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# Retinoids as a potential treatment for experimental puromycin-induced nephrosis

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**1** Puromycin aminonucleoside (PAN)-induced nephrosis is a model of human minimal change disease. In rats, PAN induces nephrotic-range proteinuria, renal epithelial cell (podocyte) damage, infiltration of mononuclear leukocytes, and apoptosis of several renal cell types.

**2** Retinoic acid (RA) modulates a wide range of biological processes, such as inflammation and apoptosis. Since renal damage by PAN is characterized by inflammatory infiltration and epithelial cell death, the effect of treatment with all-*trans* RA (tRA) was examined in the PAN nephrosis model and in the cultured differentiated podocyte.

**3** Treatment with tRA 4 days after PAN injection did not inhibit the proteinuria peak but reversed it significantly. However, treatment with tRA both before and 2 days after the injection of PAN protected the glomerular epithelial cells, diminishing the cellular edema and diffuseness of the foot process effacement. Preservation of the podocyte architecture correlated with the inhibition of proteinuria. The anti-inflammatory effect of tRA was evidenced by the inhibition of PAN-induced interstitial mononuclear cell infiltration and the decreased renal expression of two molecules involved in monocyte infiltration: fibronectin and monocyte chemoattractant protein-1. TUNEL assays showed that tRA inhibited the PAN-induced apoptosis of cultured differentiated mouse podocytes.

**4** We conclude that tRA treatment may prevent proteinuria by protecting the podocytes from injury and diminishing the interstitial mononuclear infiltrate in the model of PAN nephrosis. Retinoids are a potential new treatment for kidney diseases characterized by proteinuria and mononuclear cell infiltration.

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**Keywords:** Puromycin aminonucleoside; tretinoin (all-trans retinoic acid); proteinuria; inflammation; adhesion molecules; podocyte; kidney; nephrosis

**Abbreviations:** ACE, angiotensin converting enzyme; AP-1, activating protein-1; FN, fibronectin; HMC, human mesangial cells; JNK, Jun NH2-terminal kinase; MCP-1, monocyte chemoattractant protein-1; NF-$\kappa$B, nuclear factor-$\kappa$B; OP, osteopontin; PAN, puromycin aminonucleoside; RA, retinoic acid; tRA, all-*trans* retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor

## Introduction

Retinoic acid (RA), one of the active metabolites of vitamin A, regulates a wide range of biological processes including cell proliferation (Sun *et al*., 2000), differentiation (Wang *et al*., 1985), and morphogenesis (De Luca, 1991). Results from several *in vivo* studies support the use of all-*trans*-retinoic acid (tRA) in treating various disease states including neoplasia (Lotan, 1996; Wu *et al*., 1997) and inflammation (Larsen *et al*., 1992; Moreno-Manzano *et al*., 2000). For example, tRA prevents the structural and functional changes associated with experimental mesangioproliferative glomerulonephritis (Wagner *et al*., 2000). Other inflammatory diseases of the kidney are characterized by mononuclear cell infiltrates that execute much of the damage. Since tRA strongly inhibits the chemotaxis and adhesion of monocytes, it has been used to treat inflammatory diseases with success (Larsen *et al*., 1992; Desreumaux *et al*., 2001). Our group has demonstrated in cultured human mesangial cells (HMC) that RA inhibits the mRNA expression of osteopontin (OP) (Moreno-Manzano *et al*., 1999b), fibronectin (FN) (Moreno-Manzano *et al*., 2000), and monocyte chemoattractant protein type 1 (MCP-1) (Lucio-Cazaña *et al*., 2001). These molecules are involved in monocytic infiltration because of their adhesive properties (OP, FN) (Magil *et al*., 1997; Rovin & Phan, 1998; Chana & Wheeler, 1999) or chemotactic properties (MCP-1) (Tang *et al*., 1997). Since infiltration by monocytes and macrophages plays a key role in the renal injury caused by inflammation (Kitamura & Fine, 1999), one of the more relevant therapeutic

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effects of RA is the significant inhibition of monocyte adhesion to HMC (Moreno-Manzano *et al*., 2000).

There is evidence that apoptosis of renal cells occurs in the glomerulus and tubulointerstitium in several forms of glomerulonephritis (Harrison, 1988; Sugiyama *et al*., 1996a). RA is known to induce apoptosis in various cell types including tumor cells and embryonic cells (Okazawa *et al*., 1996; Altucci *et al*., 2001). In contrast, little is known about the anti-apoptotic effects of RA in mammalian cells. Previous studies have shown that RA can inhibit apoptosis in several cell types (Yang *et al*., 1993; Ketley *et al*., 1997), and we have shown that tRA prevents, through an anti-activating protein 1 (AP-1) mechanism, the oxidative stress-induced apoptosis of HMC (Moreno-Manzano *et al*., 1999a).

