Inhibition of Interleukin-10 by the Immunomodulator AS101 Reduces Mesangial Cell Proliferation in Experimental Mesangioproliferative Glomerulonephritis

ASSOCIATION WITH DEPHOSPHORYLATION OF STAT3*

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Mesangial cell (MC) proliferation is essential for the pathogenesis and progression of glomerular disease. Using an acute model of mesangial proliferative glomerulonephritis (Thy1 GN), we show that neutralization of interleukin (IL)-10 greatly ameliorated the disease as expressed by both decreased MC expansion and proteinuria. Treatment with the tellurium compound AS101 (ammonium trichloro(dioxoethylene- o-o')tellurate) resulted in favorable effects provided that the compound was administered 24 h before insult, whereas partial effects were obtained when administered after insult. We identified STAT3 as playing a pivotal role in IL-10-induced MC proliferation in vitro and in vivo. IL-10 activated MC STAT3 in vitro as expressed by its phosphorylation and nuclear translocation. The role of STAT3 in MC proliferation induced by IL-10 was deduced from results showing that IL-10-induced proliferation was abrogated if MC transfected with STAT3 antisense oligonucleotides were used or if cells were incubated with inhibitors of STAT3. AS101 deactivates STAT3 in control but not in MC transfected with IL-10 antisense oligonucleotides. Inactivation of STAT3 prevents reduction of MC proliferation by AS101. We further demonstrate the role of STAT3 in the regulation of cell cycle and survival regulatory proteins by AS101 in MC via inhibition of IL-10. IL-10 increased MC expression of Bel-2 and Bcl-XI and simultaneously decreased the levels of p27kip1. These survival factors were decreased by AS101 in a STAT3- and IL-10-dependent manner, whereas p27kip1 was similarly increased. In Thy1 GN, phosphorylated STAT3 in glomerular MC peaked at day 6 and correlated with MC expansion. Neutralization of IL-10 or its inhibition by AS101 abolished phosphorylation of STAT3. This effect positively correlated with amelioration of the disease. These in vitro and in vivo studies indicate that the autocrine MC growth factor IL-10 induces MC proliferation via STAT3. We suggest that IL-10 or its downstream target STAT3 might be therapeutic targets for kidney diseases induced by mesangial proliferation.

Cellular proliferation accompanies a wide variety of renal diseases. This is especially true for proliferative forms of glomerulonephritis (GN)1 (1). Excessive proliferation of mesangial cells is usually associated with matrix expansion, leading to the development of glomerular sclerosis (2). Mesangial cells proliferate in response to a variety of growth factors and cytokines such as platelet-derived growth factor (PDGF), basic fibroblast growth factor, and interleukin-6 (3–5). Recently, we showed that IL-10 is a potent mesangial autocrine growth factor that plays a pivotal role in rat MC proliferation in vitro (6). Therefore, its inhibition by the non-toxic tellurium anti-IL-10 compound, ammonium trichloro(dioxoethylene-o-o')tellurate (AS101), extensively decreased proliferation of these cells (6).

IL-10 first recognized for its ability to inhibit activation and effector function of T cells, monocytes and macrophages, is a multifunctional cytokine with diverse effects on a variety of cell types. The principal function of IL-10 appears to be limitation and ultimately termination of inflammatory responses. In addition, IL-10 regulates growth and/or differentiation of B cells, natural killer cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells (7). The best-characterized IL-10 signaling pathway is the JAK/Signal transducer and activator of transcription (STAT) system. The IL-10/IL-10R interaction engages the Jak family tyrosine kinases JAK1 and Tyk2 and induces tyrosine phosphorylation and activation of the latent transcription factors STAT3 and STAT1 (8). The STATs comprise a family of functionally related proteins that play key roles in a variety of biological activities such as cell differentiation and proliferation. They exert their activities through cytokine and growth factor receptors and are believed to be involved in determining the biological specificity of specific cytokines on various target tissues (9). The molecular events that underlie cell proliferation are tightly regulated in a cell cycle-dependent manner. The cell division cycle is coordinated by the activation and inactivation of the cyclin-dependent kinases (CDKs) (10). The G1 to S phase transition in the cell cycle is thought to be controlled by CDKs that are sequentially regulated by cyclins D, E, and A. These are negatively regulated by two distinct families of CDK

