1 (+) cells has been demonstrated in the acute phase of anti-Thy1.1-nephritis. The present study in the chronic model suggests (*a*) that monocytes/macrophages play an important role in the chronic phase as well and (*b*) that retinoids interfere with this process.

The chronic model of mAb 1-22-3 glomerulonephritis is characterized by an elevated expression of TGF-β1, a cytokine that is closely associated with the development of glomerulosclerosis and interstitial fibrosis [27]. The increase in renal TGF-β1 and TGF receptor II gene expression found in vehicle-treated nephritic rats was almost normalized by retinoid-treatment. Retinoids have a direct effect on TGF-β1 gene expression via inhibition of AP-1 [47], since the TGF-β1 promoter contains three AP-1 binding sites. Nevertheless, the effects of retinoids depend on the cellular context, since in cell cultures both increased or decreased TGF-β1 expression was seen under the influence of retinoids [48]. In contrast, retinoids consistently inhibit the Ang II-dependent increase in TGF-β1 gene expression in vascular smooth muscle cells [21]. The reduction of TGF-β1 gene expression in the nephritic kidney may be due to a direct suppression of TGF-β1 expression, but also may reflect to some extent the lower number of monocytes and macrophages, since these cells are prominent sources of TGF-β1 [20]. Renal expression of fibronectin-1 and pro-collagen-1 in the nephritic kidney was reduced after isotretinoin treatment, reflecting the lesser degree of interstitial fibrosis and glomerulosclerosis. This also was indicated by the fewer number of tubulointerstitial cells and a reduction in tubulointerstitial lesions. This observation is in line with the previous documentation of a possible antifibrotic action of retinoids [49].

In summary, we have established that in the rat mesangioproliferative glomerulonephritis model, retinoids in doses used in human disease markedly reduce renal damage and normalize both glomerular and interstitial structure. The present study provides further evidence for anti-inflammatory, antiproliferative and antifibrotic actions of retinoids in glomerulonephritis. Although the renal effects of retinoids and the mechanisms of their

action have not yet been examined in detail, the therapeutic efficacy of these drugs in reducing renal damage in experimental models may open interesting perspectives for the treatment of glomerulonephritis.

