American Journal of Pathology, Vol. 148, No. 1, January 1996

Interferon-Inducible Protein-10 Is Highly Expressed in Rats with Experimental Nephrosis

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Interferon-inducible protein (IP)-10 is a small glycoprotein member of a family of chemotactic cytokines structurally related to interleukin-8. We have recently described the induction of IP-10 mRNA in mouse mesangial cells stimulated with lipopolysaccharide, interferon-γ, and tumor necrosis factor-α. To further evaluate a possible role for this chemokine in renal injury, we have studied IP-10 in an experimental model of nephrosis induced in rats by adriamycin. High levels of glomerular IP-10 mRNA expression and glomerular and tubulointerstitial IP-10 protein were seen on day 21, coinciding with maximal proteinuria, glomerular tumor necrosis factor mRNA expression, and interstitial cellular infiltrates. Maintenance on a low protein diet not only delayed the appearance of proteinuria and interstitial cellular infiltrate but also decreased glomerular IP-10 mRNA expression. Isolated normal glomeruli and cultured glomerular epithelial and mesangial cells from normal rats expressed IP-10 mRNA upon stimulation with 100 U/ml interferon or 1 μg/ml lipopolysaccharide for 3 hours. IP-10 mRNA expression was also inducible by lipopolysaccharide and cytokines in NRK 49F renal interstitial fibroblasts and, to a lesser extent, in NRK 52E tubular epithelial cells. Furthermore, IP-10 protein was inducible in murine mesangial cells. We conclude that IP-10 is highly inducible* in vitro *and* in vivo *in resident glomerular and tubulointerstitial cells. IP-10 may participate in the modulation of renal damage in experimental nephrosis. (Am J Pathol 1996, 148:301–311)

Although the precise mechanisms responsible for the induction and progression of renal disease have not been elucidated, they are probably multifactorial. A role for a cascade of inflammatory mediators has been suggested on the basis of multiple *in vitro* and *in vivo* studies, including some that show a protective effect by different interventions.[1] Adriamycin (ADR)-induced nephrosis is characterized by the development of heavy proteinuria and glomerular epithelial cell damage in the absence of leukocytic infiltration within the glomeruli and provides a model to study the participation of mediators released by resident glomerular cells in the pathogenesis of proteinuria.[2] We have suggested that ADR might interact with indigenous glomerular cells, inducing the release of inflammatory mediators like platelet-activating factor and cytokines (tumor necrosis factor (TNF)-α) that could be responsible for a cascade of local events leading to glomerular epithelial cell damage.[3]

Recently, several cytokines structurally related to neutrophil-activating protein/interleukin (IL)-8 have been grouped into the chemokine family (for reviews see Refs. 4 and 5). Chemokines share a conserved motif containing four cysteine residues and have been divided into two subfamilies based on chromosomal location and the presence (α or CXC chemokines) or absence (β or CC) of an amino acid located

Supported in part by grants from Fondo de Investigaciones Sanitárias de la Seguridad Social (91/0162, 92/0592, 93/0834, and 94/0370), Ministerio de Educación (89/0065 and 92/042), Fundación Conchita Rábago, Instituto Reina Sofia de Investigación Nefrológica and Sandoz Pharma. M. Gomez-Chiarri was a fellow of Ministerio de Educación y Ciencia.

Accepted for publication October 2, 1995.

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This study was presented as an oral communication at the American Society of Nephrology meeting, Baltimore, 1992.

between the two amino-terminal cysteines. Chemokines appear to be involved in proinflammatory and/or restorative functions. Most of them are potent chemoattractants and some are activating agents or mitogens for specific cell types. Several cell types, including leukocytes, synovial and endothelial cells, fibroblasts, platelets, chondrocytes, and keratinocytes express and secrete chemokines.[4,5] Chemokines have also been implicated in renal disease.[6,7] Within the kidney, cortical tubular epithelial cells[8,9] and glomerular mesangial and epithelial cells[10–12] express and/or release some of these proteins when stimulated with exogenous and endogenous proinflammatory agonists like lipopolysaccharide (LPS), TNF, IL-1β, and interferon (IFN)-γ.

The IFN-inducible protein IP-10 belongs to the α (CXC) subfamily of chemokines, which also includes platelet factor-4, β-thromboglobulin, the melanoma-growth stimulatory activity (MGSA/gro/KC), and IL-8.[6] The expression of IP-10 mRNA is inducible in multiple cell types, including murine peritoneal macrophages and fibroblasts, by a variety of stimuli like IFN[13–14] and LPS[15] as well as growth factors such as platelet-derived growth factor and IL-2.[15,16] IP-10 has been implicated in delayed-type hypersensitivity[17] and in experimental autoimmune encephalomyelitis.[18,19] We have previously reported that LPS and IFN and, to a lesser extent, soluble immune complexes and TNF induced the expression of murine IP-10 mRNA in mouse mesangial cells in culture.[20] We speculated that IP-10 may play a role in the progression of inflammation in renal disease. Therefore, we have studied the expression of IP-10 in the experimental nephrosis induced in rats by ADR.

Materials and Methods

Experimental Nephrosis Model

A single dose of ADR (Farmiblastina, Farmitalia Carlo Erba, Milan, Italy), 7.5 mg/kg body weight, was injected into female Wistar rats weighing 200 to 225 g via the tail vein. All animals were allowed unlimited access to both water and conventional rat chow throughout the study. To evaluate different degrees of proteinuria, rats were subject to dietary intervention. Rats (n = 24) were divided into three groups, each fed a diet with a different protein content (Panlab, Barcelona, Spain) from 15 days before the induction of the disease until the moment of sacrifice. Group I received a standard diet (23% protein content), group II a low protein diet (9%), and group III a high protein diet (40%). The diets were rendered isocaloric by adjusting the content of carbohydrates and contained the same amount and type of fats. Urine was collected once a week and the amount of proteinuria was quantified by turbidimetry with sulfosalicylic acid. On days 0, 7, l4, and 21 after ADR injection, groups of four animals fed a standard diet were anesthetized and the kidneys were perfused with cold saline; samples were obtained for histology and glomerular isolation. In addition, rats that had received ADR and were fed a low (n = 4) or high (n = 4) protein diet were sacrificed on day 21, as were a group of unmanipulated age-matched controls (n = 4). Blood was collected through the aorta, and serum creatinine, total serum proteins, and cholesterol were determined by an automated serum chemistry analyzer.