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1 The abbreviations used are: GN, glomerulonephritis; PDGF, platelet-derived growth factor; IL, interleukin; STAT, signal transducers and activators of transcription; AS101, ammonium trichloro(dioxoethylene-o-o')tellurate; JAK, Janus kinase; CDK, cyclin-dependent kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; FITC, fluorescein isothiocyanate; αSMA, α-smooth muscle actin; Ab, antibody; I.P., intraperitoneal; RMC, rat mesangial cell; MC, mesangial cell; p, phospho; ODN, oligonucleotide.
inhibitors. One of these includes p21<sup>cip</sup>, p27<sup>kip1</sup>, and p57<sup>kip2</sup>, which bind and inhibit cyclin D-, E-, and A-dependent kinases (11). STAT3 has been previously reported to play an important role in the G<sub>1</sub> to S phase transition through the up-regulation of cyclins D and A and the concomitant down-regulation of p21 and p27 (12). STAT proteins are latent transcription factors that become activated by phosphorylation of a single tyrosine, which causes STAT proteins to dimerize. STAT dimerization is usually followed by translocation to the nucleus (13). Within the nucleus, STAT proteins recognize and bind to consensus DNA binding sites that represent enhancer sequences for a variety of genes. STAT3 activation has been implicated in the proliferation of cells (14).

The non-toxic immunomodulator AS101 first developed by us has been shown to have beneficial effects in diverse preclinical and clinical studies. Most of its activities have been primarily attributed to the direct inhibition of the anti-inflammatory cytokine IL-10 (15–17). This immunomodulatory property was found to be crucial for the clinical activities of AS101, demonstrating the protective effects of AS101 in parasite and viral-infected mice models (18), in autoimmune diseases (19), in septic mice (20), and in a variety of tumor models in mice and humans where AS101 had a clear anti-tumoral effect (21–23). More importantly, AS101 was previously shown to delay the onset of autoimmune manifestations in a murine model of lupus erythematosus, reduce the level of immune complex deposition in the glomeruli, reduce proteinuria, prevent glomerular hypercellularity and mesangial expansion, and reduce the mean glomerular volume of treated mice (19). Furthermore, in a murine model of septic peritonitis, AS101 was recently shown to prevent kidney damage of septic mice (20).

Given our recent findings that IL-10 is a potent mesangial autocrine growth factor and the ability of AS101 to reduce MC proliferation in vitro via inhibition of IL-10, this study primarily aimed to explore the role of each one of them in experimental mesangioproliferative glomerulonephritis. In addition, because it has been recently demonstrated that in the Thy1 model STAT3 is phosphorylated in glomerular mesangial cells (24), this study also aimed to examine whether the beneficial effects of AS101 are associated to the deactivation of STAT3 via IL-10 inhibition. Furthermore, we studied the implications of this activity on the regulation of cell cycle regulatory proteins in vivo.

**MATERIALS AND METHODS**

**Animals**

Male Wistar rats (8–10 weeks old, 150–170 g) were purchased from Harlan Laboratories (Jerusalem, Israel). Animal experiments were performed in accordance with approved institutional protocols and approved by the Institutional Animal Care and Use Committee.

