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ORIGINAL ARTICLE

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Retinoic acid receptor α and retinoid X receptor specific agonists reduce renal injury in established chronic glomerulonephritis of the rat

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Abstract Retinoids, derivatives of vitamin A, inhibit mesangial cell proliferation, glomerular inflammation, and extracellular matrix deposition in acute anti-Thy1.1 glomerulonephritis (Thy-GN) of the rat. We examined a model, chronic mesangioproliferative Thy-GN (MoAb 1-22-3), which is more akin to human disease. Treatment started on day 23 when Thy-GN had already been established. Nonnephritic control and Thy-GN rats were treated orally for 67 days with vehicle or with two doses of either the retinoic acid receptor α-specific agonist AGN 195183 (RARα agonist) or the retinoid X receptor specific agonist AGN 194204 (RXR agonist). Doses of either the RARα or the RXR agonist significantly reduced albuminuria and normalized blood pressure during the course of treatment. The glomerulosclerosis index, glomerular cell and interstitial cell counts, and area of the interstitial space were significantly lower in nephritic rats treated with the RARα agonist or RXR agonist than with vehicle. The RARα and RXR agonist significantly reduced the infiltration of the glomerulus by macrophages. The increase in glomerular TGFβ1 and prepro-ET_1 gene expression in vehicle-treated nephritic rats was significantly attenuated by RARα or RXR agonists. Glomerular expression of RXRα and RARα receptor mRNA was significantly greater in vehicle-treated nephritic rats than in nonnephritic controls. Treatment with RARα or RXR agonists tended to normalize retinoid-receptor gene expression. Our data indicate that both RARα agonists and RXR agonists reduce renal damage in rats with established chronic glomerulonephritis. Receptor-specific retinoids may provide a novel therapeutic approach for the treatment of chronic glomerulonephritis.

Keywords Chronic glomerulonephritis · Retinoids · Retinoid receptor expression

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Abbreviations *AP:* Activator protein · *MoAb:* Monoclonal antibody · *PAS:* Periodic acid–Schiff · *RA:* Retinoic acid · *RAR:* Retinoic acid receptor · *RXR:* Retinoid X receptor · *TGF:* Transforming growth factor · *Thy-GN:* Anti-Thy1.1 glomerulonephritis

Introduction

Retinoids are derivatives of vitamin A which exist as natural retinoid acids or synthetic compounds [1]. Retinoids are used in the treatment of proliferative disorders such as psoriasis and acne conglobata in dermatology and lymphomas, promyelocytic leukemia, and solid tumors in oncology [2, 3, 4, 5]. The natural retinoic acids (RA) exist in different forms, including all-*trans* RA, 9-*cis* RA, and 4-oxo-RA. These retinoid isomers may exhibit receptor-specific binding to so-called RA receptors (RAR) and retinoid X receptors (RXR) with the isoforms α, β, and γ. These receptors are expressed in the kidney and play a role in renal development, particularly formation of the ureteral bud, and codetermine the final number of glomeruli per kidney [6]. The natural retinoids isomerize in vivo and thus preclude analysis of receptor-specific effects. High doses provoke symptoms of hypervitaminosis A. To improve the therapeutic margin synthetic retinoids were developed which exhibit receptor specificity, for example, AGN 195183 with high specificity to the RARα receptor and AGN 194204 with high specificity to RXR receptors.

We have previously demonstrated that natural retinoids such as all-*trans* RA and non-receptor-specific retinoids such as 13-*cis* RA (isotretinoin) significantly decrease renal damage in established models of acute or chronic mesangioproliferative glomerulonephritis in the rat (anti-Thy1.1 glomerulonephritis, Thy-GN) [7, 8]. Retinoids maintained glomerular structure and reduced tubulointerstitial damage through antiproliferative and anti-inflammatory actions [9, 10, 11, 12]. The use of natural or non-receptor-specific retinoids in these models did not allow receptor specific effects to be distinguished. The present study was designed to answer the following questions: (a) Are receptor-specific retinoid agonists similarly renoprotective in experimental glomerulonephritis as natural or non-receptor-specific retinoids? (b) Do RAR agonists differ from RXR agonists with respect to renoprotection? (c) Are retinoid receptor specific agonists effective in established chronic glomerulonephritis?

Material and methods

Experimental protocol

Male Wistar rats weighing 145–150 g (Charles River Sulzfeld, Germany) were used. Monoclonal antibody (MoAb) 1-22-3 was prepared as previously described [13, 14] to induce the rat anti-Thy 1.1 model of chronic mesangioproliferative Thy-GN. Forty rats were intravenously injected with 500 μg MoAb 1-22-3 twice on days 0 and 14 [15, 16]. Prior to the start of the oral therapy on day 23 albuminuria was determined in nephritic rats by 24-h urine collection. The animals were then stratified into the different experimental groups to ensure comparable degrees of albuminuria. The nephritic animals were then divided into five groups (n=8). Treatment was started 23 days after the first injection of Moab 1-22-3. Rats were treated per os as follow:

- Group 1: 2×0.5 mg MoAb 1-22-3 + vehicle (Thy-GN/vehicle)
- Group 2: 2×0.5 mg MoAb 1-22-3 + 4 mg/kg per day AGN 195183, a selective RARα agonist (Allergan, Irvine, Calilf., USA; Thy-GN/AGN 195183-low)
- Group 3: 2×0.5 mg MoAb 1-22-3 + 20 mg/kg per day AGN 195183 (Thy-GN/AGN 195183-high)
- Group 4: 2×0.5 mg MoAb 1-22-3 + 0.4 mg/kg per day AGN 194204, a selective RXR agonist (Allergan; Thy-GN/AGN 194204-low)
- Group 5: 2×0.5 mg MoAb 1-22-3 + 2 mg/kg per day AGN 194204 (Thy-GN/AGN 194204-high)

The five nonnephritic groups (n=6) received injections of phosphate-buffered solution instead of MoAb 1-22-3 and were treated with the same agents described above. The experiment was terminated 90 days after the first injection of MoAb 1-22-3.

Rats were fed after 6 pm when lights were turned off in the animal facility. Animals were pair-fed to ascertain comparable calorie and receptor-specific retinoid intakes in nephritic animals and nonnephritic controls. The dose of retinoids was adjusted by offering the amount of pellets calculated to deliver the respective dose. The rats had free access to tap water. Animal experimentation was performed according to German laws on animal protection.

Galenic preparation of receptor specific retinoids

To improve homogeneity and oxidative stability receptor-specific retinoids were first incorporated into a lactose-gelatin granular carrier substance including 5% ascorbic acid (Sigma-Aldrich Chemie, Deisenhofen, Germany) using the wet-granulation method [17]. For preparation of the carrier substance we used an oscillating damp-granulating machine (Frewitt). Subsequently the receptor-specific retinoid carrier was pressed into standard rat chow (Altr. 1324, Altromin, Lage, Germany). The chow of vehicle rats consisted of carrier substance including 5% ascorbic acid incorporated into standard rat chow without receptor-specific retinoids. The receptor-specific retinoid carrier substance was produced with the friendly support of Dr. M. Bultmann (Institute of Pharmaceutical Technology, University of Heidelberg). Rat chow was stored portionized, packed in vacuumized light-tight sealed plastic bags at −20°C.

Blood pressure measurement

Systolic blood pressure was determined on days 0, 14, 33, 40, 55, 65, 75, and 90 after the first injection of MoAb 1-22-3 by tail cuff plethysmography under light ether anesthesia. The systolic blood pressure for each rat was calculated as the average of three separate measurement at each session.

Measurement of urinary albumin and creatinine clearance

For determination of albumin in urine the rats were placed in metabolic cages, and urine was collected for 24 h. Urine was stored at −20°C until measurement. Albuminuria in rats was determined as described by Magnotti et al. [18] on a 96-well enzyme-linked immunosorbent assay plate using 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 urine collection 24-h before the animals were killed; Creatinine Kit, Hoffmann La Roche, Basel, Switzerland) on a Hitachi autoanalyzer (Hitachi, Frankfurt, Germany).

Table 1 Sequence and GeneBank accession number of the primers used for reverse transcriptase PCR

Gene	Primer sequence (5′→3′)	GeneBank accession no.
TGFβ1	S: CAC CAT CCA TGA CAT GGA CC AS: TCA TGT TGG ACA ACT GCT CC	X 52498 [27]
Prepro-ET-1	S: TGG CTT TCC AAG GAG CTC C AS: GCT TGG CAG AAA TTC CAG C	M 64711 [30]
RARα	S: ATC GAG ACC CAG AGC AGC AG AS: TGT TCT GAG CTG TTG TTC G	X 06614
RARβ	S: TTG CTG TGG AGC AG AS: TCC TCA TGT CAG GCA	AJ 002942
RARγ	S: AAC AAG GTG ACC AGG AAT CG AS: AGA AGG TCA TGG TGT TCT GC	M 34476
RXRα	S: TCC TCA GGC AAG CAC TAT GG AS: GCA TCT TGG ACA CAA GCT CC	X 52773

Processing of renal tissue

At the time of killing animals were injected intramuscularly with xylazine (5 mg/kg BW; Bayer Vital, Leverkusen, Germany) and ketamine (10%, 100 mg/kg BW; WDT, Garbsen, Germany). Rats were saline-perfused with 0.5 g/l procaine hydrochloride at a defined pressure of 110 mmHg by retrograde insertion of a cannula into the abdominal aorta [19]. The kidneys were removed immediately and processed further for histological studies and RNA extraction. Glomeruli were isolated by a fractionated sieving technique as described previously [20]. The yield and purity of isolated glomeruli were comparable between groups (purity greater than 90%).

Renal morphology

Tissue for light microscopy was fixed in 10% buffered formalin and embedded in paraffin. Sections of 4 μm were stained with periodic acid–Schiff (PAS) reagent and counterstained with hematoxylin. The investigator was unaware of the treatment protocol in all morphological determinations. To check reproducibility the same stains were reexamined by a second investigator.

