Retinoid receptor-specific agonists alleviate experimental glomerulonephritis

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Lehrke, Ingo, Matthias Schaiser, Kerstin Schade, Christian Morath, Ruediger Waldherr, Eberhard Ritz, and Juergen Wagner. Retinoid receptor-specific agonists alleviate experimental glomerulonephritis. Am J Physiol Renal Physiol 282: F741–F751, 2002.—Retinoids are potent antiproliferative and anti-inflammatory compounds. We previously demonstrated that the natural pan-agonists all-trans retinoic acid (RA) and 13-cis RA efficiently preserve renal structure and function in rat mesangioproliferative glomerulonephritis. We examine effects of synthetic retinoid receptor-specific agonists 1) to identify common and receptor subtype-specific pathways in this model and 2) to characterize effects of retinoids on the renal endothelin (ET) system. Vehicle-injected control rats were compared with rats treated with daily subcutaneous injections of agonists specific for retinoid A (Ro-137410) and retinoid X (Ro-257386) receptors and the complex anti-activator protein-1 active retinoid BMS-453 7 days after induction of anti-Thy1.1 nephritis (n = 7–9/group). The different retinoids lowered glomerular ET-1 and ET type A and B receptor gene expression in control and nephritic rats with comparable efficacy. Reduction of glomerular c-Fos and GATA-2 mRNA expression levels suggests downregulation of transcription factors required for ET expression. The different retinoids were similar in their action on the glomerular capillary occlusion score, number of total glomerular cells, and glomerular infiltrating macrophage count. They differed in their ability to normalize blood pressure (Ro-257386 > BMS-453 > arotinoid), albuminuria (BMS-453 > Ro-257386 > arotinoid), and creatinine clearance (arotinoid > BMS-453 > Ro-257386). No signs of toxicity were observed. We conclude that all retinoid agonists with different subtype specificity are highly efficient in reducing renal damage and proliferation of mesangial cells. Retinoid X and A receptor-specific pathways are apparently involved in the antiproliferative, anti-inflammatory, and anti-ET action. Further studies are indicated to define the potential use of retinoid agonists in inflammatory renal disease.

THE RETINOIDS, derivatives of vitamin A, comprise natural and synthetic compounds, which, unlike retinol, act selectively via retinoid A receptor (RAR) or retinoid X receptor (RXR) subtypes (39) The retinoid receptors belong to the steroid receptor supergene family and heterodimerize with each other (RAR-RXR) or steroid receptors, such as vitamin D receptor, thyroid receptor, peroxisome proliferator-activated receptors (PPAR), or others (24, 27, 55). RARs and RXRs are expressed in the rat kidney (17, 50). All-trans retinoic acid (RA) is the prototype for an RAR-specific agonist, whereas RXRs respond to 9-cis RA (6, 31). In vivo, however, these substances may isomerize into different retinoids. Retinoid receptor-specific agonists have been developed to identify receptor-specific pathways and to achieve an improved efficacy-to-toxicity ratio. Newer synthetic retinoids such as Ro-137410 (arotinoid), which is RAR specific, or Ro-257386, which is RXR specific, are less toxic and do not isomerize. They allow studies on retinoid receptor subtype-specific pathways. The retinoid BMS-189453 (BMS-453) differs from the above compounds, in that it is a combined agonist-antagonist and dissociates retinoid-dependent transactivation from activator protein-1 (AP-1) transrepression activity (7). This compound, therefore, differs from the other retinoid agonists, in that BMS-453 does not induce gene expression via binding of retinoid-receptor complexes to retinoid-responsive elements on the promoters of retinoid-dependent genes. In other aspects, it still acts as a retinoid, i.e., inhibits AP-1.

The anti-inflammatory and antiproliferative actions of retinoids have long been known. They have only been used, however, for treatment of hyperplastic skin disease or malignancies (39, 42). Retinoids interfere with factors that contribute to renal damage, such as endothelin (ET), angiotensin II [via angiotensin subtype 1 (AT1) receptor], platelet-derived growth factor, nitric oxide, transforming growth factor-β (TGF-β), and others (1, 9, 18–21, 32).

We previously demonstrated that retinoids are highly efficient in limiting renal damage in a rat model of mesangioproliferative glomerulonephritis (51). Retinoids inhibited proliferation of mesangial cells, reduced extracellular matrix (ECM) deposition, and lowered the number of infiltrating monocytes.

It has not been shown which retinoid receptor subtype is involved in the renoprotective effects of retinoids. The first aim of this study was to identify the

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receptor subtypes involved in prevention of renal lesions by retinoid receptor-specific agonists.