The present study evaluates the effect of tRA treatment on puromycin aminonucleoside (PAN)-induced nephrosis. The model of PAN nephrosis involves structural alterations that are characterized by retraction of foot processes in the podocytes and interstitial infiltration of monocytes and macrophages (Eddy & Michael, 1988; Eddy *et al*., 1991). The functional alterations include the early development of proteinuria and later development of acute renal insufficiency that is often reversible. Given the properties of tRA to reduce inflammation and to inhibit apoptosis of mesangial cells, we studied the effects of tRA treatment on the course of PAN-induced interstitial mononuclear infiltration, epithelial cell death, and proteinuria.

## Methods

### *Experimental design* in vivo

A first study was performed in five groups ($n=6$ in each group) of female Wistar rats: control, PAN, tRA, tRA + PAN (treatment with tRA 1 day before PAN injection), and PAN + tRA2 (treatment with tRA 2 days after PAN). The PAN-treated groups received a single intraperitoneal injection of PAN at 100 mg kg$^{-1}$ on day 1 (Sigma Chemical Co., St Louis, MO, U.S.A.). Animals from the Control and PAN groups were fed standard chow. The tRA and tRA + PAN groups were fed standard chow mixed with tRA at 10 mg kg$^{-1}$ body weight (RocheFarma S.A., Spain) from day 0, while the PAN + tRA2 group did not receive this dose of tRA until 2 days after PAN administration. In effect, tRA + PAN is a preventive treatment with tRA whereas PAN + tRA2 is a therapeutic treatment with tRA 2 days after PAN injection. This timing was chosen because PAN, 2 days after injection, causes foot process effacement of glomerular epithelial cells (Ryan & Karnovsky, 1975; Inokuchi *et al*., 1996). At 1 day before killing, the rats were individually housed in metabolic cages, and 24-h urine samples were collected. The urine was centrifuged to remove contaminants and stored at −20°C until analysis for protein content. The animals were killed 16 days after PAN administration and the kidneys were processed as indicated below. Samples of whole blood and serum from every rat were also obtained. Once the results were analyzed, a second study in rats was performed (in a similar way than the one described above). The goal was to assess the effect of the treatment with tRA when it was started later enough (4 days after PAN administration) to allow the induction of maximal proteinuria by PAN.

Food containing tRA was prepared daily in the following way: a solution of tRA in 100% ethanol (1.68 mg ml$^{-1}$) was mixed in a dark cold room with standard chow (0.8 ml 15 g$^{-1}$). After the ethanol evaporated, 15 g of food per day was given to each rat so that the daily intake of tRA was approximately 10 mg kg$^{-1}$ of body weight. Control and PAN animals ate food treated in the same way with 0.8 ml ethanol 15 g$^{-1}$ chow.

The physical condition of each animal was good, and no change in the food intake was observed during the study. Decreases in erythrocyte counts or body weight and signs of cheilitis or hair loss were used to gauge the presence of tRA toxicity (Thacher *et al*., 2000).

### *Experimental design* in vitro

Cultivation of conditionally immortalized mouse podocytes was performed as described (Mundel *et al*., 1997). Briefly, podocytes were maintained in RPMI 1640 (GIBCO BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% fetal calf serum, 100 U ml$^{-1}$ penicillin, and 100 $\mu$g ml$^{-1}$ streptomycin. To propagate podocytes, cells were cultivated at 33°C (permissive conditions), and the culture medium was supplemented with 10 U ml$^{-1}$ of mouse recombinant $\gamma$-interferon (GIBCO BRL) to enhance expression of the large T antigen. To induce differentiation, podocytes were maintained on a bed of type I collagen at 37°C without $\gamma$-interferon (nonpermissive conditions) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, penicillin, and streptomycin.

For TUNEL assays, cells were cultured in chamber slides under nonpermissive conditions for at least 14 days to allow for full differentiation. Culture media were changed every 5 days during this time. After the differentiation period, cells were pretreated with 50 nM tRA (Sigma Chemical Co., St Louis, MO, U.S.A.) for 16 h and then subjected to 50 $\mu$g ml$^{-1}$ PAN (Sigma) for 72 h, a dose sufficient to cause severe injury to podocytes (Reiser *et al*., 2000). TUNEL assays were carried out according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany).

### *cDNA probes and antibodies*

The FN probe was synthesized by polymerase chain reaction (PCR) using murine kidney cDNA as templates and specific oligonucleotide primers based on the published cDNA sequences (Sharma & Ziyadeh, 1994). The PCR products were cloned into the pCRII TA cloning system (Invitrogen, La Jolla, CA, U.S.A.), and the identity of the probe was confirmed by nucleotide sequencing. The MCP-1 probe was a 0.8 kb Pst-I fragment of the mouse MCP-1 cDNA (Rollins *et al*., 1988).