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REFERENCES

1. Ots M, Pechter U, Tamm A: Characteristics of progressive renal disease. *Clin Chim Acta* 297:29–41, 2000
2. Raffo P, Emionite L, Colucci L, *et al*: Retinoid receptors: Pathways of proliferation inhibition and apoptosis induction in breast cancer cell lines. *Anticancer Res* 20:1535–1543, 2000
3. Wozel G, Chang A, Zultak M, *et al*: The effect of topical retinoids on the leukotriene-B4-induced migration of polymorphonuclear leukocytes into human skin. *Arch Dermatol Res* 283:158–161, 1991
4. Scita G, Darwiche N, Greenwald E, *et al*: Retinoic acid down-regulation of fibronectin and retinoic acid receptor alpha proteins in NIH-3T3 cells. *J Biol Chem* 271:6502–6508, 1996
5. Sugawara A, Sanno N, Takahashi N, *et al*: Retinoid X receptors in the kidney: their protein expression and functional significance. *Endocrinology* 138:3175–3180, 1997
6. Yang T, Michele DE, Park J, *et al*: Expression of peroxisomal proliferator-activated receptors and retinoid X receptors in the kidney. *Am J Physiol* 277:F966–F973, 1999
7. Kliewer SA, Umesono K, Mangelsdorf DJ, Evans RM: Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* 355:446–449, 1992
8. Mendelsohn C, Lohnes D, Decimo D, *et al*: Function of the retinoic acid receptors during development. (II) Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* 120:2749–2771, 1994
9. Mendelsohn C, Batourina E, Fung S, *et al*: Stromal cells mediate retinoid-dependent functions essential for renal development. *Development* 126:1139–1148, 1999
10. Burrow CR: Retinoids and renal development. *Exp Nephrol* 8: 219–225, 2000
11. Bennett RT, Mazzaccaro RJ, Chopra N, *et al*: Suppression of renal inflammation with vitamins A and E in ascending pyelonephritis in rats. *J Urol* 161:1681–1684, 1999
12. Leville CD, Osipov VO, Jean-Claude JM, *et al*: All-trans-retinoic acid decreases cell proliferation and increases apoptosis in an animal model of vein bypass grafting. *Surgery* 128:178–184, 2000
13. De Luca LM: Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J* 5:2924–2933, 1991
14. Simonson MS: Anti-AP-1 activity of all-trans retinoic acid in glomerular mesangial cells. *Am J Physiol* 267:F805–F815, 1994
15. Lin F, Xiao D, Kolluri SK, Zhang X: Unique anti-activator protein-1 activity of retinoic acid receptor beta. *Cancer Res* 60: 3271–3280, 2000
16. Na SY, Kang BY, Chung SW, *et al*: Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFkappaB. *J Biol Chem* 274:7674–7680, 1999
17. Donjerkovic D, Mueller CM, Scott DW: Steroid- and retinoid-mediated growth arrest and apoptosis in WEHI-231 cells: Role of NF-kappaB, c-Myc and CKI p27(Kip1). *Eur J Immunol* 30:1154–1161, 2000
18. Chakravarti D, LaMorte V, Nelson M, *et al*: Role of CBP/P-300 in nuclear receptor signalling. *Nature* 383:99–103, 1996
19. Wagner J, Dechow C, Morath C, *et al*: Retinoic acid reduces glomerular injury in a rat model of glomerular damage. *J Am Soc Nephrol* 11:1479–1487, 2000
20. Morath C, Dechow C, Lehrke I, *et al*: Effects of retinoids on the TGF-beta system and extracellular matrix in experimental glomerulonephritis. *J Am Soc Nephrol* 12(11) 2001 (in press)
21. Haxsen V, Adam-Stitah S, Ritz E, Wagner J: Retinoids inhibit the actions of angiotensin II on vascular smooth muscle cells. *Circ Res* 88:637–644, 2001
22. Shimizu F, Kawachi H, Orikasa M: Role of mesangial cell damage in progressive renal disease. *Kidney Blood Press Res* 22:5–12, 1999
23. Cheng QL, Orikasa M, Morioka T, *et al*: Progressive renal lesions induced by administration of monoclonal antibody 1–22–3 to unilaterally nephrectomized rats. *Clin Exp Immunol* 102:181–185, 1995
24. Kawachi H, Iwanaga T, Toyabe S, *et al*: Mesangial sclerotic change with persistent proteinuria in rats after two consecutive injections of monoclonal antibody 1–22–3. *Clin Exp Immunol* 90:129–134, 1992
25. Kawachi H, Orikasa M, Matsui K, *et al*: Epitope-specific induction of mesangial lesions with proteinuria by a MoAb against mesangial cell surface antigen. *Clin Exp Immunol* 88:399–404, 1992
26. Nakamura T, Obata J, Kimura H, *et al*: Blocking angiotensin II ameliorates proteinuria and glomerular lesions in progressive mesangioproliferative glomerulonephritis. *Kidney Int* 55:877–889, 1999