It has been shown that retinoids inhibit expression of ET-1 (20). ET-1 is also among the key factors involved in the pathogenesis of renal damage in this model (13, 35). Therefore, we have studied the effects of retinoid agonists on this system to clarify whether the beneficial effect of retinoid agonists may be explained, at least in part, by inhibition of the ET system.

The model of acute anti-Thy1.1 nephritis is a well-established model that is characterized by a marked proliferation of mesangial cells (37). To assess the antiproliferative action of the retinoid receptor-specific agonists, we examined this model on day 8, when mesangial cell proliferation is markedly enhanced. At this time point, glomerular damage and inflammation are also prominent, so the effects of the different retinoids on cell proliferation, monocyte/macrophage migration, expression of ECM proteins, and albuminuria can be quantified. This allows comparison of the efficacy of the different compounds on various aspects of renal damage. Because anti-Thy1.1 nephritis is a self-limited disease, no estimation of the effects of retinoids in long-term treatment is possible (22, 37).

MATERIALS AND METHODS

Experimental Protocol

The rat anti-Thy1.1 model of mesangiproliferative glomerulonephritis was induced by injection of OX-7 (1 mg/kg body wt iv; kindly provided by Dr. Jürgen Flöge, Dept. of nephrology, University of Aachen), a monoclonal antibody against the Thy1.1 antigen (European Collection of Animal Cell Cultures, Salisbury, UK) (4), into 180- to 200-g male Wistar rats (Charles River, Sulzfeld, Germany). All animal experimentation was performed according to the German animal protection laws.

Treatment started 1 day before injection of the Thy antibody. The retinoids were dissolved in arachis oil and 10% DMSO. Glomerulonephritis was induced in four groups (n = 9) by injection of the Thy antibody. Animals were treated with daily subcutaneous injections of Ro-137410 (8 mg/kg body wt), a selective RARα agonist (arottinoid; Hoffmann-La Roche, Basel, Switzerland); Ro-257386 (80 mg/kg body wt), a selective RARβ antagonist (Hoffmann-La Roche); BMS-189453 (20 mg/kg body wt), an AP-1 transrepressing substance, weak RARβ agonist, and RARα and RARγ antagonist (Bristol-Myers Squibb, Buffalo, NY); or arachis oil with DMSO alone. The four nonnephritic control groups (n = 7) were injected with PBS instead of Thy antibody and treated with the agents described above.

Blood pressure was determined on days 0, 5, and 7 by tail-cuff plethysmography under light ether anesthesia. The experiment was terminated 7 days after administration of the antibody by injection of xylazine (5 mg/kg body wt im; Bayer Vital, Leverkusen, Germany) and 10% ketamine (100 mg/kg body wt im; WDT, Garbsen, Germany). Rats were perfused with saline containing procaine hydrochloride (0.5 g/l) at a defined pressure of 110 mmHg after retrograde insertion of a cannula into the abdominal aorta (53). The inferior vena cava was incised to drain blood or perfusate. Glomeruli were isolated using a fractionated sieving technique as described elsewhere (49). The yield and purity of isolated glomeruli were comparable between groups (purity >90%). Creatinine clearance was calculated after enzymatic determination of serum and urinary creatinine (from 24-h urine collection, creatinine kit, Hoffmann-La Roche) on a Hitachi auto analyzer.

Renal Morphology by Light Microscopy

Tissue for light microscopy was fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm thick) 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 sections were reexamined by a second investigator.

In PAS-stained sections, the area of capillary tuft occlusion was determined using a semiquantitative score system: little or no occlusion was scored 0 and ≤25, 25–50, 50–75, and >75% occluded capillary tuft area of ≥30 cortical glomeruli was scored +1, +2, +3, and +4, respectively.

Total glomerular cell count was determined in PAS-stained sections in 30 cortical glomeruli/kidney with a diameter of ≥100 μm (12), and the mean number of cells per glomerulus was calculated.

RNA Isolation and Reverse Transcription

The TRIzol (Life Technologies, Gaithersburg, MD) 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 and 280 nm. Reverse transcription was performed as described elsewhere (52). For each biopsy, reverse transcription was carried out three times, and the resulting cDNA was pooled.

Quantitative PCR Assay

Quantification of specific mRNA was performed essentially as described by Paul et al. (40) and Wagner et al. (52). For each gene, a DNA deletion mutant was cloned (5). These mutants had the same sequences as the wild-type genes, with identical primer binding sites but a deletion of ≤20% resulting in a shorter amplification product. Reverse-transcribed 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 as described in Table 1 (28).