Rat lymphohematopoietic cell surface markers were identified with the following monoclonal antibodies: OX-1 (Serotec, Oxford, UK), specific for the pan-leukocyte CD45 antigen; OX-19 (Serotec), specific for the CD5 antigen; and ED-1 (Serotec), which reacts with monocytes and macrophages (Eddy *et al*., 1986). For Western blot analysis, four primary antibodies were used: anti-human FN cell binding domain (Chemicon, Temecula, CA, U.S.A.) monoclonal antibody, anti-rat MCP-1 polyclonal antibody (Chemicon, Temecula, CA, U.S.A.), anti-rat OP monoclonal antibody (MPIIIB10 obtained from the Developmental Studies Hybridoma Bank,

The University of Iowa), anti-mouse ß-tubulin (Sigma Chemical Co., St Louis, MO, U.S.A.) monoclonal antibody. Secondary antibodies conjugated to horseradish peroxidase were added as follows: anti-mouse IgG (Sigma Chemical Co., St Louis, MO, U.S.A.) against the FN, OP and ß-tubulin antibodies and anti-rabbit IgG (Chemicon, Temecula, CA, U.S.A.) against the MCP-1 antibody. Western blots identified polypeptide bands of 69, 220, 25 and 55 kDa for OP, FN, MCP-1, and ß-tubulin respectively.

### *RNA extraction and Northern blot*

One piece of kidney cortex was kept in denaturing solution and homogenized for extraction of total RNA and Northern analysis, as previously described (Chomczynski & Sacchi, 1987). Total RNA was electrophoresed in a denaturing 1% agarose gel, transferred on to a nylon membrane (Hybond D, Amersham Iberica, UK), and hybridized with $^{32}$P-labelled-probes. The membranes were washed for 10 min twice at room temperature with 2 × standard saline citrate (1 × standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). This was followed by two washes of 5 min each at 65°C with 1 × standard saline citrate containing 1% sodium dodecyl sulfate and, if necessary, two more washes of 5 min at 65°C with 0.1 × standard saline citrate containing 0.1% sodium dodecyl sulfate. Blots were subsequently probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The blots were exposed to film (XOMAT UV film, Kodak Industry, France) for 6–24 h and analyzed with the NIH Image 1.62c program. For quantitation, the densitometric ratios of FN to GAPDH and MCP-1 to GAPDH were used.

### *Protein extraction and Western blot*

One piece of kidney cortex was homogenised in a combination of detergents and protease inhibitors for protein extraction and Western blot analysis, as previously described (Shih *et al*., 1999). The protein solution was boiled for 5 min before being separated on a SDS–PAGE gel, ranging from 6.5 to 12% polyacrylamide. The proteins were then transferred onto a nitrocellulose membrane (Optiman Ba-S 85, Cheicher and Chevell, Germany) that was incubated for 1 h with the primary antibody at a concentration of 1–2 $\mu$g ml$^{-1}$. After incubation with the appropriate secondary antibody, the band was visualized using the Super Signal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL, U.S.A.). Films (Kodak) exposed for 1–15 min were scanned by densitometry as above. Blots were subsequently probed with monoclonal anti-ß-tubulin (Sigma Chemical Co, St Louis, MO, U.S.A.) as a loading control.

### *Light microscopy*

Kidney tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections (4 $\mu$m) were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. The cellularity was determined by counting the total number of nuclei in each glomerular cross section.

### *Immunohistochemical staining*

Pieces of renal tissue were snap-frozen in isopentane, precooled in liquid nitrogen, and stored at −70°C until use. Frozen kidney tissue sections were stained by an indirect immunophosphatase method (APAAP) to detect OX-1-, OX-19-, and ED-1-positive cells in the interstitium (Cordell *et al*., 1984). Normal rat spleen served as the positive control of these antibody reagents. The specificities were assessed by incubating the kidney sections with normal mouse serum, normal IgG, and hybridoma-induced ascitic fluids containing irrelevant antibodies, as previously described (Mampaso & Wilson, 1983). The inflammatory mononuclear cells were counted by determining the total number of positive-labelled cells with each antibody. A total of 10 randomly chosen high-power fields ( × 630) of interstitium were examined as previously described (Mampaso & Wilson, 1983). The ratio of positive-stained cells to the total number of infiltrating cells was then obtained.

### *Electron microscopy*

For ultrastructural studies, the renal cortex was immediately fixed in 2.5%. glutaraldehyde in 1.1 M cacodylate buffer for 2 h, followed by post-fixation in osmium tetroxide. The tissue was then dehydrated in increasing concentrations of ethanol and embedded in Epon 812. Thin sections for ultrastructural examination were stained with lead citrate and examined at 75 kV with a Jeol 100 CX electron microscope.

### *Statistics*

The multiple mean values obtained in the *in vivo* studies were compared using ANOVA followed by *post hoc* comparisons performed with a Bonferroni/Dunn test. For the TUNEL assay (Figure 5), results are shown as the mean ± s.e.m., and $n$ = number of experiments (see Figure legends) and unpaired, one-tailed Student's $t$-test was used to compare PAN with the other groups. $P < 0.05$ was considered significant.