Renal Histopathological Studies

For histology, renal tissue was fixed in buffered formalin and embedded in paraffin. Sections (2 to 3 μm thick) were prepared and stained with hematoxylin and eosin (H&E), periodic acid-Schiff, and Masson trichrome. Tissue (1 mm^3 cubes) for electron microscopy was fixed in 4% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.2, and postfixed in 1% osmium tetroxide in veronal buffer, followed by staining with 1.5% uranyl acetate in 0.05 mol/L maleate buffer, pH 6.2, for 1 hour at 4°C. After dehydration in graded alcohol, the tissue cubes were embedded in Epon 8l2 resin. Sections (0.5 to 1.0 μm thick), stained with toluidine blue, screened by light microscopy, were used to select representative areas for ultrathin sections. These sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in a Zeiss M109 electron microscope. At least four glomeruli were screened from each preparation.

The characterization of infiltrating glomerular and interstitial cells was performed with an avidin-biotin technique.[21] The monoclonal primary antibodies employed were 0X1 (leukocyte common antigen; Seralab, Crawley Down, UK), W3/13 (T lymphocytes; Seralab), and anti-ED1 (macrophages; Serotec, Oxford, UK).[22–24] Enumeration of cells in 15 randomly chosen interstitial fields (0.45 mm^2) and 15 glomeruli in each sample was performed, and results were expressed as the number of positive cells/mm^2 of cortical interstitium or as the number of positive cells per glomerular cross section.

Glomerular Isolation and Cultures of Glomerular Cells

Renal glomeruli were isolated based on the ability of glomeruli to pass through a 105-μm sieve and to be

retained on a 75-μm sieve.[3] The suspension obtained was washed in cold phosphate-buffered saline (PBS), pH 7.2, treated with diethylpyrocarbonate (Sigma Chemical Co., St. Louis, MO), and resuspended in lysis buffer for RNA extraction.

Cultures of mesangial cells were obtained as previously described.[3] Glomeruli from normal male Sprague-Dawley rats were incubated with 750 U/ml collagenase (type IA; Sigma) at 37°C for 30 minutes, washed twice, and plated on 100-mm culture dishes, (Costar, Cambridge, MA) in RPMI 1640, 20% fetal bovine serum (FBS), 2 mmol/L glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cultures were used on days 15 to 21. Cells were morphologically characterized by phase contrast microscopy, positive staining for desmin, vimentin, and Thy-1 antigen and negative for factor-VIII-related antigen and cytokeratin, excluding endothelial and epithelial cell contamination, respectively.[25]

Cultures of glomerular epithelial cells were obtained by plating non-collagenase-digested glomeruli on 100-mm culture dishes (Costar) in RPMI 1640, 5% FBS, glutamine, and penicillin-streptomycin, as above. On day 7, glomeruli were removed by lightly scraping the dishes with a cell lifter (Costar), leaving the epithelial cell outgrowth on the plate. These cultures, used on days 11 and 12, were 90% homogeneous, as determined by their early outgrowth (24 to 48 hours) from the isolated glomeruli, their ability to grow in low serum concentrations (5%), the polygonal shape of the cells, and positive staining for cytokeratin antibodies.[3]

For *in vitro* studies, glomerular epithelial or mesangial cells and whole glomeruli were incubated with recombinant IFN (Boehringer Ingelheim, Barcelona, Spain), LPS (Sigma), or ADR in RPMI 1640, 0.5% FBS. At the end of the incubation period, the cells were washed with cold PBS-diethylpyrocarbonate and lysed for RNA extraction.

Cultures of Tubulointerstitial Cells

Renal interstitial NRK 49F fibroblasts (American Type Culture Collection (Rockville, MD) CRC 1570) and tubular epithelial NRK 52E cells (American Type Culture Collection CRC 1571)[26] were grown in RPMI 1640, 5% FBS, 2 mmol/L glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin, stimulated with LPS, IFN, TNF, or ADR, and processed as described above.

Preparation of RNA and Northern Analysis

Total cellular RNA was extracted by the guanidine-phenol-chloroform method.[27] Equal amounts of RNA (10 to 25 μg) were denatured and subjected to electrophoresis in a 1% agarose-formaldehyde gel. The RNA was then blotted by capillary transfer onto Genescreen Plus membranes (New England Nuclear, Boston, MA). The blots were prehybridized for 6 hours at 42°C in 50% formamide, 1% sodium dodecyl sulfate (SDS), 5X standard saline citrate (SSC), 1% Denhardt's (0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 0.25 mg/ml denatured salmon sperm DNA, and 50 mmol/L sodium phosphate buffer, pH 6.5. Hybridization was carried out at 42°C overnight (16 to 18 hours) with 20% dextran sulfate and 7×10^6 cpm of denatured probe. The filters were washed in 0.1% SDS, 2X SSC for 30 minutes at room temperature and for 15 minutes at 55°C. The blots were then exposed to XAR-5 x-ray film (Eastman Kodak, Rochester, NY) with SHX intensifying screens (Valca, Madrid, Spain) at −70°C. Blots were reused by stripping and rehybridizing with different cDNA probes. The RNA load per gel was assessed by ethidium bromide staining of the original agarose gel after capillary transfer, and the expression of the 28S ribosomal RNA was used as an internal control of the amount of RNA loaded on each well. Autoradiograms were quantified with a GS350 densitometer and GS350 data system (Hoeffer Instruments, San Francisco, CA) and the results expressed as arbitrary densitometric units related to the expression of 28S.