27. Nakamura T, Obata JE, Onitsuka M, *et al*: Benidipine, a long-acting calcium-channel blocker, prevents the progression to end-stage renal failure in a rat mesangioproliferative glomerulonephritis. *Nephron* 86:315–326, 2000
28. Rudnic E, Schwartz JB: Wet-Granulation Method, in *Remington's Pharmaceutical Sciences* (18th ed), edited by Gennaro AR, Easton, Mack Publishing Company, 1990, pp 1641–1645
29. Magnotti RA Jr, Stephens GW, Rogers RK, Pesce AJ: Microplate measurement of urinary albumin and creatinine. *Clin Chem* 35:1371–1375, 1989
30. Wagner J, Haufe C, Klotz S, *et al*: Accelerated progression of chronic renal failure in transgenic rats carrying an additional renin gene. *J Hypertension* 15:441–451, 1997
31. Raij L, Azar S, Keane W: Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. *Kidney Int* 26:137–143, 1984
32. Veniant M, Heudes D, Clozel JP, *et al*: Calcium blockade versus ACE inhibition in clipped and unclipped kidneys of 2K–1C rats. *Kidney Int* 46:421–429, 1994
33. Floege J, Eng E, Young B, *et al*: Heparin suppresses mesangial cell proliferation and matrix expansion in experimental mesangioproliferative glomerulonephritis. *Kidney Int* 43:369–380, 1993
34. Kaneto H, Morrissey J, McCracken R, *et al*: Enalapril reduces collagen type IV synthesis and expansion of the interstitium in the obstructed rat kidney. *Kidney Int* 45:1637–1647, 1994
35. Ishidoya S, Morrissey J, McCracken R, *et al*: Angiotensin II receptor antagonist ameliorates renal tubulointerstitial fibrosis caused by unilateral ureteral obstruction. *Kidney Int* 47:1285–1294, 1995
36. Wagner J, Gehlen F, Ciechanowicz A, Ritz E: Angiotensin II receptor type 1 gene expression in human glomerulonephritis and diabetes mellitus. *J Am Soc Nephrol* 10:545–551, 1999
37. Paul M, Wagner J, Dzau VJ: Gene expression of the renin-angiotensin system in human tissues. Quantitative analysis by the polymerase chain reaction. *J Clin Invest* 91:2058–2064, 1993
38. Celi FS, Zenilman ME, Shuldiner AR: A rapid and versatile method to synthesize internal standards for competitive PCR. *Nucleic Acids Res* 21:1047, 1993
39. Siegert A, Ritz E, Orth S, Wagner J: Differential regulation of transforming growth factor receptors by angiotensin II and transforming growth factor-beta1 in vascular smooth muscle. *J Mol Med* 77:437–445, 1999
40. Wagner J, Volk S, Haufe C, *et al*: Renin gene expression in human kidney biopsies from patients with glomerulonephritis or graft rejection. *J Am Soc Nephrol* 5:1469–1475, 1995
41. Shalita AR, Cunningham WJ, Leyden JJ, *et al*: Isotretinoin treatment of acne and related disorders: An update. *J Am Acad Dermatol* 9:629–638, 1993
42. Hansen LA, Sigman CC, Andreola F, *et al*: Retinoids in chemoprevention and differentiation therapy. *Carcinogenesis* 21:1271–1279, 2000
43. Paige K, Palomares M, D'Amore PA, Braunhut SJ: Retinol-induced modification of the extracellular matrix of endothelial cells: Its role in growth control. *In Vitro Cell Dev Biol* 27A:151–157, 1991
44. Miano JM, Kelly LA, Artacho CA, *et al*: All-trans-retinoic acid reduces neointimal formation and promotes favourable geometric

remodeling of the rat carotid artery after balloon withdrawal injury. *Circulation* 98:1219–1227, 1998
45. Zhou MD, Sucov HM, Evans RM, Chien KR: Retinoid-dependent pathways suppress myocardial cell hypertrophy. *Proc Natl Acad Sci USA* 92:7391–7395, 1995
46. Kreutz M, Fritsche J, Ackermann U, *et al*: Retinoic acid inhibits monocyte to macrophage survival and differentiation. *Blood* 91: 4796–4802, 1998
47. Morishita R, Gibbons GH, Horiuchi M, *et al*: Role of AP-1 complex in angiotensin II-mediated transforming growth factor-beta expression and growth of smooth muscle cells: using decoy approach against AP-1 binding site. *Biochem Biophys Res Commun* 243:361–367, 1998
48. Jakowlew SB, Cubert J, Danielpour D, *et al*: Differential regulation of the expression of transforming growth factor-beta mRNAs by growth factors and retinoic acid in chicken embryo chondrocytes, myocytes, and fibroblasts. *J Cell Physiol* 150:377–385, 1992
49. Shigematsu T, Tajima S: Modulation of collagen synthesis and cell proliferation by retinoids in human skin fibroblasts. *J Dermatol Sci* 9:142–145, 1995