## Results

No statistically significant differences in the creatinine clearance were found between the five groups of rats at the moment of killing (data not shown). These results confirm the reversible nature of the decrease in creatinine clearance found within the first week after a single injection of PAN (Ryan & Karnovsky, 1975). Administered orally, tRA can cause toxic side effects such as weight loss and decrease in the erythrocyte number (Teelmann *et al*., 1993). Neither of these parameters was changed in the tRA-treated animals. Furthermore, no cheilitis or hair loss was seen with tRA treatment (data not shown), ruling out tRA toxicity with reasonable certainty.

The groups of rats that received a single intraperitoneal injection of PAN (PAN group) showed glomerular epithelial cell damage (Figure 1), with edema of the epithelial cell cytoplasm and foot process effacement. Pretreatment with tRA (tRA + PAN group) resulted in very mild degenerative changes of the glomerular epithelial cell (Figure 1). In addition, tRA treatment after PAN injection (PAN + tRA2 group) reduced the severity of foot process effacement

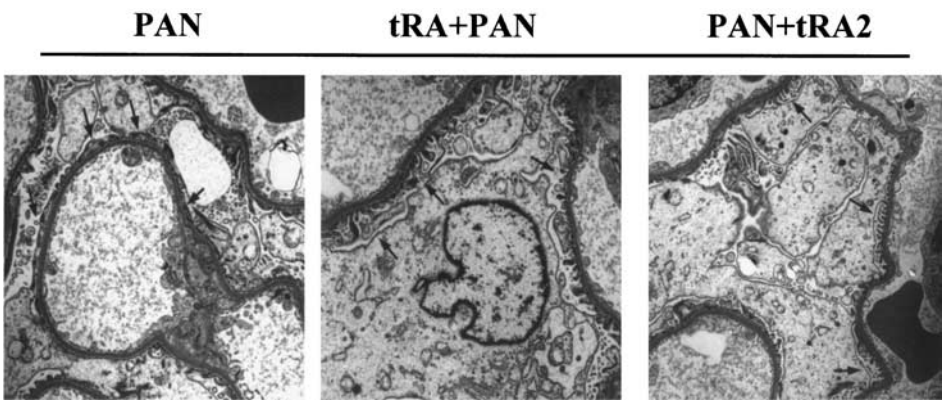


**Figure 1** Effects of tRA on the structure of glomerular epithelial cells: In all, 30 female Wistar rats were divided into the following groups: control, tRA, PAN, tRA + PAN, and PAN + tRA2 (see Methods). *Left panel*: Glomerular epithelial cell damage in a PAN-treated animal consists of edema of the epithelial cell cytoplasm with diffuse and prominent foot process obliteration (black arrows). *Central panel*: Very mild degenerative changes of glomerular epithelial cells can be seen in an animal with tRA pretreatment (tRA + PAN group). *Right panel*: Glomeruli from an animal with tRA treatment 2 days after PAN injection (PAN + tRA2 group) show much less intense foot process obliteration (black arrows) than in the PAN group. (Magnification: $\times 5200$).

compared with PAN injection alone (PAN group). The initial phase of the disease was also characterized by heavy proteinuria that was sustained at day 16 in the PAN group (Figure 2a). When tRA treatment was started before the onset of nephrosis (tRA + PAN group), proteinuria was completely prevented to control values (Figure 2a). However, when tRA was administered 2 days after PAN injection (PAN + tRA2 group), proteinuria was significantly greater than control at both 9 and 16 days. Nonetheless, protein excretion in that group remained significantly lower than in the PAN group (Figure 2a). In the two tRA-treated groups, the reduction in proteinuria correlated with the preservation of podocyte architecture (Figure 1). Given that nephrosis by day 9 after PAN injection was very mild in the PAN + tRA2 group, we studied in an additional experiment (involving control rats and PAN-treated rats) the effect of tRA when treatment started 4 days after PAN administration (PAN + tRA4). In these conditions, proteinuria 9 days after PAN injection was not statistically different between PAN and PAN + tRA4 groups, but still on day 16 it was possible to see an important protective effect of tRA (Figure 2b). Therefore, tRA reduces nephrosis even in the presence of heavy proteinuria.

Renal infiltration by monocytes and macrophages correlates closely with the amount of proteinuria in patients with several types of primary and secondary nephritides (Kitamura & Fine, 1999). In the PAN-treated animals, staining of the kidney sections revealed an influx of mononuclear cells into the interstitium 16 days after PAN administration (Figure 3a). Both pretreatment (tRA + PAN group) and post-treatment (PAN + tRA2 group) with tRA completely inhibited the mononuclear infiltration induced by PAN, normalizing the number of infiltrating cells to control levels (Figure 3b). The photomicrograph of the tRA + PAN group is not shown because it is similar to that of the PAN + tRA2 group (Figure 3a).

