Interferon-β Reduces Proteinuria in Experimental Glomerulonephritis

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ABSTRACT

Interferon-β (IFN-β) is a multifunctional cytokine with immunomodulatory properties. We examined the effect of IFN-β in three separate rat models of glomerular injury and in cultured human glomerular endothelial cells and podocytes. In nephrotoxic nephritis in WKY rats, recombinant rat IFN-β started either at induction or after establishment of disease significantly reduced 24-h proteinuria by up to 73% and 51%, respectively, but did not affect serum creatinine. There was a slight reduction in numbers of glomerular macrophages, but no difference in glomerular or tubulointerstitial scarring. In Thy-1 nephritis in Lewis rats, IFN-β started at induction of disease reduced proteinuria by up to 66% with no effect on numbers of glomerular macrophages, but a reduced number of proliferating cells. In puromycin nephropathy in Wistar rats, IFN-β started at induction of disease reduced proteinuria by up to 93%, but had no effect on glomerular histology. In cultured cells, human IFN-β-1a had a dramatic effect on barrier properties, increasing electrical resistance across monolayers of either glomerular endothelial cells or podocytes and decreasing trans-monolayer passage of albumin. In conclusion, these results show that IFN-β reduces proteinuria in three different rat models of glomerular injury and that its anti-proteinuric action may result from direct effects on cells that comprise the glomerular filtration barrier. These data indicate that IFN-β may have potential as a therapeutic agent in proteinuric renal disease.


IFN-β, a type-1 IFN, is produced in response to viral infection and other biologic stimuli, mainly by fibroblasts,1 macrophages, and dendritic cells.2 IFN-β is a 20-kD glycoprotein containing 166 amino acids. It binds to the same receptor as IFN-α, and as a highly pleiotropic cytokine, IFN-β has a broad range of immunomodulatory effects on the cells of the innate and adaptive immune systems.3,4 As a consequence, IFN-β has been used as a therapy for several diseases, including viral infections, cancer, and multiple sclerosis. Several studies have shown that IFN-β can downregulate IFN-γ-induced MHC class II expression on monocytes in vitro,5 inhibit T cell proliferation,6 and stimulate NK cells and hence antibody-dependent cytotoxicity. IFN-β has also been reported to reduce the production of IL-12, which may inhibit Th1 development.

Recently, IFN-β was shown to be effective in the treatment of renal disease in a mouse model of lupus in MRL-Faspr mice.7 In this model, IFN-β reduced proteinuria, improved renal function, and prolonged survival. This was associated with re-
duced glomerular hypercellularity and tubular damage. In addition to its role in inflammation, IFN-β may modulate mediators of fibrosis by inhibiting fibroblast proliferation and collagen synthesis. In vivo, IFN-β inhibited fibrosis in a mouse model of bleomycin-induced pulmonary fibrosis.

In view of the potential benefits of IFN-β on glomerular injury and fibrosis, we examined its effects in three distinct models of glomerular injury in rats: Nephrotoxic nephritis (NTN), Thy-1 nephritis, and puromycin aminonucleoside nephrosis (PAN). Our results show a striking reduction of proteinuria in all three models, but this was not accompanied by major effects on glomerular inflammation. We therefore developed the hypothesis that there may be direct effects of IFN-β on cells of the glomerular filtration barrier, glomerular endothelial cells (GEnC), and podocytes, and we went on to test this hypothesis in vitro. Monolayers of human cells were grown on porous supports or electrode arrays, and the effects of IFN-β1a on barrier properties were assessed by measurement of transmonolayer electrical resistance (TMER) and passage of labeled albumin across the cell monolayers.