**Experimental Rat Anti-Thy1.1-induced Glomerulonephritis**

Anti-Thy1.1 glomerulonephritis was induced by an intravenous injection of 1 mg/kg anti-Thy1.1 (OX-7, Cedarlane, Ontario, Canada). Rats were treated as follows: daily I.P. injection with PBS with no OX-7 administration (vehicle control) (n = 6); daily injection with PBS (control anti-Thy1) (n = 8); daily I.P. injection with AS101 (0.5 mg/kg) (supplied by Prof. M. Albeck from the Department of Chemistry, Bar-Ilan University, Ramat Gan, Israel) starting 24 h before OX-7 administration (AS101 before) (n = 10); daily I.P. injection with AS101 (0.5 mg/kg) starting 24 h after OX-7 administration (AS101 after) (n = 9); daily I.P. injection with goat anti-rat IL-10-neutralizing IgG (R&D Systems, Minneapolis, MN) (250 μg/injection) starting 24 h before OX-7 administration (anti-IL-10 before) (n = 3); daily I.P. injection with goat anti-rat IL-10-neutralizing IgG (250 μg/injection) starting 24 h after OX-7 administration (anti-IL-10 after) (n = 3); and daily I.P. injection with isotype-matched goat anti-rat IgG (250 μg/injection) (R&D Systems) starting 24 h before OX-7 administration (goat IgG) (n = 3).

Rats were housed in individual metabolic cages with free access to food and water. The weight of rats in the various groups was similar at day 0 and at day 6 after OX-7 administration. A 24-h urine collection was obtained for determination of protein excretion on days 0, 2, 4, and 6. Following nephrectomy, tissues were fixed in formalin for histology and immunohistochemistry or were further processed for isolation of glomeruli.

**Isolation of Glomeruli**

Glomeruli were isolated from the renal cortex of rats using the differential sieving method. The purity of glomeruli was >95% (9).

**MC Cultures**

Two sources of rat glomerular MC were used for in vitro studies. 1) An immortalized rat mesangial cell line (RMC) was kindly provided by Dr. M. Allenberg (Department of Medicine, Toronto University, Toronto, Canada). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. 2) Primary rat glomerular mesangial cells of glomeruli from rat kidneys were isolated. RMCs were cultured in DMEM supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. RMCs were used between passages 5 and 10.

**Clonogenic Assay**

Following 24 h of serum starvation, cells were disrupted by repeated aspiration through a 26-gauge needle, counted, and plated at 1000 cells/plate in DMEM supplemented with 10% fetal calf serum and incubated for macroscopic colony formation. Following 4–5 days of incubation, colonies were fixed with methanol, stained with Giemsa, and counted.

**<sup>3</sup>H/Thymidine Uptake**

RMCs were cultured in flat-bottomed 96-well plates at 1.10<sup>5</sup> cells/well in medium containing 10% fetal calf serum. After 24 h, cells were growth-arrested for 48 h in medium containing 0.5% fetal calf serum. MCs were then exposed for 24 h to fresh medium without or with 2% fetal calf serum in the presence of specific components. Cells were pulsed with 1 μCi/ml [3H]thymidine for the last 24 h.

**Immunoprecipitation and Western Blot Analysis**

Total, cytoplasmic, or nuclear cell extracts and immunoprecipitation were performed as described previously (22). P27<sup>kip1</sup>, Bcl-2, and Bcl<sub>x</sub><sup>L</sup> antibodies were from Santa Cruz Biotechnology with the exception of STAT3 and pSTAT3 (New England Biolabs, Beverly, MA).

**Transfection of Antisense ODN**

Phosphothioate-modified antisense or mismatch control ODNs were purchased from MWG-Biotech AG (Ebersberg, Germany) and dissolved in water. The ODNs had the following sequences and positions: AS IL-10, 5′-CATTTCCTGACAAGGCTTGG-3′; Control to AS IL-10, 5′-CCCAAGCCTTGGCAAGAATTG-3′; AS STAT3, 5′-GCAAGGAATCGCGAGACGAC-3′; and Control to AS STAT3, 5′-AGTTCAAGGCCAGGTCCG-3′. For assessment of transfection efficiency, cells lysates were harvested and analyzed by Western blot analysis for STAT3 protein expression. Polycationic transfection reagent (LipofectAMINE, Invitrogen) was used to facilitate uptake of ODN according to the protocol recommended by the manufacturer.