The PCR mix contained 0.25 mM dNTP (Promega, Madison, WI), 2.5 mM MgCl2, 20 mM Tris-HCl (pH 8.4), 50 mM KC1, sense and antisense primers (Life Technologies) at 80 mM, and 1 U of Taq polymerase (Life Technologies). The thermal profile consisted of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s carried out 27 times for ET-1, 30 times for the ET type A (ETα) receptor and 27 times for the ET type B (ETβ) receptor. In all experiments, possible contamination with genomic DNA was excluded by PCR amplification in the 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 software (National Institutes of Health, Bethesda, MD). The ratio of the optical density of the endogenous cDNA to the optical density of the mutant DNA was determined. Each sample was measured in triplicate individual PCR assays for each gene.
Table 1. Sequence and hybridization position of the primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer Sequence (5′ → 3′)</th>
<th>Hybridization Position</th>
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<tr>
<td>Prepro-ET-1</td>
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<tr>
<td>Sense</td>
<td>TGG CTT TCC AAG GAG CTC C</td>
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<tr>
<td>Antisense</td>
<td>GCT TGG CAG AAA TTC CAG G</td>
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<tr>
<td>ETαR</td>
<td></td>
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<tr>
<td>Sense</td>
<td>TCC ACA TTA AGA TGG GTG TCC</td>
</tr>
<tr>
<td>Antisense</td>
<td>TCA ATG CGG GTA ATC AAG G</td>
</tr>
<tr>
<td>ETβR</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
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</tr>
<tr>
<td>Antisense</td>
<td>CAG TGG AGC CAT GTC GAT ACC</td>
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<tr>
<td>c-Fos</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
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</tr>
<tr>
<td>Antisense</td>
<td>ACA GCT TGG TGT TCA CGG</td>
</tr>
<tr>
<td>GATA-2</td>
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<tr>
<td>Sense</td>
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</tr>
<tr>
<td>Antisense</td>
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</tr>
<tr>
<td>TGFB-3</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
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<tr>
<td>Antisense</td>
<td>TCA TGG TGG ACA ACT GAT GG</td>
</tr>
<tr>
<td>Procollagen I</td>
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<tr>
<td>Fibronectin I</td>
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</tr>
<tr>
<td>Antisense</td>
<td>AGG AGG TGG CCA CAT GAT CAT G</td>
</tr>
</tbody>
</table>

ET, endothelin; ETαR and ETβR, ET Type A and B receptors; TGFB-3, transforming growth factor β1.

Immunohistochemistry

Renal tissue was fixed in 10% buffered formalin (ET-1, Ki-67, and fibronectin 1) or methyl Carnoy’s solution (ED-1), embedded in paraffin, and cut into 4-μm slices. The primary antibodies were MIB5, a murine monoclonal antibody against rat Ki-67 (Dianova, Hamburg, Germany); a rabbit anti-rat fibronectin 1 antibody (Chemicon, Temecula, CA); MCA341R (Serotec, Oxford, UK), a murine IgG monoclonal antibody against ED-1; and a rabbit polyclonal antibody against ET-1 (Biotrend, Cologne, Germany). The ET-1 antibody showed no cross-reactivity with Big-ET, ET-2, or ET-3. Before incubation with primary antibodies against ET-1 or Ki-67, microwave pretreatment was performed in citrate buffer (pH 6.0) for 6 min at 750 W. The staining procedure was done by the labeled avidin-biotin method with 3-aminobenzidine hydrochloride as substrate using the Histostain-SP kit (Zymed, San Francisco, CA) according to the manufacturer’s recommendations. Sections were counterstained with Mayer’s hemalum (Merck, Darmstadt, Germany) and mounted under glass coverslips. Negative-control experiments were performed by substitution of the primary antibody with PBS or normal mouse serum.

For each biopsy, 20 cross sections of consecutive cortical glomeruli with a diameter of ≥100 μm were evaluated by one of the authors who was unaware of the protocol. Mean values per glomerular cross section were calculated for the number of proliferating (Ki-67-positive) cells and monocytes/macrophages (ED-1-positive). In immunoperoxidase stains for ET-1 and fibronectin 1, 20 glomeruli were graded semiquantitatively as described previously (12), with very weak or absent staining of the glomerular tuft scored 0, diffuse weak staining with <25% of the glomerular tuft showing focally increased staining scored +1, 25–50% of the glomerular tuft demonstrating a focal strong staining scored +2, 50–75% of the glomerular tuft stained strongly in a focal manner scored +3, and >75% of the glomerular tuft stained strongly scored +4. For evaluation of the tubular staining for ET-1, each of 20 proximal tubules was graded according to the intensity of staining of tubular epithelial cells, with no staining (grade 0) or weak (grade 1), moderate (grade 2), intense (grade 3), or maximal (grade 4) staining. The mean score per biopsy was calculated.