The semiquantitative *glomerular sclerosis index* was used to evaluate the degree of glomerular sclerosis according to the method of Raij et al. [21]. The severity of the lesions was examined in 75 glomeruli selected at random, graded from 0 to 4 points according to the percentage of morphological changes on each glomerulus (0=0%, 1+=1–25%, 2+=26–50%, 3+=51–75%, 4+=76–100%). 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. Seventy-five glomeruli were examined independently, and the glomerular sclerosis index was obtained by the following formula: $(0\times n_0+1\times n_1+2\times n_2+3\times n_3+4\times n_4)/75$.

The *total glomerular cell count* was determined in PAS-stained sections in 60 cortical glomeruli per kidney with a diameter of at least 100 μm [22], and the mean number of cells per glomerular cross section was calculated.

For evaluation of *interstitial area* and *number of interstitial cells* PAS staining was performed. Cross-sections were analyzed using a grid containing 121 fields (Leica, Wetzlar, Germany) [23, 24]. In each kidney at least 30 nonoverlapping cortical areas of two different sections were evaluated. The proportion of interstitial vs. tubular area was quantified, and the number of interstitial cells per grid were counted.

Immunohistochemistry

Renal tissue was fixed in methyl Carnoy's solution, paraffin-embedded and cut into 4-μm slices. The primary antibody was a mouse anti-rat ED-1 antibody (Serotech, Oxford, UK). 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, Calif., USA) according to the manufacturer's recommendations. Sections were counterstained with Mayer's hemalum (Merck, Darmstadt, Germany) and mounted under glass cover-slips. In each biopsy specimen 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 monocytes/macrophages (ED-1$^+$).

RNA isolation and reverse transcription

Total RNA was isolated using the Trizol (Life Technologies, Gaithersburg, Md., USA) method according to the manufacturer's recommendations. The concentrations of total RNA were calculated by spectrophotometric measurements at 260/280 nm wavelength. Reverse transcription was performed as previously described [25].

Quantitative PCR assay

Quantification of specific mRNAs was carried out in triplicates using strand-specific primers as described by Paul et al. [26] and Wagner et al. [25]. Table 1 summarizes the primers used for amplification. For quantification we used deletion mutants of the respective genes sharing the same primer sequences [27]. The results were expressed as ratio of the optical densities of wild-type vs. mutant cDNA signal [27].

Serum analyses

Serum parameters were determined using standard clinical analyzing methods on a Hitachi autoanalyzer.

Statistical analysis

Results are presented as mean ±SEM. The statistical significance of parameters with Gaussian distribution was evaluated using analysis of variance and Bonferroni's multiple comparison test. Parameters with nonnormal distribution were evaluated using the Kruskal-Wallis test and Dunn's multiple comparison test. The null hypothesis was rejected at $P < 0.05$.

Results

Effects of AGN 195183 and AGN 194204 on blood pressure, albuminuria, and creatinine clearance

After injection of the MoAb1-22-3 antibody blood pressure (Fig. 1A) rose significantly compared to non-nephritic controls. The blood pressure decreased after institution of treatment with either AGN 195183 or AGN 194204 in Thy-GN rats but rose further in the vehicle-treated Thy-GN group. The blood pressure decreased and almost normalized at the end of the experiment in the different treatment groups. In contrast, in the Thy-GN/vehicle group systolic blood pressure remained elevated throughout the entire experimental period. AGN 195183 and AGN 194204 did not influence blood pressure in nonnephritic rats (data not shown).

Urinary albumin excretion (Fig. 1B) was elevated in Thy-GN rats on day 16. Albuminuria decreased when Thy-GN rats were treated with either a low or a high dose of AGN 195183 and AGN 194204. It is of note that albuminuria levels were very low on day 40 when blood pressure values had not yet normalized. Albuminuria was minimal in nonnephritic controls and was not influenced by treatment with retinoids. The creatinine clearance was not significantly lower in vehicle-treated nephritic rats (1.9±0.23 ml min^{-1} kg^{-1} BW) than in nonnephritic controls (2.7±0.5 ml min^{-1} kg^{-1} BW). Treatment with AGN195183 or AGN 194204 at neither dose affected the creatinine clearance.