**RESULTS**

**NTN**

As described previously, control rats developed focal and segmental necrotizing glomerulonephritis with extensive cellular crescents on day 14 (Figure 1) and progression to fibrocellular crescents and tubulointerstitial scarring by day 28 (Figure 2). In experiment A, in which rats were treated with IFN-β from days 0 to 28, there was a significant reduction, of up to 73%, in proteinuria at day 14 (treated 86 ± 0.4 mg/µmol; control 235 ± 17 µmol/L; control 266 ± 17 µmol/L; P < 0.001) and day 28 (treated 0.5 ± 0.3 mg/µmol; control 1.2 ± 0.2 mg/µmol; P < 0.001) (Figure 3A). Urinary protein creatinine ratio was also significantly reduced at day 14 (treated 3 ± 0.1 mg/µmol; control 3 ± 0.1 mg/µmol; P < 0.001) and day 28 (treated 0.5 ± 0.3 mg/µmol; control 1.8 ± 0.4 mg/µmol; P < 0.01). At day 14, there was a slight but significant reduction in glomerular macrophage numbers and bromodeoxyuridine (BrdU)-positive cells but no difference in histologic appearance or staining for α-smooth muscle actin (α-SMA) or fibronectin (Table 1, Figure 1). There was no difference between the groups in serum creatinine measured at day 14 (treated 86 ± 4 µmol/L; control 80 ± 5 µmol/L) or at day 28 (treated 235 ± 12 µmol/L; control 266 ± 17 µmol/L). At day 28, there was no difference in the amount of glomerular or tubulointerstitial scarring between the treated and control rats (mean tubulointerstitial scarring score 3.6 in each group). Neither was there any difference in glomerular staining for BrdU-positive cells or fibronectin, but there was an increase in macrophage numbers and a slight reduction in α-SMA staining (Table 1, histology not shown).

In experiment B, we tested whether delaying treatment until after disease was established would also affect proteinuria. As can be seen in Figure 3B, treatment that was started at day 14 led to a significant reduction, of up to 51%, in proteinuria at days 21 and 28; however, we saw no significant effects on histology, macrophage numbers, or α-SMA or fibronectin staining at day 28 (Figure 2, Table 1). Again, there was no difference between the groups in serum creatinine measured at day 14 (treated 79 ± 5 µmol/L; control 78 ± 6 µmol/L) or at day 28 (treated 159 ± 17 µmol/L; control 138 ± 17 µmol/L).

**Thy-1 Nephritis**

The results in the model of NTN indicated that IFN-β had a marked effect on proteinuria. We therefore tested whether the antiproteinuric effect would also be seen in a different immunologically mediated model of glomerulonephritis. Thy-1 nephritis is induced by an antibody that binds to glomerular mesangial cells, leading to complement-mediated mesangiolysis followed by mesangial repair. In rats that were treated with IFN-β, there was a significant reduction, of up to 66%, in proteinuria at days 7 and 10 (Figure 4). Urinary protein creatinine ratio was also reduced at day 10 (treated 0.4 ± 0.1 mg/µmol; control 1.1 ± 0.1 mg/µmol; P < 0.01). Histologic studies showed mesangial hypercellularity as anticipated, but IFN-β treatment had no effect on these appearances (Figure 5). No effects were seen on glomerular macrophage numbers, but there was a reduction in proliferating cells as identified by...
staining for BrdU and a modest reduction in α-SMA staining (Table 2).

PAN

PAN is a model in which direct toxic injury to glomerular cells leads to proteinuria. We were interested in determining whether IFN-β could also lead to a reduction in proteinuria in this model, which lacks a significant immunologic component. As shown in Figure 6, treatment with IFN-β led to a significant reduction, of up to 93%, in proteinuria at days 4 and 8. There was no difference between the groups in serum creatinine measured at day 8 (treated 77 ± 13 μmol/L; control 73 ± 5 μmol/L). No differences in glomerular histology were seen by light microscopy (data not shown). Inflammatory indices were not studied in detail in this model given its nonimmune pathogenesis. Electron microscopy revealed podocyte abnormalities including foot process effacement as expected, but there were no differences between IFN-β–treated and control rats (Figure 7). GEnC morphology seemed normal with preservation of fenestrations.