Preparation of Rat IP-10 cDNA Probe

Kidney RNA from a rat injected with 10,000 U of IFN was isolated following the guanidine-cesium chloride method.[28] Total RNA (1 μg) was reverse transcribed and amplified by the polymerase chain reaction (PCR) in a Thermal Cycler (Perkin Elmer Cetus, Emeryville, CA). The primers used, designed from the mouse IP-10 sequences,[29] define a fragment of 487 bp from the beginning of the coding region and have the following sequences: sense primer, 5′ ACC ATG AAC CCA AGT GCT GCC GTC 3′; antisense primer, 5′ CTC AGG ACC ATG GCT TGA CCA TCA 3′. The amplified fragment obtained by PCR was purified and cloned in a Bluescript plasmid (Stratagene, La Jolla, CA) in an *Eco*RV site using standard protocols for blunt end cloning.[30]

Preparation of Plasmid DNA

Plasmid DNA encoding the genes for TNF (American Type Culture Collection) and rat IP-10 were prepared as described previously.[28] One microgram of plasmid DNA was radiolabeled by nick translation

(Boehringer Mannheim, Mannheim, Germany), with [^{32}P]dCTP (DuPont NEN Research Products, Boston, MA). The resultant specific activity was approximately 10^8 cpm/μg.

IP-10 Protein

Murine mesangial cells, obtained from E. G. Neilson,[31] were grown in eight-well Titertek slides and stimulated with either control media, 300 U/ml IFN, 100 U/ml TNF, or LPS in RPMI 1640, 5% FBS. For the *in vivo* study of IP-10, control and nephrotic rats (n = 3) were sacrificed on day 21 after ADR injection and frozen kidneys sections were used. Immunofluorescence was carried out as previously described[32] in cells or tissues fixed with methanol-acetone at −20°C. Polyclonal rabbit anti-murine IP-10 antibody (5 μg/ml) in PBS (a gift of Dr. Luster, Harvard University), pH 7.2, or nonimmune serum were used as primary antibodies, and a 1:300 dilution of fluorescein isothiocyanate-goat anti-rabbit antibody (The Binding Site, Birmingham, UK) was used as secondary antibody. After washing with PBS, preparations were mounted with a 90% glycerol solution and examined in a Zeiss microscope (Carl Zeiss, Thornwood, NJ). Images were photographed on Kodak TMAX 3200 film and printed at equivalent exposures.

Statistical Analysis

Results were expressed as the mean ± SD. Significance was established by R-Sigma (Horus, Madrid, Spain) statistical software. Fisher's protected *t*-test was used to compare means among groups. Linear regression analysis was performed to establish correlation between proteinuria and interstitial cell infiltrates. The differences were considered to be statistically significant when $P < 0.05$.

Results

Evolution of Nephrosis

At 21 days after the injection of ADR, rats had intense proteinuria (259 ± 35 *versus* 7 ± 5 mg/24 hours in control rats; $P < 0.0005$) and increased serum cholesterol levels (218 ± 43 *versus* 45 ± 12 mg/dl; $P < 0.01$) accompanied by a decrease in total serum proteins (4.3 ± 0.4 *versus* 6.5 ± 0.5 g/dl; $P < 0.02$). At this point, glomeruli were microscopically normal, but a mild to moderate, cortical, focal interstitial nephritis was observed (Figure 1). Interstitial inflammation was not especially prominent in the periglomerular areas. Electron microscopy showed striking abnormalities of glomerular epithelial cells, including extensive fusion of foot processes, an increased number of resorption vacuoles, and segmental detachment of epithelial cells from the underlying basement membrane (not shown).

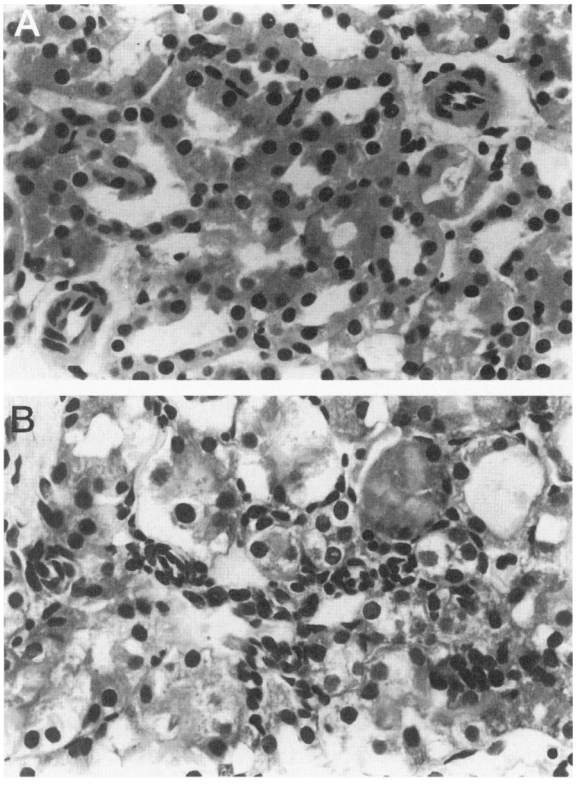

Figure 1. *Interstitial nephritis in ADR-nephrosis.* A: *Normal kidney.* B: *Light micrograph showing interstitial mononuclear cell infiltrates in rat kidney tissue at day 14 after ADR injection. H&E; magnification, ×216.*

Dietary intervention by a low protein diet has been shown to prevent proteinuria and glomerular damage in ADR-treated rats.[33] In this study, a significant delay in the increase of proteinuria levels was observed in rats injected with ADR and fed a low protein diet. Maximal values, evaluated on day 21, were significantly lower (133 ± 24 mg/24 hours; $P < 0.005$) than in rats fed a standard diet (259 ± 35 mg/24 hours) or a high protein diet (392 ± 40 mg/24 hours). A normalization to almost control values in serum cholesterol and total proteins was also observed in rats fed a low protein diet. There were no significant differences in body weight or serum creatinine among the three groups of rats fed different diets (not shown).