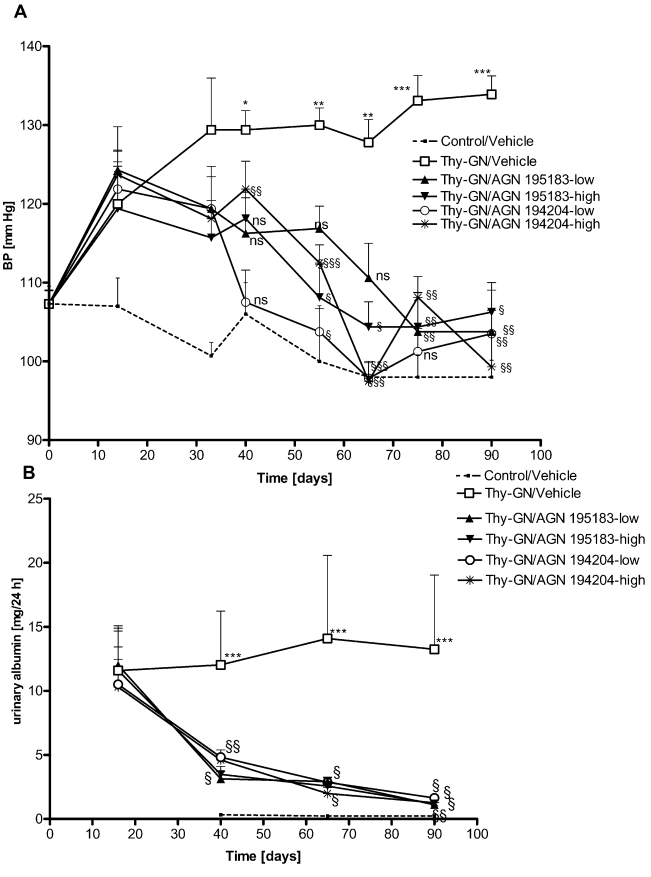

Fig. 1 Effects of AGN 195183 and AGN 194204 on blood pressure and albuminuria. **A** Time course of systolic blood pressure (*BP*). Both low- and high-dose RARα and RXR agonists significantly nearly normalized elevated blood pressures. Data are presented as mean ±SEM. $^{\S}P$ <0.05, $^{\S\S}P$ <0.01, $^{\S\S\S}P$ <0.001 vs. Thy-GN/Vehicle, $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 vs. control/vehicle (Kruskal-Wallis and Dunn's multiple comparison test). **B** Time course of albuminuria. Elevated urinary albuminuria in nephritic rats decreased after institution of treatment with both synthetic retinoid agonists. Data are presented as mean ±SEM. $^{\S}P$ <0.05, $^{\S\S}P$ <0.01, $^{\S\S\S}P$ <0.001 vs. Thy-GN/vehicle, $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 vs. control/vehicle (Kruskal-Wallis and Dunn's multiple comparison test)

Effects of AGN 195183 and AGN 194204 on glomerular expression of retinoid receptors

The expression of glomerular retinoid receptors was differentially regulated in Thy-GN. RARα expression was markedly greater in vehicle-treated nephritic glomeruli than in nonnephritic controls (Fig. 2A). Treatment with either AGN 195183 or AGN 194204 dose-dependently normalized glomerular RARα gene expression. Glomerular expression of RARβ was not significantly greater in vehicle-treated nephritic rats than in controls, but low-dose treatment with either AGN 195183 or AGN 194204 enhanced glomerular RARβ expression (Fig. 2B). Figure 2C demonstrates that expression of RARγ was lower in nephritic glomeruli of vehicle-treated rats than in those of nonnephritic controls. It was markedly lower after administration of AGN 195183 or AGN 194204 in nonnephritic controls. In glomeruli of nephritic rats treated with AGN195183 and AGN 194204 RARγ gene expression was lower only at the high, not at the low, dose (Fig. 2C). Gene expression of RXRα was markedly higher in glomeruli of vehicle-treated nephritic rats than in nonnephritic controls (Fig. 2D). Expression of RXRα was significantly lower after low- or high-dose treatment with AGN 195183 and AGN 194204 (Fig. 2D).

Effects of retinoid agonists on histological markers of renal damage

Figure 3 presents representative PAS stains of vehicle-treated nonnephritic (Fig. 3A) and nephritic rats that were treated with vehicle (Fig. 3B), low-dose AGN 195183 (Fig. 3C), or low-dose AGN 194204 (Fig. 3D). AGN 195183 and AGN 194204 improved the pathological changes such as mesangial cell proliferation and mesangial matrix expansion which were seen in vehicle-treated nephritic rats (Table 2).

The glomerular sclerosis index was markedly higher in vehicle-treated nephritic rats than in nonnephritic controls. After treatment with either AGN 195183 or AGN 194204 the glomerulosclerosis index was signifi-