Chemotactic and adhesive molecules also play a role in the pathogenesis of leukocyte infiltration in PAN-associated nephrosis (Wu *et al*., 1996; Magil *et al*., 1997; Tang *et al*., 1997). Recently, we have demonstrated in cultured glomerular HMC that RA inhibits the expression of (1) OP and FN (Moreno-Manzano *et al*., 2000), two cell adhesion molecules, and (2) MCP-1 (Lucio-Cazaña *et al*., 2001), a chemotactic peptide that specifically attracts monocytes. In the present study, we found that the mRNA expression of FN and MCP-1 were significantly higher in the renal cortex of PAN-treated rats (Figure 4) and that pretreatment with tRA completely abolished these PAN-induced changes, while there were not statistically significant changes among the different experimental groups in OP protein expression (Figure 4 inset). It is possible to speculate that the active inhibition of FN and MCP-1 may underlie the ability of tRA to dampen mononuclear infiltration and thus protect the kidney from damage by PAN.

Podocyte depletion marks the histological picture of both primary and secondary forms of glomerulosclerosis and is now considered a critical factor in the progression of renal diseases (Fries *et al*., 1989; Kriz *et al*., 1998). To explore the underlying mechanisms of podocyte damage in PAN nephrosis, we determined the rates of apoptosis in cultured podocytes that were exposed to PAN. Maintained under nonpermissive culture conditions, fully differentiated podocytes were incubated with 50 nM tRA for 16 h and then with 50 $\mu$g ml$^{-1}$ PAN for 72 more hours. A cell was scored as apoptotic if its nucleus was labelled by the TUNEL assay. Many of the labelled podocytes also showed the characteristic morphological features of apoptosis, including condensed chromatin or fragmented nuclei (Schrantz *et al*., 1999). As expected, PAN significantly increased the prevalence of apoptosis in podocytes compared with control (Figure 5). Pretreatment with tRA, while having no adverse consequence of its own, completely blocked the effect of PAN to induce apoptosis in cultured differentiated podocytes (Figure 5).

## Discussion

This study investigated the effects of tRA in the rat model of PAN-induced nephrosis, chosen because it reproduces the pathological features of certain proteinuric nephropathies in the human such as minimal change disease and focal segmental glomerulosclerosis. In fact, several types of human nephrotic syndromes, regardless of their etiologies, share the same

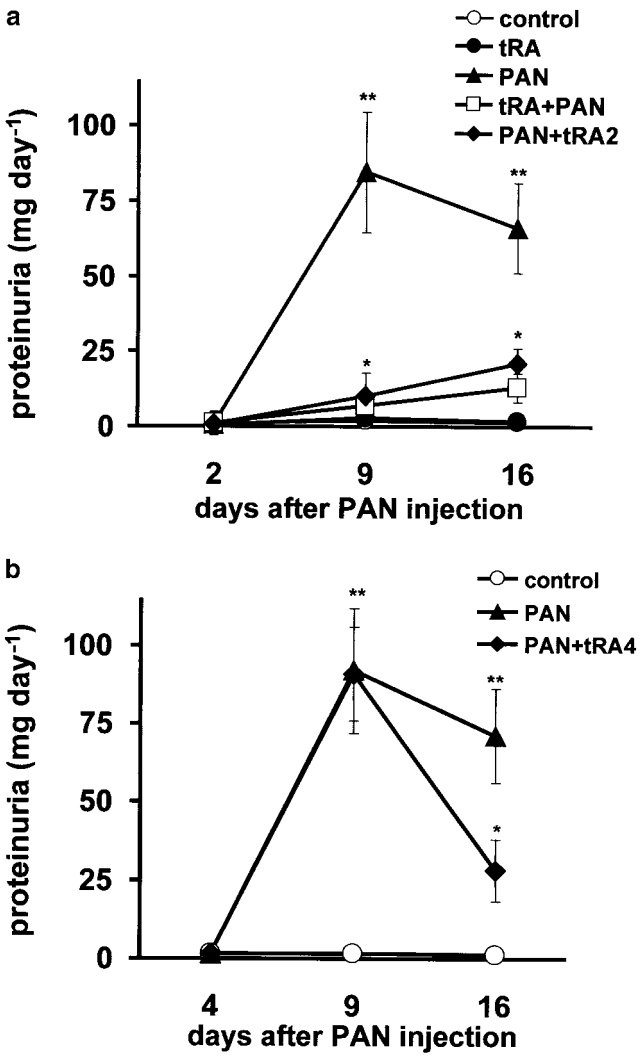


**Figure 2** Effects of tRA on PAN-induced proteinuria. (a) Increased urinary protein excretion in PAN-treated animals (closed triangles) compared with Control (open circles). Both pretreatment (tRA + PAN, open squares) and treatment with tRA 2 days after PAN injection (PAN + tRA2, rhombus), significantly prevented the increase in proteinuria. tRA alone did not have any significant effect on proteinuria (closed circles). (b) Treatment with tRA 4 days after PAN injection. The study on three experimental groups, control (open circles), PAN (closed triangles) and PAN + tRA4 (rhombus) shows that tRA did not inhibit PAN-induced maximal proteinuria (day 9), However, at day 16 proteinuria was significantly lower in the PAN + tRA4 group as compared to the PAN group. $^{**}P<0.01$ vs other groups; $^{*}P<0.05$ vs control.