Immunophenotype of Infiltrating Cells

Rats with ADR-induced nephrosis developed a tubulointerstitial nephritis characterized by tubular cell

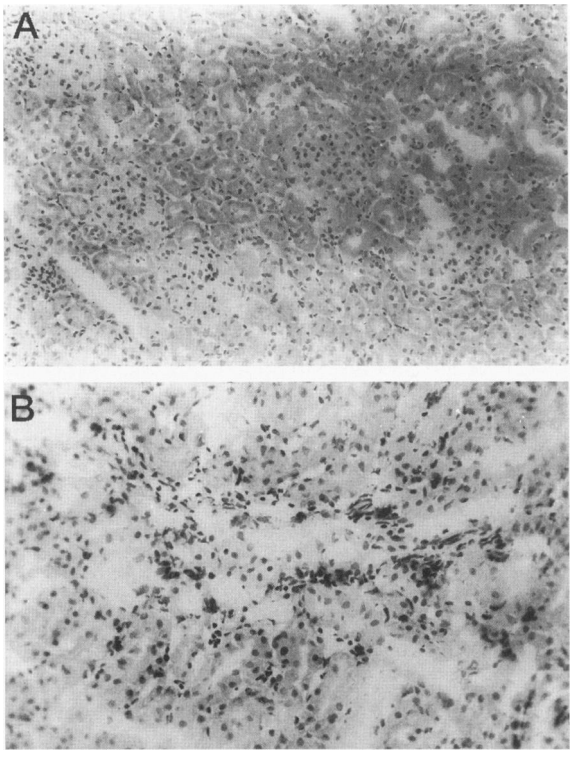

Figure 2. *Immunohistochemistry of interstitial cell infiltrates in ADR-nephrosis.* A: *Section stained with nonimmune serum.* B: *Immunoperoxidase staining showing mononuclear leukocytes (OX1$^+$) distributed throughout the interstitium at day 14 after ADR injection. Peroxidase; magnification, ×123.*

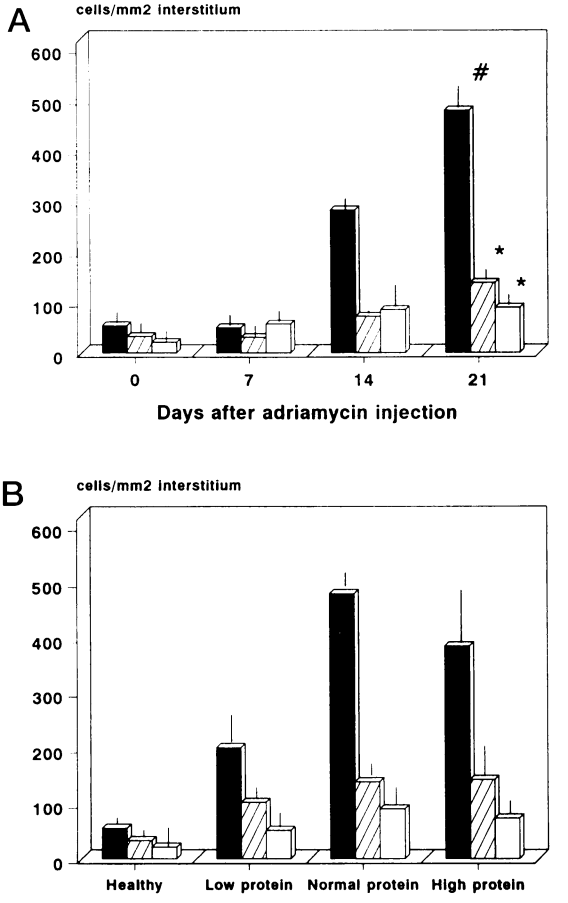

Figure 3. *Characterization of interstitial inflammatory cells in ADR-induced nephrosis.* A: *Evolution of interstitial cell infiltrates in ADR-induced nephrosis. Mean ± SD of total leukocytes (■), T lymphocytes (▨), and macrophages (□), in groups of four rats sacrificed 0, 7, 14, and 21 days after ADR injection are shown. *P < 0.05, #, P < 0.01 compared with cellular infiltrates on day 0.* B: *Effect of dietary protein content on interstitial cell infiltrates in ADR-induced nephrosis. Mean + SD of total leukocytes (■), T lymphocytes (▨), and macrophages (□) in healthy rats and ADR-treated rats fed three diets with different protein content are shown. Both total leukocytes and macrophages were significantly decreased in rats fed a low protein diet (P < 0.05). Four rats group were sacrified on day 21 after ADR injection.*

injury and an increase in mononuclear cells that infiltrate the interstitium (Figure 2). Within the interstitium, levels of total leukocytes (OX1$^+$) slowly increased after ADR injection and were maximal on day 21 (Figure 3A; leukocyte common antigen, 480 ± 78 *versus* 54 ± 9 cells/mm^2 interstitium; $P < 0.01$). Increased numbers of T lymphocytes (W3/13$^+$, 139 ± 32 *versus* 16 ± 3 cells/mm^2 interstitium; $P < 0.05$), and macrophages (ED1$^+$, 97 ± 12 *versus* 11 ± 8 cells/mm^2 interstitium; $P < 0.05$) also reached significance and peaked on day 21. There was a positive correlation between the number of total interstitial leukocytes and the severity of proteinuria when measured on days 7, 14, and 21 ($r = 0.64$; $P = 0.005$). By contrast, the number of leukocytes infiltrating the glomeruli remained within normal limits. Although ED1-positive cells were slightly increased in glomeruli on day 21 (0.82 ± 0.5 *versus* 0.2 ± 0.2 cells/glomerulus; $P = 0.06$), the difference did not reach statistical significance and there was no correlation with the magnitude of proteinuria (not shown).