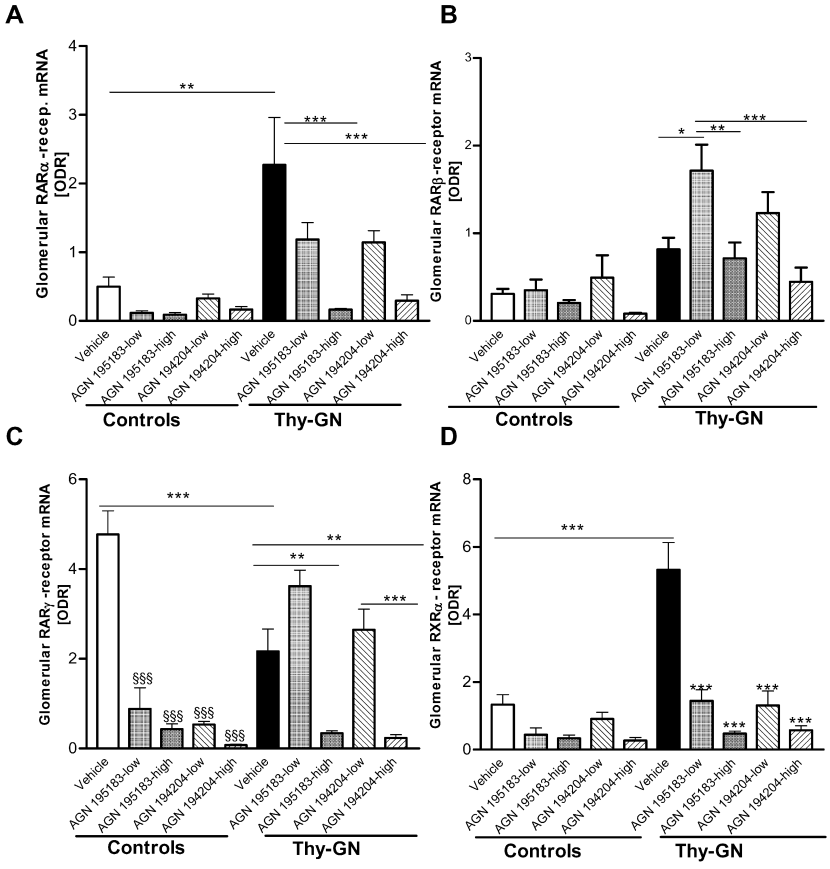

Fig. 2 Glomerular gene expression of retinoid receptors after treatment with AGN 195183 and AGN 194204. **A** Expression of glomerular RARα receptor. RARα receptor mRNA was significantly higher in nephritic rats than in nonnephritic controls. Both AGN 195183 and AGN 194204 dose-dependently suppressed glomerular RARα receptor overexpression in nephritic rats. No significant changes in RARα receptor expression were seen in nonnephritic rats. Data are presented as mean ±SEM. **B** Expression of glomerular RARβ receptor. RARβ receptor expression did not significantly differ between vehicle-treated nephritic rats and controls. In contrast, low-dose treatment with AGN 195183 significantly enhanced glomerular RARβ-receptor expression. **C** Expression of glomerular RARγ receptor. Glomeruli expression of RARγ receptor was significantly lower in vehicle-treated nephritic rats than in nonnephritic controls. Glomerular RARγ receptor mRNA was lower only with high-dose, not with the low-dose, treatment with AGN 195183 and AGN 194204. **D** Expression of glomerular RXRα receptor. RXRα receptor gene expression was significantly higher in vehicle-treated rats than in controls. Both doses of AGN 195183 and AGN 194204 reduced glomerular gene expression of RXRα receptor in nephritic groups

cantly lower. It was similar with the high and the low dose of either compound. There was a trend to AGN 195183 being more effective than AGN 194204, but this difference did not reach statistical significance. The glomerular cell count per glomerular cross-section was significantly higher in vehicle-treated nephritic rats than in nonnephritic controls. It was markedly lower in rats treated with AGN 195183 or AGN 194204. There was no difference between the low and the high doses. The retinoid agonists did not influence glomerular cell count in nonnephritic rats.

The tubulointerstitial area was larger in nephritic rats treated with vehicle than in nonnephritic controls. The area was significantly smaller in rats treated with AGN195183 or AGN 194204 than in vehicle-treated rats. Similarly, the interstitial cell count was less in rats treated with AGN 195183 or AGN 194204 than in vehicle-treated nephritic rats.

Effects of AGN 195183 and AGN 194204 on glomerular inflammation and marker genes of renal damage

The number of monocytes/macrophages per glomerular cross-section was higher in vehicle-treated nephritic rats than in nonnephritic controls. In rats treated with AGN 195183 or AGN 194204 the number of glomerular monocytes/macrophages was almost normalized. The higher doses of AGN 195183 or AGN 194204 lowered glomerular ED-1^+ cells more than the lower doses. Expression of the gene for transforming growth factor (TGF) β_1 was significantly higher in the glomeruli of nephritic rats than in nonnephritic controls. Both AGN 195183 and AGN194204 significantly lowered TGFβ1 gene expression in nephritic glomeruli. The decrease in glomerular TGFβ1 was significantly more pronounced with high dose than with low dose treatment.