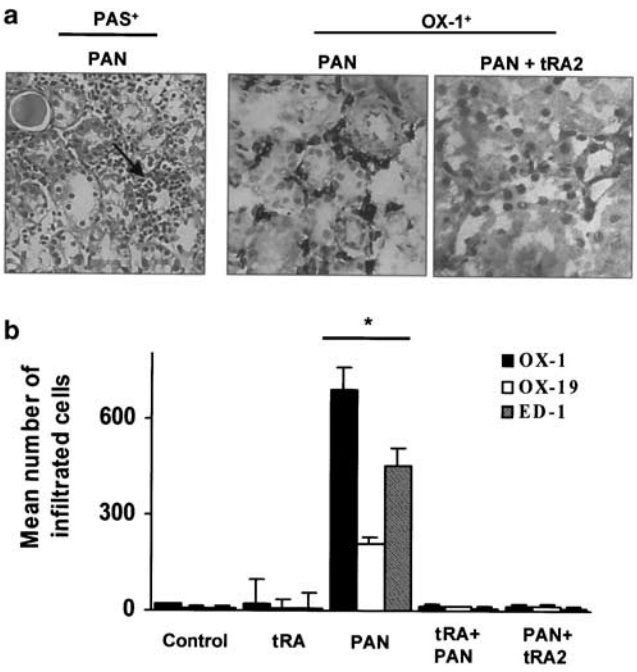


**Figure 3** Effects of tRA on interstitial mononuclear cell infiltration. Rats were treated as described in Methods. (a) Light micrographs (magnification $\times 630$) from slices of renal cortex. *Left panel*: PAS stain that illustrates infiltrating mononuclear cells in the interstitium (black arrow) in a PAN-treated animal. *Right and central panels*: OX-1 immunophosphatase stain showing the absence of interstitial infiltrating leukocytes in a PAN + tRA2-treated rat (right panel) compared with a PAN-treated rat (central panel). (b) Number of interstitial leukocytes (OX-1 positive cells); monocytes/macrophages (ED-1 positive cells); and both $CD4^+$ and $CD8^+$ cells (OX-19 positive cells) counted in 10 fields randomly chosen in each group of animals. Results are shown as the mean ± s.e.m., $^{*}P< 0.01$ vs other groups.

characteristics as those of nephrosis induced by a single injection of PAN: glomerular epithelial cell damage (Figure 1), manifesting as edema of the cytoplasm with diffuse and prominent foot process obliteration, and proteinuria (Figure 2). We show that tRA treatment 2 days after PAN injection significantly reduces the renal damage in rats with PAN-induced nephrosis, indicated by the inhibition of proteinuria, reduction of interstitial mononuclear cell infiltration, and the prevention of apoptosis in cultured differentiated podocytes.

Alterations in the morphology of the podocyte, from the disruption of foot process architecture to the loss of entire podocytes, are associated with significant proteinuria in many renal diseases (Kriz *et al*., 1994; Bjorn *et al*., 1995; Barisoni *et al*., 1999). Several investigators have correlated podocyte abnormalities with the onset of proteinuria in PAN-induced nephrosis (Messina *et al*., 1987), and the evidence to date favors the hypothesis that the glomerular visceral epithelial cell is the initial target of injury (Ryan & Karnovsky, 1975; Inokuchi *et al*., 1996). Protecting the glomerular epithelial cell is probably the mechanism by which tRA inhibits PAN-induced proteinuria when treatment with tRA starts 2 days after PAN injection. To our knowledge, we have shown for the first time that tRA protects the glomerular epithelial cell from injury due to PAN. This effect is critical because podocyte loss is considered an irreversible event that sets into motion the relentless progression of many glomerular diseases (Kriz *et al*., 1998). In this context, the protective effect of tRA in other animal models of glomerular epithelial cell damage deserves further investigation. Of note, when treatment with tRA starts 4 days after PAN injection, there was no protective effect on PAN-induced proteinuria (Figure 2b, day 9). However, it was still possible to see (Figure 2b, day 16) an important protective effect of tRA. Therefore, tRA reduces nephrosis even in the presence of heavy proteinuria.