Effects of IFN-β1a on Cell Monolayers

GEnC monolayers developed a resistance of 13.1 Ω after 5 d, comparable with previous results, whereas conditionally immortalized podocytes (ciPod) at 33°C developed a resistance of 30.3 Ω. IFN-β1a caused an increase in TMER at 24 h in GEnC and ciPod monolayers of 9.7 and 9.0 Ω, respectively, relative to controls (Figure 8). IFN-β1a caused a corresponding decrease in passage of FITC-labeled BSA (FITC-BSA) across monolayers of both cell types (Figure 9). Passage over 3 h was reduced by 54.8 and 29.8% in GEnC and ciPod, respectively. IFN-β1a did not effect cell numbers of GEnC (treated 14,500 ± 1800 cells/cm²; control 13,600 ± 1500 cells/cm²) or podocytes (treated 24,800 ± 3000 cells/cm²; control 25,700 ± 1900 cells/cm²) over 24 h, confirming that observed effects on TMER were not attributable to effects of IFN-β on cell density. Treatment with EGTA substantially reduced the TMER of treated and control monolayers of GEnC and podocytes, confirming that the barrier properties of IFN-β–treated monolayers remain dependent on cell–cell adhesion.

Time course analysis of the effect of IFN-β1a on TMER of GEnC monolayers using the Electrical Cell-Substrate Impedance Sensing (ECIS) system demonstrated an initial decrease in resistance, persisting for the first 5 h, followed by a gradual increase in TMER during the subsequent 19 h (Figure 10). These results are consistent with those from the insert experiments. Further experiments showed that this enhanced resistance reaches a maximum at 54 h and is maintained for at least 96 h (data not shown). Resistance modeling showed that the
paracellular resistance (Rb) in monolayers that were treated with IFN-β1a for 24 h was 6.4 ± 0.5 Ω/cm² compared with 0.6 ± 0.1 Ω/cm² in untreated monolayers, whereas α (the resistance between the cells and the electrode) was 6.0 ± 0.2 and 7.3 ± 0.2 cmΩ^0.5^, respectively (Figure 11).

**DISCUSSION**

The established immunomodulatory and antifibrotic effects of IFN-β led us to examine whether it would improve disease in glomerular injury in the rat. We initially studied NTN in the WKY rat, a model of crescentic glomerulonephritis mediated by antibody deposition on the glomerular basement membrane with subsequent macrophage influx and crescent formation. We hypothesized that IFN-β would lead to a reduction in glomerular inflammation and in subsequent tubulointerstitial scarring. In fact, we found only very minor effects on inflammation, with small and inconsistent effects on glomerular macrophage numbers, and no effect on renal function or scarring; however, there was a large and sustained reduction in proteinuria. We also found that when treatment was delayed until disease was established, there was a rapid reduction in proteinuria once IFN-β was given. We therefore investigated whether this effect on proteinuria would be seen in other models and confirmed that in both Thy-1 glomerulonephritis and puromycin-induced glomerular injury, IFN-β reduced proteinuria with only minor effects on histology. Because PAN is the result of direct toxicity to cells of the glomerular filtration barrier, with no significant immunologic component, the antiproteinuric effect of IFN-β in this model at least may be independent of its immunomodulatory actions.

These experiments were designed to assess the effects of IFN-β in the acute stage of glomerulonephritis in terms of histologic changes and proteinuria. The question of whether the profound reduction in proteinuria observed will translate into improved recovery of glomerulonephritis remains open.