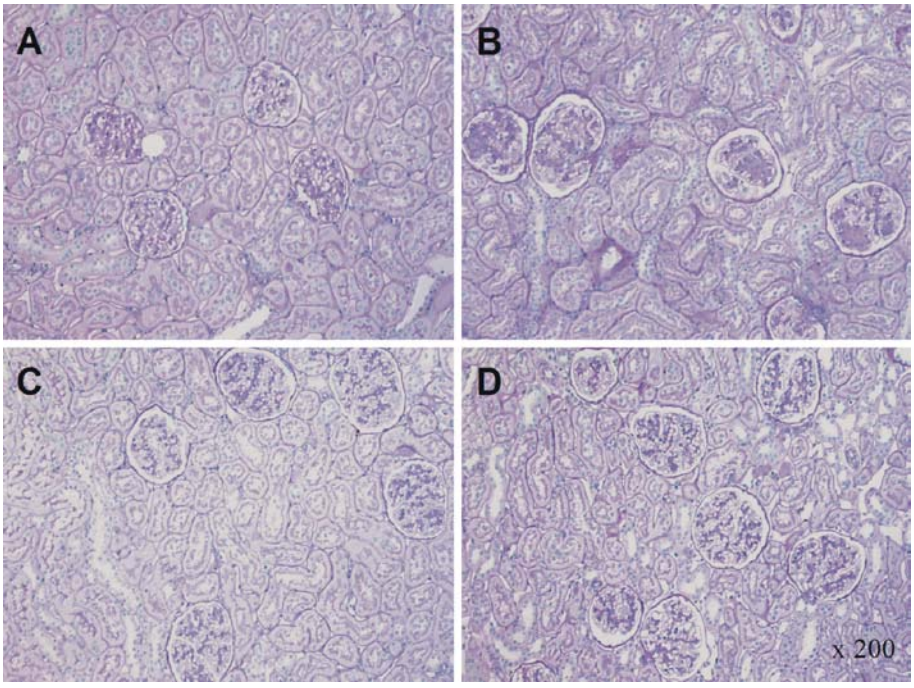

Fig. 3 Representative examples of PAS stains of glomeruli. Typical PAS-stains of glomeruli of vehicle-treated nonnephritic rats (**A**) and nephritic rats that were treated with vehicle (**B**) or low-dose AGN 195183 (**C**) or low-dose AGN 194204 (**D**). Note the reduction in mesangial cell proliferation and mesangial matrix expansion in receptor-specific retinoid-treated animals

Similarly to TGFβ1, glomerular ET-1 gene expression was significantly higher in vehicle-treated nephritic rats than in nonnephritic controls. It was dose-dependently lowered by treatment with AGN195183 or AGN194204.

Effects of AGN195183 and AGN 194204 on serum parameters

There was no difference in the various parameters between vehicle-treated nonnephritic and nephritic rats. High-dose treatment with AGN 195183 raised serum triglyceride levels in both in nephritic rats and non-nephritic controls, whereas the low dose had no effect. No effect on cholesterol levels was observed. In contrast, the high dose of AGN 194204 had no effect on serum triglyceride levels but raised serum cholesterol in nephritic and nonnephritic control animals (Table 3).

Discussion

The main findings of this study are (a) that retinoid agonists reduce renal damage in a model of chronic glomerulonephritis as determined by functional, morphological, and immunohistological parameters, and (b) that there is no clearcut retinoid receptor specific difference with respect to the renoprotective effect.

Systolic blood pressure values decreased progressively in nephritic animals treated with retinoids and were almost normalized at the end of the treatment period. This was seen with both the RARα and with the RXR agonist. The slow and gradual decrease in blood pressure argues against a direct antihypertensive effect of retinoids. It is more likely that blood pressure normalization reflects the gradual improvement in renal damage. An alternative, or complementary, explanation is the known effect of retinoids to reduce the activity of renin-angiotensin system, particularly the expression of AT-1 receptors [28]. It is unlikely that the attenuation of renal damage results only from lowering of blood pressure. Albuminuria as a surrogate marker of glomerular damage decreased rapidly after administration of either retinoid agonist, even though blood pressure values had not yet been decreased in most treatment groups. At the end of the experimental period there was no difference between the high and low doses of either retinoid compound, indicating that even low doses of the retinoids are effective.

The magnitude of albuminuria, i.e., 10 mg/day is comparable to that in other chronic models of renal disease [8]. In vehicle-treated nephritic animals albuminuria remained stable over the entire experimental period, indicating that there was no tendency to self-repair in this model. A marked initial decrease in albuminuria was observed in the first 20 days of treatment with retinoids; subsequently a slower but progressive reduction in albuminuria was noted throughout the rest of the experimental period. There was no statistically significant difference between RAR and RXR agonists. The decrease in albuminuria was not due to a decrease in glomerular filtration rate; the creatinine clearance (as an admittedly insensitive index of glomerular filtration rate) remained unchanged and within the normal range in retinoid-treated nephritic rats.

At the end of the experiment the glomerular cell count was almost twice as high in vehicle-treated nephritic rats as in nonnephritic controls. These changes reflect the

Table 2 Effects of synthetic retinoid agonists on markers of renal morphology and glomerular inflammation as well as expression of genes involved in renal damage