Measurement of Urinary Albumin

For determination of albumin and ET in urine, rats were placed in metabolic cages and urine was collected for 24 h. Urine was frozen at −20°C until measurement. Albuminuria in rats was determined essentially as described by Magnotti et al. (30) on a 96-well ELISA plate using a peroxidase-conjugated anti-rat albumin antibody (ICN Biomedical, Eschwege, Germany). Measurements were performed in quadruplicate.

Statistical Analysis

Values are means ± SE. Data were analyzed using non-parametric Mann-Whitney rank sum test. The zero hypothesis was rejected at P < 0.05 (33).

RESULTS

Blood Pressure, Albuminuria, and Kidney Function: Effects of Retinoid Agonists and BMS-453 on Functional Parameters of Renal Damage

Systolic blood pressure was significantly elevated in vehicle-treated glomerulonephritic rats 7 days after induction of glomerulonephritis. Treatment with retinoids remained without effect in control rats without glomerulonephritis, but in glomerulonephritic rats, Ro-257386 completely abrogated the blood pressure increase; treatment with BMS-453 and arotinoid partially prevented the blood pressure increase (Fig. 1).

Twenty-four-hour albumin excretion rate was markedly increased in vehicle-treated glomerulonephritic rats compared with controls. In glomerulonephritic rats, 24-hour albumin excretion was significantly increased compared with control rats (33).

Fig. 1. Time course of systolic blood pressure (BP) 5 and 7 days after induction of anti-Thy1.1 glomerulonephritis (Thy GN). Retinoid treatment partially prevented blood pressure increase in Thy GN rats. Values are means ± SE of 9 (Thy GN) or 7 [control (Con)] rats/group.*P < 0.05, **P < 0.01, and ***P < 0.001 vs. untreated Thy GN rats (Thy GN/Vehicle); n.s., not significant.
Rats, treatment with BMS-453 or Ro-257386 led to a reduction of albuminuria by 80 or 68%, respectively. Arotinoid treatment was less effective; albuminuria was reduced by 22%, which was not statistically significant (Fig. 2).

Thy glomerulonephritis caused a decrease of creatinine clearance: 0.98 ± 0.11 and 2.12 ± 0.26 ml/min in vehicle-treated Thy glomerulonephritic and control rats, respectively (P < 0.005). Treatment of glomerulonephritic rats with arotinoid or BMS-453 preserved renal function so that creatinine clearance was comparable to that of control rats without glomerulonephritis: 2.02 ± 0.37 and 1.69 ± 0.14 ml/min in arotinoid- and BMS-453-treated Thy glomerulonephritic rats, respectively. In contrast, treatment with Ro-257386 had no beneficial effect: 1.02 ± 0.22 ml/min (Table 2).

No signs of retinoid toxicity or weight loss were apparent after treatment with any of the retinoids.

**Retinoids Preserve Renal Morphology**

Glomerular damage was assessed by scoring the area of capillary occlusion in PAS-stained material. Glomerulonephritic rats showed significantly more occlusion of the glomerular capillary tuft. Treatment with any of the three retinoids reduced the capillary occlusion score in nephritic animals to an almost equal

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**Table 2. Effect of retinoid treatment on creatinine clearance and markers of glomerular proliferation, ECM production, and glomerular inflammation**

<table>
<thead>
<tr>
<th>Controls (n = 7)</th>
<th>Thy GN (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>2.12 ± 0.26</td>
</tr>
<tr>
<td>Capillary occlusion score, glomerular score</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>Glomerular cell count, cells/glomerulus</td>
<td>50.10 ± 1.34</td>
</tr>
<tr>
<td>Ki-67, glomerular score</td>
<td>0.8 ± 0.16</td>
</tr>
<tr>
<td>Glomerular TGF-β1 mRNA, ODR</td>
<td>0.80 ± 0.12</td>
</tr>
<tr>
<td>Glomerular procollagen I mRNA, ODR</td>
<td>0.27 ± 0.15</td>
</tr>
<tr>
<td>Glomerular fibronectin I mRNA, ODR</td>
<td>1.27 ± 0.56</td>
</tr>
<tr>
<td>Glomerular fibronectin I immunostaining score</td>
<td>2.40 ± 0.10</td>
</tr>
</tbody>
</table>

**Values are means ± SE. ECM, extracellular matrix; Thy GN, Thy1.1 glomerulonephritis; ODR, ratio of optical density of endogenous cDNA to mutant DNA. *P < 0.05 and **P < 0.01 vs. control/vehicle; †P < 0.05, ‡P < 0.01, and ***P < 0.001 vs. Thy GN/vehicle.
extent (P < 0.001, Thy glomerulonephritic arotinoid-, Ro-257386-, and BMS-453-treated rats vs. Thy glomerulonephritic vehicle-treated rats; Table 2).