**Table 1. Effect of IFN-β treatment of NTN on glomerular injury and histology**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experiment A Day 14</th>
<th>Experiment A Day 28</th>
<th>Experiment B Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages/gcs</td>
<td>IFN-β Treated: 19 ± 0.9^b^</td>
<td>IFN-β Treated: 13 ± 1.0^b^</td>
<td>IFN-β Treated: 6.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Vehicle Treated: 22 ± 1.2</td>
<td>Vehicle Treated: 8.0 ± 0.7</td>
<td>Vehicle Treated: 5.0 ± 0.4</td>
</tr>
<tr>
<td>α-SMA %area/gcs</td>
<td>IFN-β Treated: 29 ± 2.0</td>
<td>IFN-β Treated: 30 ± 1.2^b^</td>
<td>IFN-β Treated: 34 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Vehicle Treated: 29 ± 2.5</td>
<td>Vehicle Treated: 37 ± 3.7</td>
<td>Vehicle Treated: 34 ± 2.8</td>
</tr>
<tr>
<td>ED(A)/FN %area/gcs</td>
<td>IFN-β Treated: 36 ± 5.0</td>
<td>IFN-β Treated: 38 ± 3.5</td>
<td>IFN-β Treated: 35 ± 3.9</td>
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<tr>
<td></td>
<td>Vehicle Treated: 36 ± 5.0</td>
<td>Vehicle Treated: 41 ± 3.1</td>
<td>Vehicle Treated: 32 ± 2.6</td>
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<tr>
<td>BrdU^+ cells/gcs</td>
<td>IFN-β Treated: 2.5 ± 0.3^b^</td>
<td>IFN-β Treated: 1.4 ± 0.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Vehicle Treated: 4.0 ± 0.4</td>
<td>Vehicle Treated: 1.4 ± 0.2</td>
<td>–</td>
</tr>
</tbody>
</table>

^a^Rats received rat serum albumin control or 6 × 10⁵ U/d rat IFN-β from day 0 (experiment A) or from day 14 (experiment B). gcs, glomerular cross-section. ^b^P < 0.05.
because rats were not studied for long enough to address this issue. In NTN and PAN, there were no significant differences in serum creatinine between treatment and control groups, consistent with the similarity in histologic appearances; however, there is considerable evidence that proteinuria itself is important in progression of renal disease. IFN-β therefore would be expected to improve long-term renal function at least through reduction in proteinuria if not also by other potential mechanisms, including the presumably glomerular pathways through which IFN-β reduced proteinuria. In the murine model of lupus referred to previously, IFN-β improved renal function and prolonged survival in addition to reducing proteinuria.

In view of consistent effects in reducing proteinuria in these animal models but modest effects on markers of inflammation, we hypothesized that IFN-β may have direct effects on the cells of the glomerular filtration barrier. Using techniques that we developed previously using human GEnC monolayers, we studied the barrier properties of human podocytes in culture for the first time and the responses of both GEnC and podocytes to human IFN-β1a.

IFN-β1a caused a marked increase in TMER of monolayers of both GEnC and podocytes. TMER is a measure of ion flux and is inversely related to the fractional area of pathways open to water and small molecules across a cell monolayer. An increase in resistance suggests a reduction in such pathways. The observed increase of 9.7 Ω in the GEnC monolayers compares...
favorably to the maximal response to angiopoietin-1 (5.7 μg), one of few other mediators that are known to reduce endothelial permeability. IFN-β1a caused a corresponding reduction in FITC-BSA passage across GEnC monolayers with a 54.8% reduction (n = 7; P < 0.001) and 29.8% (n = 5; P < 0.05) in GEnC and ciPod, respectively.

figures 9, 10, 11

Figure 9. The affect of 24-h treatment with IFN-β1a (1000 IU/ml) on passage of FITC-labeled BSA through GEnC or ciPod monolayers over time, measured using a tissue culture insert system. IFN-β1a reduced passage of FITC-BSA over 3 h by 54.8% (n = 7; P < 0.001) and 29.8% (n = 5; P < 0.05) in GEnC and ciPod, respectively.

Figure 10. Effect of treatment with IFN-β1a (1000 IU/ml) on TMER of GEnC monolayers over time measured using the ECIS biosensor. IFN-β1a or control was added to monolayers at time 0, and resistance was measured every 10 min for 24 h. IFN-β1a caused an initial decrease and subsequent substantial increase in resistance relative to control (n = 8; P < 0.001).

Figure 11. Effect of 24 h treatment with IFN-β1a (1000 IU/ml) on relative contributions to the overall resistance of GEnC monolayers of the resistance of the paracellular pathway (Rb; A) and the resistance between the cell surface and the electrode (α; B). Note that Rb and α are not measured in the same units and therefore cannot be compared directly. After IFN-β1a treatment, Rb in treated monolayers was 6.4 ± 0.5 Ω/cm² compared with 0.6 ± 0.1 Ω/cm² in untreated monolayers (n = 8; P < 0.001), whereas α was 6.0 ± 0.2 and 7.3 ± 0.2 cmΩ⁰.⁵ (n = 8; P < 0.005), respectively. IFN-β1a treatment increased Rb but not α.