Treatment	Controls					Thy-GN				
	Vehicle	RARα-specific agonist AGN 195183		RXR-specific agonist AGN 194204		Vehicle	RARα-specific agonist AGN 195183		RXR-specific agonist AGN 194204	
		Low dose	High dose	Low dose	High dose		Low dose	High dose	Low dose	High dose
Markers of renal morphology										
Glomerulosclerosis index (glom. score)	0.12±0.02	0.13±0.02	0.13±0.02	0.15±0.04	0.10±0.03	2.2±0.13[4]*	0.91±0.19[4]*	1.1±0.16[4]*	1.3±0.19[4]*	1.3±0.14[4]*
Glomerular cell count (cells per glom.)	52±0.8	52±0.6	54±1.9	47±0.6	47±0.9	95±2.1[4]*	69±2.3[4]*	75±3.3[4]*	83±3.3***	82±2.7***
Renal interstitial area (% of tubuloint. area)	5.4±0.31	5.5±0.22	5.7±0.07	5.9±0.13	5.4±0.07	14±0.34[4]*	8.6±0.23[4]*	7.7±0.31[4]*	11±0.17[4]*	8.2±0.25[4]*
Interstitial cell count (interst. cells per grid)	20±0.3	23±0.9	22±0.2	23±0.3	22±0.2	38±1.3[4]*	26±0.9[4]*	25±0.7[4]*	29±0.8[4]*	28±1.9[4]*
Marker of glomerular inflammation										
ED-1 (positive cells per glom.)	0.77±0.06	0.56±0.06	0.57±0.04	0.40±0.06	0.31±0.04*	1.8±0.14[4]*	0.79±0.08[4]*	0.50±0.04[4]*	0.68±0.15[4]*	0.47±0.04[4]*
Expression of genes involved in renal damage										
Glom. TGFβ1 mRNA (ODR)	1.5±0.41	0.89±0.39	0.38±0.11	0.39±0.11	0.16±0.04	3.9±0.66**	2.9±0.32	0.49±0.09[4]*	2.7±1.0	0.27±0.02[4]*
Glom. Prepro-ET-1 mRNA (ODR)	0.92±0.19	0.35±0.19	0.17±0.02	0.33±0.09	0.25±0.05	2.1±0.57**	1.1±0.09**	0.32±0.04[4]*	0.94±0.22**	0.39±0.11[4]*

*P<0.05 vs. control/vehicle, **P<0.05, ***P<0.01, [4]*P<0.001 vs. Thy-GN/vehicle (analysis of variance and Bonferroni's multiple comparison test)

Table 3 Effects of retinoid-receptor specific agonists on markers of toxic side effects

Treatment	Controls					Thy-GN				
	Vehicle	RARα-specific agonist AGN 95183		RXR-specific agonist AGN 194204		Vehicle	RARα-specific agonist AGN 195183		RXR-specific agonist AGN 194204	
		Low dose	High dose	Low dose	High dose		Low dose	High dose	Low dose	High dose
Calcium (mmol/l)	2.5±0.04	2.6±0.28	2.4±0.07	2.2±0.06	2.3±0.06	2.4±0.08	2.3±0.04	2.3±0.05	2.5±0.04	2.4±0.05
Alkaline phosphatase (U/l)	180±26	200±37	220±17	190±11	310±32*	180±15	260±28	270±27	240±21	440±52[4]*
Triglyceride (mg/dl)	63±19	54±13	231±116	34±5	34±8	62±16	75±10	189±63	54±9	45±15
Cholesterol (mg/dl)	39±3	30±5	29±3	51±5	63±4*	38±2	36±2	38±2	46±3	62±6[4]**
Aspartate aminotransferase (U/l)	61±8	72±5	83±12	58±11	50±7	76±13	75±10	74±10	74±11	69±7
Alanine aminotransferase (U/l)	33±9	27±5	44±13	24±2	31±2	25±2	31±2	32±2	35±2	40±3
Bilirubin (mg/dl)	0.41±0.05	0.50±0.03	0.37±0.06	0.38±0.05	0.34±0.07	0.41±0.05	0.29±0.03	0.35±0.02	0.32±0.04	0.33±0.03

*P<0.001 vs. control/vehicle, **P<0.05, ***P<0.01, [4]*P<0.001 vs. Thy-GN/vehicle (analysis of variance and Bonferroni's multiple comparison test)

chronicity of the Thy-GN model using MoAb 1-22-3; in contrast, in the acute Thy-GN model the initially increased glomerular cell count normalizes rapidly [15, 29]. In the chronic Thy-GN model glomerular cell counts were lower in retinoid treated animals, similar to what has been seen in the acute Thy-GN model [7, 8, 30]. Nevertheless the mechanisms underlying this observation presumably differ between the two models. In acute Thy-GN the reduction in mesangial cell numbers by retinoids reflects inhibition of proliferation, while in the chronic model reduction in initially elevated glomerular cell counts presumably reflects cell loss by apoptosis or other mechanisms. Retinoids inhibit both apoptosis and mitosis of mesangial cells [31]. Xu et al. [32] documented that in mesangial cells all-*trans* RA inhibits apoptosis induced by oxidative stress, since antiapoptotic action of retinoids was mediated at least in part by induction of mitogen-activated protein kinase phosphatase 1 via both nuclear receptor dependent and independent mechanisms. The high doses of the retinoids were not more effective than the low doses, but the RARα agonist was slightly more effective than the RXR agonist. Firm conclusions are not possible, however, in the absence of full dose-response curves.