The number of cells per glomerulus was significantly elevated in vehicle-treated glomerulonephritic rats compared with control rats. Treatment with retinoids did not influence the glomerular cell count in control rats, but in glomerulonephritic rats the glomerular cell counts were significantly less after retinoid pretreatment, with arotinoid being the most effective agent (Table 2).

Effect of Retinoids on the Renal ET System

ET-1 is a major proproliferative factor for mesangial cells and is actively involved in the pathogenesis of renal damage and serves as a marker of renal lesions in a number of experimental models of renal disease. Inhibition of ET-1-dependent cell growth by retinoids has been described. Therefore, we examined 1) whether ET-1 expression is enhanced in the proliferative phase of anti-Thy1.1 nephritis and 2) whether retinoids inhibit the components of this system in this model.

ET-1. Expression of glomerular prepro-ET-1 mRNA was increased 2.5-fold in glomerulonephritic rats compared with control rats (Fig. 3A). All three retinoids significantly reduced glomerular prepro-ET-1 expression in control as well as nephritic rats. All three retinoids were capable of reducing glomerular prepro-ET-1 expression in nephritic rats to a level lower than that in untreated control rats.

ETA receptor. The level of glomerular ET$_A$ receptor mRNA was eightfold higher in glomerulonephritic than in control rats (Fig. 3B). In all retinoid-treated control groups, mRNA expression was below detection limits. Treatment of nephritic rats with arotinoid or Ro-257386 resulted in complete normalization of ET$_A$ receptor expression, whereas BMS-453 was less effective.

ET$_B$ receptor. In contrast to ET-1 and ET$_A$ receptor, the glomerular mRNA expression of ET$_B$ receptor was significantly reduced (-33%) in glomerulonephritic rats compared with controls (Fig. 3C). In all retinoid-treated nephritic and control rats, ET$_B$ receptor expression was significantly reduced.

Fig. 3. Glomerular mRNA expression of endothelin (ET)-1 and ET type A (ET$_A$) and ET type B (ET$_B$) receptors (ET$_A$R and ET$_B$R) measured using quantitative RT-PCR. ODR, optical density ratio of wild-type to mutant cDNA. Values are means ± SE of 9 (Thy GN) or 7 (Con) rats/group. A: induction of Thy GN caused a 2.5-fold increase in glomerular ET-1 mRNA expression on day 7 (Thy GN/Vehicle vs. Con/Vehicle). Treatment with any of the 3 retinoid compounds significantly reduced ET-1 gene expression in Thy GN and control rats. B: glomerular ET$_A$ receptor mRNA was 8-fold higher in Thy GN than in control rats. In all retinoid-treated control groups, mRNA expression was below the threshold level of detection. Treatment of Thy GN rats with arotinoid or Ro-257386 resulted in complete normalization of ET$_A$ receptor expression; BMS-453 was not as effective. C: glomerular ET$_B$ receptor mRNA expression was significantly reduced in untreated Thy GN rats compared with controls (-33%). All retinoid-treated rats, glomerulonephritic or not, exhibited markedly lower ET$_B$ receptor expression. §§P < 0.01 vs. Con/Vehicle; **P < 0.01 and ***P < 0.001 vs. Thy GN/Vehicle.
Immunohistochemistry for ET-1. Because changes in mRNA levels may not correspond to protein expression, we also examined immunohistochemical staining for ET-1 and ETB and ETA receptors in this model (Figs. 4 and 5). This was done because the expression of the components of this system may be altered in the course of the disease and may be influenced by the action of retinoid agonists.