These observations of a reduction in two indicators of permeability in both types of cell of the glomerular filtration barrier support the hypothesis that some of the effects of IFN-β on proteinuria in these animal models may be mediated by direct effects on these cell types.

Further detailed time course analysis using the ECIS biosensor showed interestingly that IFN-β1a caused an initial decrease in TMER followed by a substantial increase, maintained for at least 96 h. The explanation for this initial decrease is not apparent but will be the subject of further study. Resistance modeling confirmed that IFN-β1a increased monolayer integrity through increasing resistance of the paracellular pathway (Rb, dependent on intercellular junctions) rather than by increasing resistance between the cell and the electrode (α).

IFN-β was previously shown to have similar effects in stabilizing the barrier properties of brain EnC¹⁴ and human umbilical vein EnC¹⁵ in culture. These effects seem to be mediated through increased integrity of intercellular junctions by up-regulation of junctional adhesion molecules¹⁶ or resistance to their downregulation by proinflammatory mediators.¹⁵,¹⁷
Such mechanisms are thought to be important in the beneficial effects of IFN-β in multiple sclerosis, reducing both edema formation and recruitment of activated T cells by preserving the integrity of the blood–brain barrier.\textsuperscript{14,18} Although this pathway of enhanced intercellular junction integrity in response to IFN-β1a is likely to be important in the GEnC studied, other possible mechanisms for the observed effects on barrier properties in these cells should also be considered. A decrease in the density of fenestrations would also be expected to cause an increase in TMER and possibly a reduction in macromolecular passage; however, we saw no effects of IFN-β1a on fenestrations in the \textit{in vivo} studies. Another possibility is that IFN-β1a has enhanced the barrier properties of the GEnC glycocalyx, which also contributes to the filtration barrier but is not preserved during standard fixation techniques for electron microscopy.\textsuperscript{19}

Studies of podocyte barrier function \textit{in vitro} are problematic because podocytes tend to dedifferentiate in culture. Although the human ciPod that we developed can be induced to differentiate at the nonpermissive temperature,\textsuperscript{20} they do not form restrictive monolayers in this state. One group\textsuperscript{21} studied monolayers of rat podocytes and demonstrated that similar experiments can successfully be used to study podocyte behavior despite podocyte dedifferentiation. We have taken a similar approach with human ciPod, using the cells at the permissive temperature, in a relatively undifferentiated form, because they produce a monolayer with a measurable resistance in this state. Although it is uncertain how closely the barrier properties of these cells \textit{in vitro} parallel behavior of podocytes \textit{in vivo}, our observations clearly demonstrate that podocytes can respond directly to IFN-β. Furthermore, this is in a manner that tends to reduce permeability of a cell layer and hence is consistent with an action in reducing proteinuria \textit{in vivo}.

As with GEnC, the most likely mechanism of enhanced barrier properties of podocytes \textit{in vitro} is through enhanced cell–cell junction integrity. Because the specialized cell–cell junction of the podocyte, the slit diaphragm, is thought to be the most restrictive barrier to macromolecules, maintenance of this structure is a candidate for the mechanism of action of IFN-β on podocytes \textit{in vivo}; however, we did not see evidence of an effect of IFN-β on foot process effacement. Future studies will be designed to address in more detail these questions regarding the mechanism of action of IFN-β at the ultrastructural level in both GEnC and podocytes.