The administration of a low protein diet not only delayed the onset of proteinuria with respect to nephrotic rats fed a standard or a high protein diet but also significantly decreased the number of total leukocytes and macrophages infiltrating the renal interstitium on day 21 after ADR injection (201 ± 48 *versus* 480 ± 78 leukocytes/mm^2 interstitium, $P < 0.05$; 49 ± 8 *versus* 97 ± 12 ED1$^+$ cells/mm^2 interstitium, $P < 0.05$; Figure 3B). ADR-treated rats fed a high protein diet developed interstitial cell infiltrates similar to rats fed a standard diet.

Glomerular Expression of IP-10 mRNA in Rats with Nephrosis

Maximal glomerular expression of mRNA for both TNF (134 ± 30 *versus* 15 ± 15 densitometric units in diseased and control rats, respectively; $P < 0.05$)

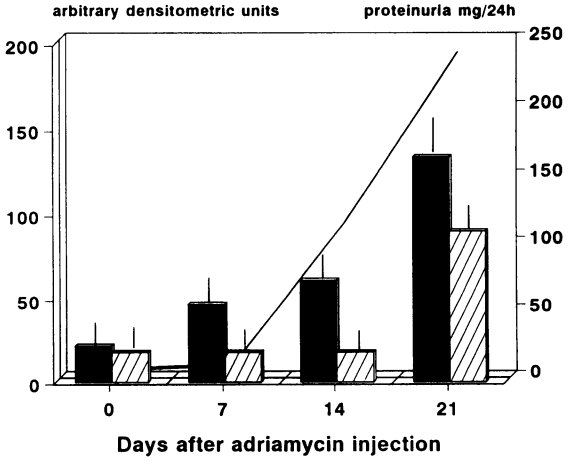

Figure 4. *Relationship between IP-10 and TNF mRNA expression and proteinuria in ADR nephosis. Proteinuria levels* (line, right axis; *mean ± SD) are compared with TNF* (■) *and IP-10* (▨) *mRNA expression* (left axis, *expressed as densitometric units related to the expression of 28S; mean ± SD), in glomeruli from four rats/group sacrificed 7, 14, and 21 days after ADR injection. Both TNF and IP-10 mRNA expression were maximal 21 days after injection, coinciding with peak proteinuria.* ($P < 0.05$ *compared with day 0).*

and IP-10 (90 ± 17 *versus* 18 ± 10 densitometric units; $P < 0.05$) occurred 21 days after ADR injection (Figure 4), coinciding with maximal proteinuria, interstitial leukocyte infiltration, and, as we have previously reported,[3] with peak TNF production by glomeruli of rats with experimental nephrosis.

We have also evaluated the effect of dietary intervention on glomerular IP-10 and TNF mRNA expression 21 days after ADR injection (Figure 5). Rats fed a low protein diet showed lower levels of TNF and IP-10 mRNA expression than rats fed normal or high protein diets. Similar to the case with interstitial infiltrates, no significant difference was noted in IP-10 mRNA between rats fed a normal or high protein diet.

Renal Expression of IP-10 Protein

Immunofluorescence performed with anti-IP-10 antibodies showed that IP-10 protein was absent from normal kidneys (Figure 6A). However, IP-10 immu-