Weak staining for immunoreactive ET-1 was observed in some glomeruli of control rats. Tubular staining was faint in proximal tubular epithelial cells and rather strong in distal tubular epithelial cells. Staining was markedly enhanced in glomerular cells of the majority of glomeruli and in proximal tubular epithelial cells of vehicle-treated glomerulonephritic rats. The glomerular staining score revealed a highly significant difference between control and untreated glomerulonephritic rats: 1.01 ± 0.26 and 3.15 ± 0.09, respectively (P < 0.01). Treatment with any of the three retinoids significantly diminished glomerular staining in glomerulonephritic rats, with arotinoid being most effective: 1.85 ± 0.16 and 2.47 ± 0.23 in arotinoid- and Ro-257386-treated Thy glomerulonephritic rats, respectively (both P < 0.01) and 2.54 ± 0.21 in BMS-453-treated Thy glomerulonephritic rats (P < 0.05; Fig. 5A). The tubular staining score revealed significantly higher staining intensity for immunoreactive ET-1 in vehicle-treated glomerulonephritic rats than in vehicle-treated control rats: 1.07 ± 0.20 vs. 2.72 ± 0.16 (P < 0.01; Fig. 5B). Treatment with arotinoid or Ro-257386 significantly reduced staining intensity in proximal tubular epithelial cells, whereas the effect of BMS-453 did not reach statistical significance: 1.58 ± 0.20 and 1.83 ± 0.17 in arotinoid- and Ro-257386-treated Thy glomerulonephritic rats, respectively (both P < 0.01), and 2.35 ± 0.12 in BMS-453-treated Thy glomerulonephritic rats (not significant; Fig. 5B).

Retinoids Modulate Proliferation and Monocyte/Macrophage Infiltration

Proliferation of mesangial cells is a main characteristic of anti-Thy1.1 nephritis. Therefore, we have determined whether retinoids inhibit glomerular cell proliferation by determination of the proliferation marker Ki-67 (1). Similarly, glomerular inflammation as indica...
cated by migration of monocytes/macrophages may contribute to renal damage. For this reason, we quantified glomerular immunostaining for ED-1.

Ki-67. Intense glomerular cell proliferation was documented by a 16-fold increase in the number of Ki-67-positive cells in vehicle-treated glomerulonephritic animals compared with controls (Table 2). Treatment of glomerulonephritic rats with retinoids reduced the number of glomerular proliferating cells by 50–60%.

ED-1. The number of ED-1-positive cells (monocytes/macrophages) in glomeruli was 10-fold higher in vehicle-treated glomerulonephritic rats than in vehicle-treated control rats (Table 2). Treatment of glomerulonephritic rats with Ro-257386 or BMS-453 reduced monocyte/macrophage infiltration by 60%, and treatment with arotinoid reduced infiltration by 50%.

Retinoids Lower Expression of Molecules of the ECM on mRNA and Protein Levels

TGF-β1 is induced in acute anti-Thy1.1 nephritis (36) and stimulates the expression of molecules of the ECM. Therefore, we compared the effects of the different receptor-specific retinoids on the glomerular expression of TGF-β1 as well as procollagen I and fibronectin I.

Compared with normal rats, induction of anti-Thy1.1 nephritis led to an increase in glomerular mRNA expression of TGF-β1 (2-fold), procollagen I (20-fold), and fibronectin I (9-fold; Table 2). Treatment of glomerulonephritic rats with Ro-257386 completely inhibited induction of mRNA expression of all three genes. Arotinoid and BMS-453 were slightly less effective but still caused a significant reduction of fibronectin I and procollagen I mRNA expression (P < 0.01 for procollagen I and P < 0.001 for fibronectin I vs. vehicle-treated Thy glomerulonephritic rats).

Immunohistochemistry for fibronectin I. Similar to mRNA data, staining for fibronectin I was significantly higher in glomeruli of nephritic rats than nonnephritic control rats (Table 2). After treatment with arotinoid, Ro-257386, or BMS-453, significantly less staining was present in glomeruli of nephritic rats; however, no difference was found with respect to the various retinoids.

Glomerular TGF-β1 gene expression of nephritic rats was significantly reduced by arotinoid, whereas reduction by BMS-453 failed to reach the level of significance. In control rats, treatment with either of the three substances reduced glomerular levels of TGF-β1 mRNA (P < 0.01 for control arotinoid-, Ro-257386-, BMS-453-treated rats vs. control vehicle-treated rats).

Glomerular Expression of Transcription Factors c-Fos and GATA-2

The mode of action of retinoids in renal disease has not been elucidated. Their inhibitory action on c-Fos expression (and AP-1 activation) is, however, well known and might, at least in part, explain their antiproliferative action. Furthermore, Kawana et al. (23) and Lee et al. (25) demonstrated that the expression of ET-1 depends on the combined action of c-Fos and GATA-2, which bind to cis elements on the ET-1 promoter. Therefore, we examined the glomerular expression of these transcription factors in the presence of retinoid agonists in anti-Thy1.1 nephritis.

c-Fos. Induction of Thy glomerulonephritis resulted in a 3.8-fold higher glomerular gene expression of c-Fos on day 7 than in untreated control rats (Fig. 6A). Expression of glomerular c-Fos mRNA was prevented by treatment of glomerulonephritic rats with arotinoid, Ro-257386, or BMS-453.