IFN-β has not been studied in the treatment of human renal disease, but the related type 1 IFN, IFN-α, has been used in an attempt to treat glomerulonephritis in small uncontrolled studies with some promising results.\textsuperscript{22} IFN-α is also used in the treatment of hepatitis and hematologic malignancies. There are reports of amelioration of proteinuria in patients with hepatitis C and mesangiocapillary glomerulonephritis\textsuperscript{23} and in a patient with chronic myeloid leukemia and FSGS.\textsuperscript{24} However, in these cases, it is difficult to distinguish direct effects of IFN-α on proteinuria from those mediated through amelioration of the underlying disease. Treatment with IFN-α has been associated with development of proteinuria, although, at least in the context of hepatitis C, this may represent exacerbation of preexisting glomerulonephritis.\textsuperscript{25} Development of nephrotic syndrome with histologic types including minimal-change disease and FSGS has also been described; however, such sporadic cases seem to be due to individual idiosyncrasies rather than to predictable dosage-dependent effects. There may be important differences between IFN-β and IFN-α in this respect, because we could identify only one case of nephrotic syndrome developing in response to IFN-β despite its wide use in the treatment of multiple sclerosis.\textsuperscript{26}

We have demonstrated dramatic effects of IFN-β in the reduction of proteinuria in three distinct animal models of glomerulonephritis, and we have shown corresponding profound effects on human glomerular cells. Our observations \textit{in vivo} and \textit{in vitro}, taken together with previous reports, suggest that IFN-β has therapeutic potential for proteinuric disease. Because its beneficial effects are mediated at least in part by effects on intrinsic glomerular cells rather than on infiltrating inflammatory cells, such therapeutic benefits could extend to noninflammatory forms of proteinuric disease, including diabetic nephropathy, the single most important cause of progressive renal insufficiency. Chronic renal disease is common: Figures suggest that as many as one in nine of the adult population of the United States is affected,\textsuperscript{27} and a study from Australia reported that proteinuria is present in 6.6% of seemingly healthy adults.\textsuperscript{28} Novel therapies to reduce proteinuria are therefore urgently needed, and our results suggest that IFN-β deserves further evaluation in this regard.

\section*{CONCISE METHODS}

\subsection*{Animals}
Male Wistar-Kyoto, Lewis, and Wistar rats were obtained from Charles River (Margate, UK). All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

\subsection*{Cells}
Human GEnC that were derived from decapsulated glomeruli that were isolated from normal kidney were obtained at passage 2 from the Applied Cell Biology Research Institute (ACBRI, Kirkland, WA). We previously characterized these cells in detail.\textsuperscript{13} Cells were cultured in EGM2-MV (Cambrex, Wokingham, UK). Cells that were being prepared for or being used in experiments were cultured in EGM2-MV without vascular endothelial growth factor. Cells were used in experiments up to passage 8. Human ciPod were cultured in RPMI-1640 – based medium as described previously\textsuperscript{20} unless stated otherwise. ciPod proliferate at the permissive temperature of 33°C and enter growth arrest and become increasingly differentiated when transferred to the nonpermissive temperature of 37°C.
Reagents

Nephrototoxic serum, a rabbit antiserum to rat glomerular basement membrane, was prepared as described previously. The Thy-1 monoclonal (IgG2) antibody (ER4), raised against the rat transmembrane glycoprotein Thy-1, was provided by Dr. W.M. Bagchus (University of Groningen, Groningen, Netherlands). Puromycin aminonucleoside was purchased from Sigma (Poole, UK). Recombinant, Chinese hamster ovary–expressed, rat IFN-β and recombinant human IFN-β1a and control (human serum albumin carrier protein) were supplied by Biogen Idec (Cambridge, MA). Rat serum albumin was obtained from Sigma and was purified by size-exclusion chromatography with pyrogen-free PBS as the mobile phase to remove endotoxin.

Induction of Renal Disease

NTN was induced in WKY rats by a single intravenous injection of 0.1 ml of nephrototoxic serum. Thy-1 nephritis was induced in Lewis rats by a single intravenous injection of the mAb ER4 at a dosage of 2.5 mg/kg body wt. PAN was induced in Wistar rats by a single intravenous injection of puromycin aminonucleoside at a dosage of 100 mg/kg body wt.

Experimental Design

NTN.