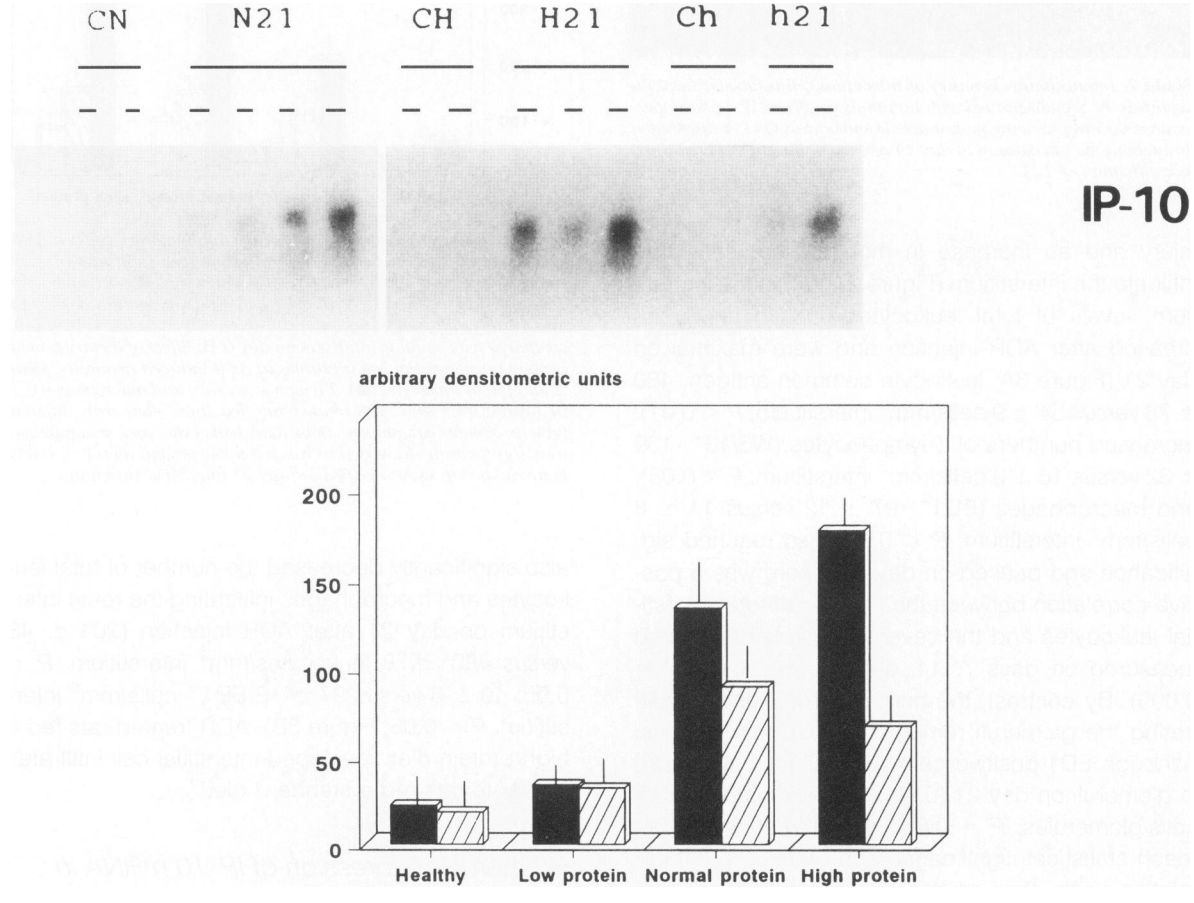

Figure 5. *Effect of dietary protein on glomerular IP-10 mRNA expression in ADR-induced nephrosis.* A: *Northern blot showing IP-10 mRNA levels in glomeruli from randomly selected control healthy rats (C) and ADR-treated rats sacrificed on day 21, fed normal protein (N), high protein (H), or low protein (h) diets.* B: *Effect of dietary protein on glomerular TNF and IP-10 mRNA expression in ADR-induced nephrosis. Mean ± SD of TNF* (■) *and IP-10* (▨) *mRNA levels (expressed in arbitrary densitometric units related to 28S expression) in glomeruli from four healthy rats and four ADR-treated rats/group fed three diets with different protein content are shown. IP-10 mRNA was significantly decreased* ($P < 0.05$) *in rats fed a low protein diet when compared with those fed the standard diet. Rats were sacrificed on day 21 after ADR injection.*

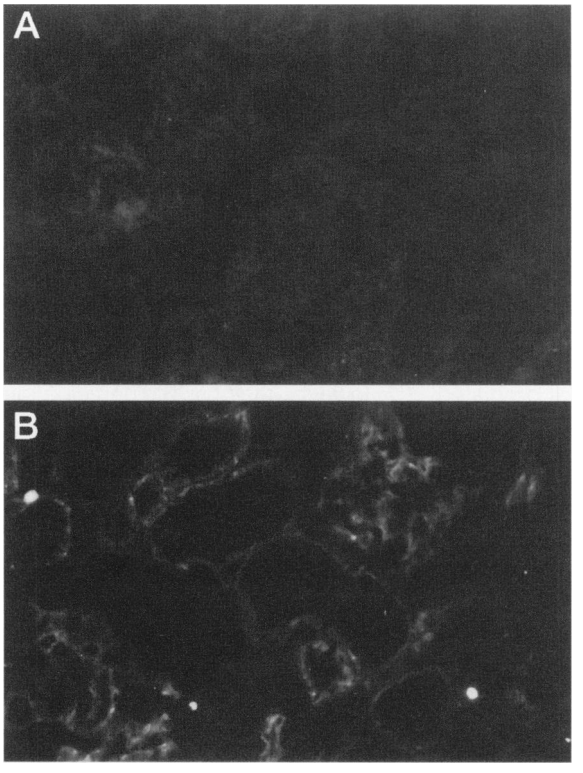

Figure 6. *IP-10 protein expression in ADR-induced nephrosis studied by immunofluorescence with a polyclonal anti-IP-10 antiserum. IP-10 immunoreactivity is absent in normal kidney* (**A**). *By contrast, immunoreactivity is present in both glomeruli and tubules of ADR-induced nephrosis on day 21 of disease* (**B**). *Magnification,* ×*160.*

noreactivity was present in glomeruli and especially in tubules in the kidneys of rats 21 days after the administration of ADR (Figure 6B). No fluorescence was observed when the first antibody was omitted.

IP-10 mRNA Expression in Cultured Glomerular and Tubulointerstitial Cells

Northern blots with RNA from normal rat glomeruli showed very low basal IP-10 mRNA expression. However, IP-10 expression was induced by stimulation with 1 μg/ml LPS, peaking at 3 hours and decreasing to almost basal levels after 18 hours (Figure 7A). As our previous work[20] demonstrated that mouse mesangial cells in culture expressed IP-10 when stimulated with LPS, IFN, TNF, and soluble immune complexes, we studied IP-10 mRNA expression in cultured rat glomerular mesangial cells and observed that these cells also expressed IP-10 mRNA when stimulated with 100 U/ml IFN or 1 μg/ml LPS (not shown). Moreover, we also explored the possibility that glomerular epithelial cells, the main target of damage in experimental nephrosis, could be a source of IP-10 mRNA. Rat glomerular epithelial cells in culture expressed IP-10 mRNA upon stimulation with 100 U/ml IFN for 3 hours (Figure 7B). However, addition of ADR (from 0.05 to 50 ng/ml) over several periods of time (between 3 and 24 hours) to either cultured glomerular mesangial or epithelial cells failed to induce a significant increase in IP-10 mRNA expression.

Figure 7. *IP-10 mRNA expression in cultured glomerular cells.* **A**: *Glomeruli from healthy rats were incubated with media alone (not shown) or 1 μg/ml LPS for 3* (**lanes 1** *and* **2**) *or 18* (**lanes 3** *and* **4**) *hours. RNA was extracted, subjected to Northern blot assay, and hybridized with the IP-10 probe. IP-10 mRNA increased after 3 hours of exposure to LPS and returned to basal levels at 18 hours.* **B**: *Glomerular epithelial cells in culture were incubated with 50 ng/ml ADR* (**lane 1**), *100 U/ml IFN* (**lane 2**), *and media alone* (**lane 3**) *for 3 hours. RNA was extracted and processed as above. Results shown are representative of two experiments.*

Cultured rat NRK 49F renal interstitial fibroblasts and, to a lesser extent, tubular epithelial NRK 52E cells expressed IP-10 mRNA. IFN (100 U/ml), LPS (1 μg/ml), or ADR (5 ng/ml) increased IP-10 mRNA expression in renal fibroblasts (2-, 14-, and 4-fold at 6 hours, respectively; Figure 8). TNF production is increased in rats with ADR-induced nephrosis, and urinary TNF has been previously reported to be increased in rats and humans with nephrotic syndrome,[34,35] suggesting that tubular cells are exposed to increased levels of TNF *in vivo*. Tubular epithelial NRK 52E cells were stimulated with 100 U/ml TNF or 1 μg/ml LPS for 6 hours. Both stimuli increased IP-10 mRNA expression in tubular cells (Figure 8). As was the case for glomerular epithelial cells, ADR failed to significantly increase IP-10 expression in NRK 52E cells (not shown).