GATA-2. Glomerular mRNA expression of transcription factor GATA-2 was not changed in glomerulonephritic rats compared with control rats on day 7 (Fig. 6B). Retinoid treatment, however, led to significantly lower glomerular mRNA expression of GATA-2 in all...
Reduction was most marked in groups treated with arotinoid.

lower gene expression of GATA-2 in all Thy GN and control groups. With control rats on
cription factor GATA-2 was not changed in Thy GN rats compared

c-Fos mRNA was prevented by treating Thy GN rats with arotinoid,
higher in Thy GN than in untreated control rats. Overexpression of
A

DISCUSSION
Our study documents that the synthetic receptor-specific retinoids uniformly alleviate renal damage and lower expression of the components of the renal ET system in anti-Thy1.1 nephritis. They differ, however, in their effects on creatinine clearance and blood pressure. Both receptor subfamilies confer retinoid-depen-
dent antiproliferation and anti-inflammation, indicating that RAR- and RXR-dependent pathways are involved in the action of retinoids on the kidney. In contrast, the differential effects on renal functional parameters suggest that some effects of retinoids are receptor subtype specific.

Retinoid inhibition of the ET system is striking. These compounds lower not only expression of ET-1 but also expression of its receptors. ET-1 is a mitogen for mesangial cells. These findings support the notion that the antiproliferative effects of retinoids are, at least in part, mediated via the ET system.

The antiproliferative action of retinoids on glomerular cells is consistent when different markers of proliferation are examined. Glomerular Ki-67 expression was comparably reduced by all retinoid compounds, and, in parallel, the number of glomerular cells and the capillary occlusion score were also comparably reduced. Retinoids inhibit the growth of most cell types by interference with (proproliferative) transcription factors (AP-1, c-Fos, nuclear factor-κB, and cyclin D1). Simonson (47) demonstrated that, in mesangial cells, retinoic acid specifically repressed serum-stimulated induction of the immediate early genes c-fos and c-jun, forming AP-1. Our data indicate that, in the kidney, retinoids lower basal and glomerulonephritis-stimu-
lated c-Fos and GATA-2 expression. Alternatively, they act via modulation of growth factors and vasoac-
tive substances. Retinoids inhibit ETs, platelet-derived growth factor (32), angiotensin II-dependent AP-1 ac-
tivation (18), and angiotensin type 1 and α-adrenergic receptors (56). All these pathways are involved in renal disease.

We found that receptor-specific antiproliferative ef-
effects of the retinoids are mediated via RAR and RXR. This is in line with earlier investigations that indicated that the inhibiting effects on cell proliferation were not retinoid receptor subtype specific (11, 16, 29, 44). The doses for the specific retinoids were selected to ensure in vivo receptor selectivity [Dr. Klaus (Hoffmann-La Roche) and Dr. Chris Zusi (Bristol-Myers Squibb), personal communication]. No signs of toxicity (body weight loss, cheilitis, hair loss, abnormal behavior, or drinking habits) were observed. The lack of receptor specificity is also indicated by the use of BMS-453. Although this substance does not activate retinoid receptor-mediated gene expression via retinoic acid response elements, it is as efficient in limiting glomerular cell number as the agonists. This indicates that AP-1 inhibition may play a role in the antiproliferative action of retinoids in this model.

The anti-inflammatory potency of the substances is confirmed by the reduction of infiltrating monocytes/macrophages. Reduction of macrophage infiltration also has an impact on ET-1 production, because macrophanes are known to produce ET-1 (10, 35). Fukuda et al. (13) demonstrated that, 1 day after induction of Thy nephritis, macrophages are the major source of ET-1 production, whereas later ET-1 production is taken over by mesangial cells. Anti-inflammatory ef-
effects of retinoids were reported in different tissues,
e.g., skin (2, 3). Effects on matrix deposition were evident. Retinoids lowered glomerular gene expression of TGF-β1, procollagen I, and fibronectin I mRNA (14, 44, 46), and immunohistochemical analysis of fibronectin I indicated that, similar to our previous study using natural pan-retinoids, not only mRNA but also protein levels are lowered by retinoids. Some differences were, however, detected concerning retinoid effects on blood pressure and renal function. BMS-453 and the RXRα agonist Ro-257386 lowered blood pressure and albuminuria very effectively, but only BMS-453 improved creatinine clearance. In contrast, the RARα agonist arotinoid was less effective in reduction of blood pressure and albuminuria but almost normalized creatinine clearance. Parallel changes of blood pressure and urinary albumin may reflect changes in glomerular filtration pressure. The comparison of RARα, RXRα agonists, and BMS-453, however, suggests that functional parameters such as albuminuria and creatinine clearance were differentially affected by the different retinoids. Final conclusions about the actions of retinoids on these functional parameters cannot be drawn at this time, however, since time-course and dose-response relationships have not been examined.