The first experiment (experiment A) was designed to test the effect of rat IFN-β given from the time of induction of NTN. NTN was induced in 32 rats. Sixteen were treated with recombinant IFN-β, 6 d/wk, at a dosage of 6 × 10^5 U/d by intraperitoneal injection, and 16 control rats received an equivalent volume of rat serum albumin carrier alone intraperitoneally. The dosage of IFN-β was established from preliminary dosage-response experiments. Proteinuria was measured weekly. Half of each group were killed at day 14 and half at day 28. Experiment B was designed to examine the effect of delayed treatment started at the peak of glomerular crescent formation. NTN was induced in 16 rats; eight were treated with IFN-β 6 × 10^5 U/d intraperitoneally from days 14 to 28, and eight received rat serum albumin intraperitoneally.

Thy-1 Nephritis.

Thy-1 nephritis was induced in 15 rats. Eight were treated with rat IFN-β 6 × 10^5 U/d intraperitoneally from days 0 to 10, and the others were given rat serum albumin. Proteinuria was measured on days 7 and 10, and rats were killed on day 10.

PAN.

PAN was induced in 12 rats. Six were treated with IFN-β 6 × 10^5 U/d intraperitoneally, from days 0 to 8. Control rats received rat serum albumin intraperitoneally. Proteinuria was measured on days 4 and 8, and rats were killed on day 8.

Assessment of Renal Disease

In all experiments, urine was collected at intervals by housing rats in metabolic cages for 24-h periods with free access to food and water. Urinary protein was measured by the sulfosalicylic acid method. The laboratory normal range for proteinuria in these rats is <10 mg/24 h. Serum and urinary creatinine concentrations were measured using an Olympus AU600 analyzer (Olympus, Eastleigh, UK). One hour before being killed, all rats were administered an intraperitoneal injection of 50 mg/kg BrdU (Sigma). Rats were killed while under general anesthesia induced with isoflurane, and samples of kidney, lung, liver, and spleen were fixed in both 10% formal saline and methyl Carnoy’s fixative and then embedded in paraffin. Kidney tissue was also snap-frozen in isopentane and then immersed in liquid nitrogen and stored at −70°C.

Histology, Immunohistochemistry, and Electron Microscopy

Tissue sections were stained with periodic acid-Schiff reagent and hematoxylin and eosin. Glomerular and tubulointerstitial scarring was scored on a semiquantitative scale from 0 to 4+. Paraffin-embedded sections were used for immunohistochemical detection of rat macrophages/monocytes (mAb ED1; Serotec, Oxford, UK) and α-SMA (mAb IA4; Dako, Glostrup, Denmark) as a marker of profibrotic changes, expressed by myofibroblasts. Antigen retrieval was carried out by heating paraffin sections in 10% citrate buffer in a microwave oven, three times, each for 5 min. Endogenous peroxidase and nonspecific binding were blocked. The primary antibodies were used at the following concentrations: ED1 at 1:500 and α-SMA at 1:1000. Antibodies were detected by biotinylated rabbit anti-mouse or biotinylated goat anti-rabbit secondary antibody (Dako) followed by avidin-biotin complex (Dako) with the chromogenic substrate 3,3′-diaminobenzidine (Dako). Sections were counterstained with hematoxylin and mounted. Proliferating cells were detected by immunohistochemical staining of Carnoy’s fixed tissue, and sections were preheated in 1 M HCl for 5 min followed by BrdU primary antibody (1:50), Dako, avidin-biotin complex, and 3,3′-diaminobenzidine. ED(A) fibronectin [ED(A)FN, IST-9, 1:1000; Harlan Sera-Lab, Loughborough, UK), a marker of extracellular matrix deposition, was detected by staining frozen sections. ED1- and BrdU-positive cells were counted in a blinded manner in 50 consecutive glomeruli, and results are expressed as mean number of cells per glomerular cross-section. Quantification of α-SMA and ED(A)FN was by computer-aided image analysis of 20 consecutive cross-sectional glomeruli using Image Pro-Plus software (Media Cybernetics, Silver Spring, MD).