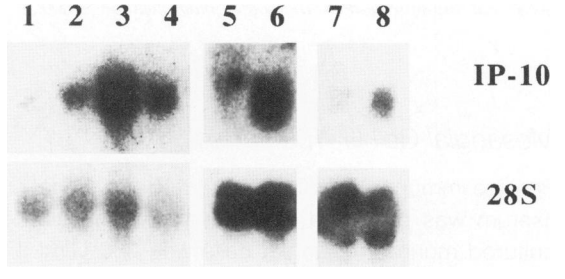

Figure 8. *IP-10 mRNA expression in cultured renal tubulointerstitial cells. Interstitial NRK 49F fibroblasts* (**lanes 1** *to* **4**) *were incubated with media alone* (**lane 1**), *100 U/ml IFN* (**lane 2**), *1 μg/ml LPS* (**lane 3**), *or 5 ng/ml ADR* (**lane 4**) *for 6 hours. NRK 52E tubular epithelial cells* (**lanes 5** *to* **8**) *were incubated with media alone* (**lanes 5** *and* **7**), *1 μg/ml LPS* (**lane 6**), *or 100 U/ml TNF* (**lane 8**) *for 6 hours. RNA was extracted and processed as above. Results shown are representative of two experiments.*

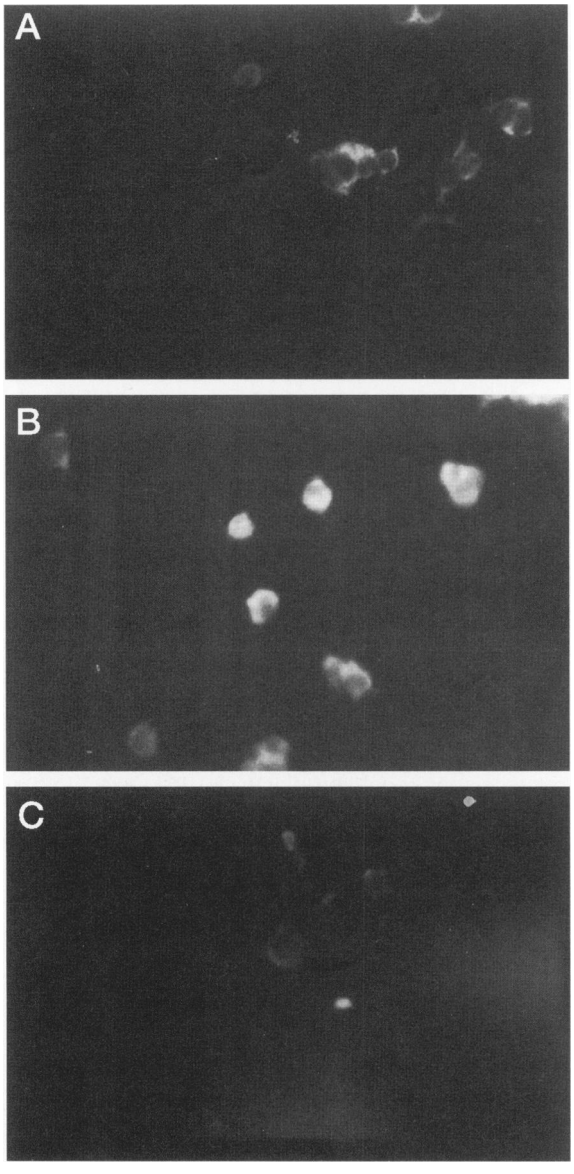

Figure 9. *IP-10 immunoreactivity was inducible in cultured murine mesangial cells 6 hours after the addition of IFN.* **A**: *Control, non-stimulated cells.* **B**: *Cells incubated for 6 hours with 300 U/ml IFN.* **C**: *Control stained with nonimmune serum. Immunofluorescence with polyclonal anti-murine-IP-10 antibodies; magnification, ×600.*

Mesangial Cell IP-10 Protein

Positive immunoreactivity to anti-IP-10 polyclonal antiserum was observed 6 hours after stimulation of cultured murine mesangial cells with 300 U/ml IFN (Figure 9), 100 U/ml TNF, or 1 μg/ml LPS, suggesting that intrinsic glomerular cells not only express IP-10 mRNA but also synthesize the protein. The cytoplasmic pattern is similar to that previously reported in astrocytes and microglia.[19] Staining with preimmune serum was negative.