The relationship of retinoids and the ET system is of special interest in this model of mesangioproliferative glomerulonephritis, since ET-1 is a mitogen of mesangial cells (15, 48) and ET-1 as well as ET receptors are activated in this model (13, 54). Retinoids lower ET expression in endothelial cells and prostatic cancer cells (9, 20). They also inhibit ET-1-induced growth of rat aortic smooth muscle cells and cardiac myocytes (8, 56). Similarly, retinoids antagonized the ET system in our model of mesangioproliferative glomerulonephritis. The ET-inhibitory action of retinoids is obviously not retinoid receptor subtype specific, since all retinoid compounds were similarly effective. Retinoids not only inhibited the nephritis-induced expression of the components of the ET system, they also reduced basal ET-1 and receptor expression in the treated control groups. This indicates that retinoids have direct effects on the components of the ET system and that reduction of expression in glomerulonephritic rats is not simply due to less renal damage.

Immunohistochemistry for ET-1 revealed a marked expression of ET-1 in glomerular and proximal tubular epithelial cells of nephritic rats, while control rats showed no or weak immunoreactivity for ET-1 in most glomeruli and in proximal tubules. Treatment of glomerulonephritic rats with retinoids reduced glomerular and proximal tubular expression of ET-1 protein, with arotinoid being slightly more effective than the other two compounds.

In anti-Thy1.1 nephritic rats, we found an induction ETα receptor mRNA, which had also been demonstrated by Fukuda et al. (13), but not by Yoshimura et al. (54). Parallel induction of ET-1 and its receptor is also found in chronic human renal disease and in aminonucleoside-induced nephrosis in the rat (26, 34). In the literature, very little is known about retinoid effects on the ET receptors, but glomerular ETα and ETβ receptors were markedly lowered by retinoid treatment. The basal expression of the ETβ receptor in retinoid-treated control groups was even below the detection limit of the assay.

Prominent ET-inhibitory effects of retinoids may be a spin-off of the potent antiangiogenic activities of these compounds (38). The level of ET-1 expression is controlled by transcription factors binding to cis-acting elements on the ET-1 promoter. The ET-1 promoter contains an AP-1 binding site as well as a GATA-2 binding site (25, 41). Reporter gene transfection experiments have indicated that both binding sites are essential for ET-1 promoter function in endothelial cells and that direct cooperative interaction exists between them (23). Retinoids, on the other hand, were shown to downregulate GATA-2 in parallel with ET-1 gene expression (9). Additionally, an anti-AP-1 activity of retinoids has been well documented. It may depend on direct protein-protein interaction of retinoid receptors with the AP-1 complex, downregulation of c-Fos and Jun-1, or other mechanisms (43, 45, 47). Therefore, we examined the mRNA expression of c-Fos and GATA-2 in the glomeruli. The expression of both factors was reduced by retinoids, even under basal conditions, i.e., in retinoid-treated control rats. Similarly, expression of these factors was even more markedly diminished in glomerulonephritic rats (whereas c-Fos, but not GATA-2, is activated in the glomerulonephritic vehicle-treated group). These findings suggest reduction of glomerular ET-1 expression and inhibition of ET-1 induction in nephritic rats due to the low expression of c-Fos and GATA-2 in the presence of retinoids. A more fundamental examination of the nuclear mechanisms is beyond the scope of this study.

In conclusion, beneficial effects of retinoids have been demonstrated in this model of renal injury. Retinoids affect renal damage by a broad spectrum of antiproliferative and anti-inflammatory actions and by reduction of ECM production. Retinoids also potently inhibit the ET system, which is a key factor for mesangial cell proliferation. These compounds also influence functional parameters such as blood pressure or creatinine clearance. In this short-term model, no signs of toxicity were observed with any of the compounds. These findings make retinoids interesting novel candidates for the treatment of renal disease.

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