The glomerulosclerosis index was significantly higher in vehicle-treated nephritic rats than in nonnephritic controls, as described by Kawachi et al. [15] in this model. The glomerulosclerosis index was significantly lower in retinoid-treated animals. These data indicate that retinoids not only reduce the extent of renal damage in acute Thy-GN but also reduce preexisting renal damage in the chronic model. Retinoids also reduce preexisting renal damage in the model of chronic Thy-GN. Again, the RARα agonist appeared to be slightly more effective than the RXR agonist, whereas no significant difference was found between high and low doses.

Tubulointerstitial lesions are known to contribute to the progression of renal disease. Retinoids reduced the expansion of both the interstitial space and interstitial cell counts.

To determine the mechanism of the renoprotective action of retinoids we measured TGFβ1 mRNA and protein expression. TGFβ1 expression was decreased in renal cortex of nephritic animals treated with retinoids [8]. Reduction in TGFβ1 expression by retinoids is relevant, since TGFβ1 is thought to be causally involved in progression of renal disease [33, 34, 35, 36, 37]. The reduction in TGFβ1 expression was dose-dependent. It was significantly more pronounced in animals treated with the high dose. The low dose decreased glomerular TGFβ1 mRNA only slightly. In contrast, the histological markers of renal damage including glomerular and interstitial cell counts showed no dose-dependent difference. The beneficial effects of retinoids may be due not only to a reduction in TGFβ1 expression. We cannot definitively exclude a difference in the time course of TGFβ1 expression and regression of histological evidence of renal damage. We did not address the issue by which mechanism retinoids reduce TGFβ1 gene expression, but Salbert et al. [38] demonstrated that TGFβ1 expression is modulated by retinoids through activator protein (AP) 1. We have previously demonstrated diminished expression of c-fos, a constituent of AP-1, in the glomeruli of rats with acute nephritis [30]. Suppression of c-Jun N-terminal kinase is another mechanism involved in the anti-AP-1 effect of retinoids [32]. It is therefore possible that the inhibition of AP-1 activation is responsible in the chronic model as well. Simultaneously with AP-1 other factors may be involved, including inhibition of cyclin D_1 and activation of antiproliferative peptides such as p21 or p27 [39].

Although a steep increase in ED-1^+ cells in glomeruli had previously been found in the acute Thy-GN model, the number of monocytes and macrophages was only moderately increased in the chronic Thy-GN model, reflecting the less marked inflammatory character of the model [15]. Low doses significantly reduced the number of ED-1^+ cells, but with the high doses the numbers were almost normalized.

Retinoids interfere with the expression and the activity of several inflammatory mediators including inducible nitric oxide synthase, osteopontin, and nuclear transcription factor κB [40]. Retinoids also inhibit monocyte-macrophage maturation and differentiation as well as their migration by inhibition of monocyte chemotactic protein 1 gene expression [41]. Further anti-inflammatory effects of retinoids include reduced expression of cytokines by macrophages, for example, interleukin-12 and TGFβ1 [40]. RAR- and RXR-specific compounds proved comparable in their ability to reduce various indices of renal damage in the chronic glomerulonephritis model, and no clear differences were found between the two receptor-specific agonists. Both compounds reduced cell proliferation, inflammation, and functional parameters of renal damage. The only differences were in that the RARα agonist increased the serum triglyceride concentration. This is a well known side effect of RAR agonists [42], while RXR agonists increase the serum cholesterol concentration [43]. These changes were seen only with the high doses whereas at low doses no significant changes of lipids were noted, although renoprotection was similar.

No information is available regarding the way in which renal retinoid receptor expression is affected by chronic glomerulonephritis. Retinoid receptor expression is altered in response to changes in retinoid status and by inflammation [44, 45]. Changes in their expression pattern may influence the tissue response to retinoids. This consideration prompted us to determine the expression of glomerular retinoid receptors in the chronic Thy-GN model. The expression of RARα and RXRα was markedly enhanced in nephritic glomeruli of vehicle-treated rats. The mechanism for this increase has not been elucidated, but it may be the result of feedback control since both retinoid agonists dose-dependently normalized the expression of RARα and RXR receptors in the glomeruli. In contrast, glomerular expression of RARβ and RARγ showed a decrease, suggesting that the various

receptor subtypes are differentially regulated and do not merely respond to the availability of ligands. The decrease in RARγ in nephritic glomeruli suggests that retinoid receptor expression does not parallel the glomerular cell count.

An important issue is whether interaction with retinoid receptor agonists modifies local synthesis of endogenous retinoids. This was not measured, but some information on this point is available from previous studies in this laboratory. Liebler et al. [46] demonstrated that glomerular retinoid synthesizing enzymes and expression of retinoid receptors are stimulated in the early phase of acute Thy-GN. The transcriptional activators mediating the effect of retinoid receptor agonists were not investigated in this study, but it is known that at least two important mediators are AP-1 and nuclear transcription factor κB [32, 40].

Taken together the data indicate that the renal retinoid system is altered in chronic glomerulonephritis. This finding supports the idea that changes in the local endogenous retinoid system are of importance for the progression of renal disease.

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