Discussion

Members of the chemokine family have been found to selectively induce the migration of various cell types. Whereas IL-8 and members of the α (CXC) subfamily attract neutrophils and T lymphocytes, members of the β subfamily attract monocytes and/or T lymphocytes (Monocyte Chemotactic protein-1, macrophage inflammatory proteins MIP-1α and MIP-1β, RANTES).[36]

Over the past few years, the potential role of the chemokines in renal pathophysiology has become appreciated.[6–12] However, the possible participation of IP-10 in renal inflammation has not been studied. IP-10 mRNA has been previously shown to be highly inducible in kidneys, liver, and spleen but not in other organs from healthy mice given IFN.[37] We report that cultured glomerular mesangial and epithelial cells and tubular and interstitial cells are sources of IP-10 when stimulated *in vitro* with proinflammatory stimuli like LPS, TNF, and IFN. Moreover, this is the first time that increased levels of IP-10 have been observed in a model of nephrosis. ADR-induced nephrosis is characterized by the absence of cellular infiltration in the glomeruli and by the presence of interstitial inflammatory infiltration. The results reported here suggest that IP-10 is highly inducible in resident glomerular and interstitial cells in renal disease. The increased renal IP-10 expression is most likely caused by the stimulation of resident cells by mediators known to be augmented in nephrosis rather than to a direct action of ADR on glomerular cells. TNF and IL-1β are some of the most common and potent stimuli of the expression and release of most of these chemokines.[4,5,8–12] Therefore, molecules of the chemokine family may mediate some of the actions originally attributed to inflammatory cytokines.

Only recently has a natural form of IP-10 been purified and identified.[15] Functional studies of this protein showed that IP-10 is not active as a chemoattractant for granulocytes.[38,39] However, human recombinant IP-10 stimulates the migration of T cells and monocytes and potentiates T cell adhesion to endothelium, suggesting that this chemokine may have an important role *in vivo* in promoting endothelial cell-lymphocyte interactions and subsequent transmigration of T cells and monocytes in various inflammatory states.[39] Recent data support this hypothesis, as mice injected with tumor cells transfected with the IP-10 gene exhibited a protective antitumor effect that appeared to require the presence of T cells.[40] However, IP-10 may have actions beyond chemotaxis. IP-10 suppressed colony formation *in vitro* by early human bone marrow progen-

itor cells that need growth factors.[41] It is conceivable that IP-10 may also have actions on renal cells. In fact, we have preliminary evidence of the existence of IP-10 receptors in renal cells (unpublished observation).

In many forms of inflammatory kidney disease, significant interstitial leukocytic infiltration is associated with chronic progression of disease.[42–44] The mechanisms responsible for the infiltration of leukocytes in the renal interstitium in nephrotic rats are not clear, but it has been suggested that interstitial cell recruitment in renal diseases is dependent upon the expression of specific cytokines[9,44] and/or lipid mediators.[45,46] Chemokines may be involved in these processes. Increased levels of IL-8 have been observed in immune-mediated glomerulonephritis[7] and in renal allograft rejection[8] associated with an increase in the infiltration of inflammatory cells. Moreover, infusion of anti-IL-8 antibodies prevented leukocyte infiltration and glomerular damage.[7] Similar to other chemokines or chemoattractants, IP-10 may participate in the recruitment of inflammatory cells in the kidney. Although we have not proven that there is a direct relationship between increased IP-10 expression and leukocytic infiltration, it is noteworthy that peak IP-10 mRNA coincides with maximal leukocyte levels in the interstitium of ADR-treated rats. Moreover, we have observed a parallel decrease in proteinuria, IP-10 mRNA expression, and interstitial cell infiltrate in ADR-nephrotic rats fed a low protein diet, again suggesting a close relationship among these events. A high protein diet did not significantly modify interstitial inflammation or IP-10 mRNA expression. As diet has been previously shown to affect the immune system,[47] we propose that the beneficial effect of dietary intervention on proteinuria and glomerular damage may be related to diminished release of inflammatory mediators by renal cells.

This is not the first time that the pattern of interstitial mononuclear cell infiltration has been related to events in the glomerulus.[44,48] Cytokines and other chemoattractant molecules produced by stimulated resident glomerular cells might diffuse down the mesangial stalk to the hilar area and attract cells from nearby capillaries and venules.[44,48] However, *in vivo,* IP-10 protein was found not only in the glomeruli of nephrotic rats but also in tubular cells. Cytokines from the glomerulus may also reach downstream tubular cells via glomerular ultrafiltrate or capillary blood. Such cytokines might then stimulate the expression of adhesion molecules and/or the release of chemokines by tubular epithelium.[9,44,49] In this sense, it is noteworthy that, besides glomerular cells, both cultured tubular epithelial cells and interstitial fibroblasts express IP-10 mRNA upon stimulation with cytokines and, for fibroblasts, with ADR. TNF, which has been shown here to be transcribed in glomeruli of ADR-treated rats and to be excreted in the urine of animals and humans with nephrotic syndrome,[34,35] increases IP-10 mRNA in rat tubular epithelial cells. In ADR-induced nephrosis and other glomerular diseases, epithelial cell damage and/or the increased permeability of the glomerular filtration barrier may especially promote entry of cytokines into the urinary space. However, increased glomerular permeability also leads to increased exposure of cortical tubules to albumin and to lipids bound to the albumin. In turn, the tubular epithelium may produce chemotactic lipids.[46] These lipids may act alone but may also synergize with other locally produced chemokines. Conceivably, the chemotactic lipids may themselves elicit IP-10 expression. At least conceptually, there are several mechanisms evoking tubulointerstitial responses that may amplify primary glomerular injury. The fact that the interstitial nephritis in this ADR model is not periglomerular cannot be construed as evidence against glomerular products eliciting interstitial nephritis.

In summary, our data support the hypothesis that the microenvironment of the injured glomerulus contains mediators and cytokines such as IL-1, TNF-α, or IFN-γ that signal glomerular mesangial and/or epithelial cells to synthesize IP-10 and other members of the chemokine family. These molecules may diffuse to the tubulointerstitial area or may be secreted by tubulointerstitial cells stimulated by other proinflammatory molecules derived from the glomerulus. In the tubular interstitium, these chemokines may synergize with other mediators to attract monocytes and lymphocytes to the interstitium, establishing a positive feedback loop. Ultimately, tubulointerstitial damage that develops in the later stages of nephrosis may contribute to progressive renal scarring. We conclude that IP-10 may be one of the mediators involved in the recruitment of inflammatory cells in the renal interstitium and therefore may participate in the progression of damage in experimental nephrosis induced by ADR.

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