For electron microscopy, 2-mm blocks of cortex were fixed in 3% glutaraldehyde, postfixed in 2% aqueous osmium tetroxide, and embedded in Spurr’s resin. Ultrathin sections (0.5 μm) were stained with 1% aqueous uranyl acetate and Reynold’s lead citrate and examined on a Philips EM300 transmission electron microscope.

Effect of IFN-β1a on Electrical Resistance of Cell Monolayers

Polycarbonate supports (0.4-μm pore size, 0.5 cm² surface area) in tissue-culture inserts (1 cm diameter; Nalge Nunc Int., Rochester, NY) were seeded with GEnC or ciPod at 33°C, at 100,000 cells/cm². After 5 d, TMER of cell monolayers was measured as described previously using an Endohm 12 electrode chamber and an EVOMx voltmeter (World Precision Instruments, Sarasota, FL). Tissue culture inserts were placed sequentially in the chamber, and the resis-
tance was recorded after 10 s. Cell monolayers were then treated with 1000 IU/ml IFN-β1a or vehicle control in usual medium for 24 h, and TMER was remeasured. ciPod were used at 33°C because in their more differentiated state at 37°C, the monolayer loses integrity (S.C.S., unpublished observations, 2006). Corresponding control experiments were set up to investigate whether IFN-β1a affected cell number because cell density may affect TMER. Cells in culture flasks were treated with IFN-β1a or control for 24 h before trypsinization and counting. In further control experiments, after treatment with IFN-β for 24 h and measurement of TMER as already described, monolayers were treated with 5 mM EGTA to disrupt calcium-dependent cell–cell adhesion, and TMER was remeasured after 10 min.

**Effect of IFN-β1a on Protein Passage across Cell Monolayers**

Transmonolayer permeability to macromolecules was assessed by measurement of passage of FITC-BSA (Sigma) across the monolayer essentially as described previously. GEnC and ciPod monolayers were prepared in tissue culture inserts and treated with IFN-β1a as already described. After 24 h, FITC-BSA was added to medium within the inserts to give a final concentration of 100 μg/ml. Unlabeled BSA was added to the medium in the well to the same concentration. At 1, 2, and 3 h, 100-μl aliquots were removed and replaced with 100 μl of serum-free medium containing unlabeled BSA (100 μg/ml). The fluorescence emission of the aliquots at 520 nm, after excitation at 490 nm, was measured on a Packard Instruments FluoroCount fluorospectro-photometer (available through PerkinElmer Life Sciences, Wellesley, MA), and the concentration of FITC-BSA was calculated by reference to a set of standard dilutions.

**Time-Course Analysis of the Effect of IFN-β1a on Electrical Resistance of GEnC Monolayers and Estimation of Cell–Cell Gaps**

Time course analysis and resistance modeling were performed using an automated cell monitoring system, ECIS 1600R (Applied Biophysics, Rochester, NY). Briefly, GEnC were seeded onto gold microelectrodes in wells of ECIS arrays (eight wells, 10 electrodes per well) at the same density as used in the insert experiments. Confluent cell monolayers were treated with 1000 IU/ml IFN-β1a or vehicle control in usual medium. With the use of the ECIS attachment mode, resistance was measured at regular time intervals, from the point of addition of IFN-β1a, for up to 96 h. The ratio of measured resistance to baseline resistance was calculated for each well and plotted as a function of time, normalized to control. The change of resistance is attributed to changes of the resistance in paracellular pathway (Rb) and the resistance between the ventral cell surface and the electrode). From the changes of Rb and α, relative changes in cell-to-cell and cell-to-substrate gaps induced by IFN-β1a may be inferred.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism (GraphPad Prism Software, San Diego, CA) or SPSS 11.0 (SPSS, Chicago, IL). Comparison between groups of rats was performed by one-way ANOVA with individual group means. Comparisons in cell culture experiments were made by t-test or repeated measures ANOVA. Results are means ± SEM.

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**DISCLOSURES**

At the time of the study, D.P.B. and R.R.L. were employees of Biogen Idec Inc.

**REFERENCES**


IFN-β Reduces Proteinuria