Apoptosis of renal cells is observed in several types of glomerulonephritis (Harrison, 1988; Takemura *et al*., 1995; Shimizu *et al*., 1996; Sugiyama *et al*., 1996a) and in PAN-induced nephrosis (Fernández *et al*., 2001). Although the

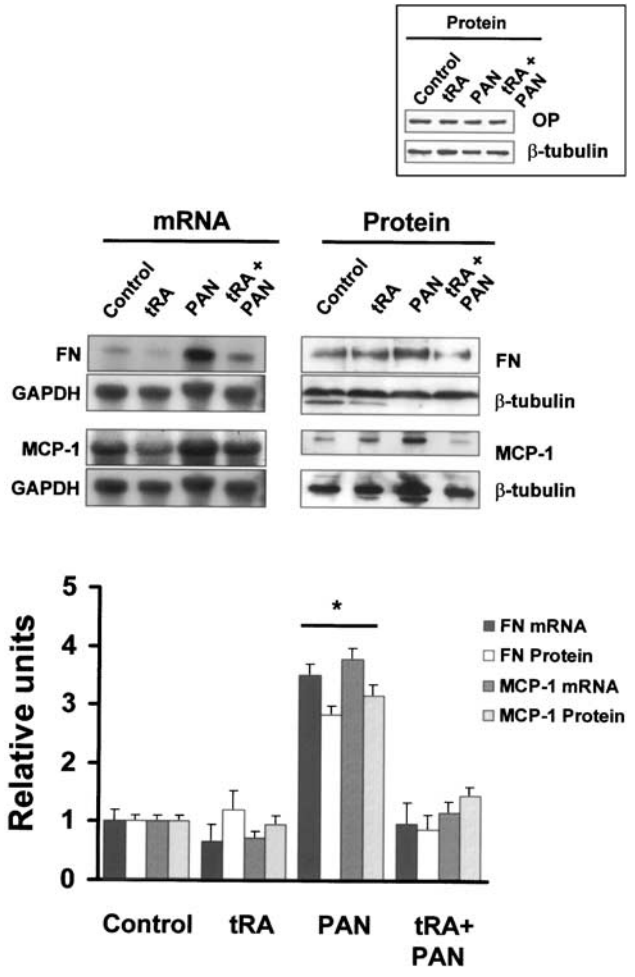


**Figure 4** Effects of tRA pretreatment on the renal cortical expression of molecules involved in leukocyte infiltration (FN, MCP-1 and OP). Upper panel: representative Western and Northern blots from one animal of each experimental group (except PAN + tRA2, which was similar to tRA + PAN group) for FN and MCP-1; *Inset:* Representative OP Western blot from the same experimental groups. RNA blots were stripped and rehybridized with a cDNA probe for the housekeeping gene GAPDH to adjust for small variations in RNA loading. For Western blot, an aliquot of every protein extract was electrophoresed and transferred onto nitrocellulose membranes. Protein blots were rehybridized with $\beta$-tubulin to adjust for small variations in protein loading. *Lower panel*: statistical analysis of mRNA and protein expressions for MCP-1 and FN. Results are shown as the mean ± s.e.m. of the densitometric values, corrected either for GAPDH (mRNA) or for $\beta$-tubulin (protein) and expressed as a percentage of the control group. $^*P<0.01$ vs other groups ($n=6$ in each group).

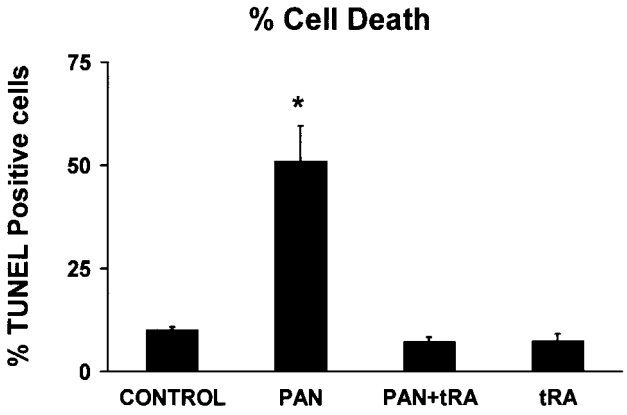


**Figure 5** Suppression of PAN-induced apoptosis of cultured, differentiated mouse podocytes by tRA. Confluent podocytes were pretreated with tRA (50 nM) for 16 h and were then exposed to PAN; (50 $\mu$g ml$^{-1}$) for another 72 h. Cells were labelled by the TUNEL reaction and examined by light microscopy. Data are expressed as the mean ± s.e.m. $^*P<0.001$ vs other groups ($n=10$ in each group).

inciting events of apoptosis have not been fully identified, several mechanisms are postulated in the model of PAN nephrosis. During initiation and progression of inflammation, toxic substances elaborated by the leukocytes may induce apoptosis of glomerular cells. Putative triggers include cytokines, nitric oxide, and reactive oxygen intermediates (Liu *et al*., 1996; Sugiyama *et al*., 1996b; Sandau *et al*., 1997; Yokoo & Kitamura, 1997). By attenuating the expression of NF-$\kappa$B and inducible nitric oxide synthase in mesangial cells (Datta & Lianos, 1999), tRA may enhance its antiapoptotic action in the kidney. Previous investigations have shown that tRA inhibits the apoptosis of T cells, leukemic cells, and hematopoietic cells (Iwata *et al*., 1992; Yang *et al*., 1993; Zauli *et al*., 1995; Ketley *et al*., 1997). In the present study, we show that tRA prevents PAN-induced apoptosis in cultured. differentiated podocytes. Although the exact mechanisms underlying this antiapoptotic effect remain unclear, suppression of a cell death pathway mediated by JNK and AP-1 appears to be involved (Moreno-Manzano *et al*., 1999a).

A wide variety of proteinuric renal diseases are associated with mononuclear cell infiltration into the interstitium (Eddy *et al*., 1991; Lan *et al*., 1991). We have found that the extent of the monocytic infiltrate correlates closely with the level of proteinuria in patients with primary and secondary nephritides (Kitamura & Fine, 1999). Other investigators have shown that interventions that decrease proteinuria, such as dietary modifications, pharmacological agents (e.g., ACE inhibitors), and irradiation-induced leukopenia, also attenuate tubulointerstitial nephritis (Diamond *et al*., 1989; Neilson, 1989; Harris *et al*., 1990; Eddy *et al*., 1991). Conversely, acute interstitial nephritis is characterized by moderate degrees of proteinuria, and treatment of the nephritis corrects the proteinuria. It should be kept in mind, however, that sustained proteinuria does not always lead to progressive interstitial fibrosis (Kikuchi *et al*., 2000). Therefore, relating proteinuria and interstitial inflammation as cause and effect has been difficult and remains to be proven. In the present study, we show that the PAN-induced proteinuria and interstitial infiltration were largely reversed by both pretreatment and post-treatment with tRA. The value of this therapeutic effect is evident, since the extent of interstitial infiltration in PAN nephrosis is the best predictor of progression to renal failure (Berens *et al*., 1998; Kriz *et al*., 1998). The capacity of tRA to reduce interstitial infiltration may contribute, at least in part, to its antiproteinuric property, but most of this effect is probably related to its ability to protect the podocyte from foot process effacement and apoptotic cell death.

We next investigated the potential mechanisms by which tRA may inhibit the interstitial mononuclear infiltration induced by PAN. This infiltration depends upon the expression levels of adhesive and chemotactic molecules involved in the process of leukocyte infiltration were investigated (Wu *et al*., 1996; Magil *et al*., 1997; Martin *et al*., 1997; Tang *et al*.,

1997; Ou & Natori, 1999). Specifically, two molecules with adhesive properties, osteopontin and FN, and one molecule with chemotactic properties, monocyte chemoattractant protein-1, were studied (Magil *et al*., 1997; Tang *et al*., 1997; Rovin & Phan, 1998; Chana & Wheeler, 1999). We previously demonstrated that the expression of these proteins was inhibited by tRA *in vitro* (Moreno-Manzano *et al*., 2000; Lucio-Cazaña *et al*., 2001). In the current study, the expression of OP protein *in vivo* was unaffected by treatment with either PAN or tRA (Figure 4 inset). However, exposure to PAN increased both the mRNA expression and protein production of FN and MCP-1 (Figure 4) and treatment with tRA completely abolished the increases in FN and MCP-1. These effects may explain how retinoids dampen the interstitial inflammation and mitigate the renal damage induced by PAN.

RTs act via retinoic acid receptors (RAR) $\alpha$, $\beta$, and $\gamma$ and retinoid X receptors (RXR) $\alpha$, $\beta$, and $\gamma$ (Lotan, 1996). These receptors are expressed in the rat and human kidney (Giguere, 1994; Yang *et al*., 1999), where they alter the expression of target genes via modulation of gene transcription (De Luca, 1991; Lotan, 1996; Thacher *et al*., 2000). tRA is known to interact specifically with the RAR and is a pan-agonist RAR. However, *in vivo*, tRA may isomerize into different retinoid forms, which may interact with RAR or RXR in a tissue-specific manner. Therefore, it is not possible to get conclusions regarding which receptors in the kidney are responsible for the tRA effects found in our experiments. An approach to identify the receptors involved is the use of synthetic retinoids which are either RAR- or RXR-specific and do not isomerize. This approach has been recently applied to the rat model of acute mesangioproliferative glomerulonephritis induced by anti-Thy1.1 using pretreatment with either a RAR$\alpha$-agonist or a RXR$\alpha$-agonist (Lehrke *et al*., 2002). In the anti-Thy1.1 nephritis, monocyte/macrophage infiltration and increased expression of FN were prevented in a similar extension by both compounds, indicating that RAR- and RXR-dependent pathways are involved in the action of retinoids in the kidney (Lehrke *et al*., 2002). However, only the RXR$\alpha$-agonist had efficacy on other parameters such the preservation of creatinine clearance and the blood pressure. A similar approach should be useful to identify retinoid receptors involved in our results. Clearly, before retinoids, and in particular tRA, can be used to treat renal diseases in humans, much work needs to be done both in cell culture and in other experimental models of renal disease. Based on the current results; however, these agents show promise in the therapy of proteinuric or inflammatory nephropathies.

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