**Histopathologic Examination**

**Light Microscopy**—Resected kidneys were cut by a coronal section through the midportion of the kidney. One-half was fixed in 10% buffered formalin, paraffin blocks were prepared, and several 3–10 μm sections were cut from each block and stained with hematoxylin-eosin and periodic acid Schiff stains.

**Morphometry**—Glomerular volumes were evaluated using stereologic methods (24). The mean glomerular tuft area (A<sub>gl</sub>) was estimated using a digitizing tablet by tracing the outline of the glomerular tuft of 30 non-globally sclerotic glomeruli from each animal on periodic acid Schiff-stained sections. A point-counting method was utilized to estimate the percentage of the glomerular tuft occupied by mesangium or sclerosis (the mesangial fractional volume V<sub>mes</sub>) and the percentage of the glomerular tuft occupied by nuclei (the nuclear fractional volume V<sub>nuc</sub>). For assessment of transfection efficiency, cells lysates have been prepared and analyzed by Western blot analysis for STAT3 protein expression. Polycationic transfection reagent (LipofectAMINE, Invitrogen) was used to facilitate uptake of ODN according to the protocol recommended by the manufacturer.

**Calculations**—Mean glomerular tuft volume (V<sub>gl</sub>) was calculated as:

\[ V_{gl} = (\beta \times A_{gl})^{3/2} \]

where \( \beta = 1.38 \) is the shape coefficient for spheres and \( k = 1.1 \) is the size distribution coefficient. The mean glomerular mesangial volume (V<sub>mes</sub>) was calculated as:

\[ V_{mes} = V_{mes} \times V_{gl} \]
Amelioration of GN by Inhibition of IL-10

Paraffin sections were prepared and stained with mouse anti-human α-smooth muscle actin (αSMA) antibody (Zymed Laboratories Inc., San Francisco, CA) and the avidin-biotin complex-staining kit. Diaminobenzidine tetrahydrochloride was used as substrate. Each slide was counterstained with hematoxylin. Controls included omitting the primary antibody or its substitution with mouse IgG.

Immunofluorescent Double and Single Staining

Double immunostaining for αSMA and phospho-STAT3 was performed on snap-frozen cryostat sections (4 μm) using the indirect immunofluorescence procedure. Both primary antibodies (αSMA (SIGMA) and pSTAT3) were incubated overnight at 4 °C followed by the incubation of Texas red-conjugated anti-rabbit IgG (for pSTAT3) and FITC-conjugated anti-mouse IgG (for αSMA) (Jackson Immunoresearch Laboratories, West Grove, PA).

To examine the glomerular binding of the Thy1 antibody and local complement activation, detection of mouse IgG and rat C3 in glomeruli was carried out on frozen cryostat sections using direct immunofluorescence staining with either FITC-conjugated anti-mouse IgG (Jackson Immunoresearch) or FITC-conjugated anti-rat C3 (ICN Radiochemicals Inc., Costa Mesa, CA).

Statistical Analysis

Data are presented as mean ± S.E. Multiple comparisons were performed with the Kruskal Wallis analysis of variance. The Mann-Whitney U test was used for comparisons between each of the two groups. For comparisons within the groups, Wilcoxon-signed Rank test was performed. Student’s two-tail t <0.05 was considered significant.

RESULTS

Inhibition of IL-10 Attenuates Glomerular Mesangial Cell Expansion in Vivo—We have previously shown that IL-10 is a potent mesangial autocrine growth factor in vitro whose inhibition by AS101 dramatically reduces MC proliferation (6). We have extended these in vitro observations to an in vivo model of glomerulonephritis. We used an acute model of mesangial proliferative glomerulonephritis known as Thy1 GN. Activated mesangial cell expansion, as illustrated by positive staining for αSMA, was compared in glomeruli of the different treatment groups at day 6, which represents the peak of mesangial cell proliferation. Fig. 1A shows a prompt abundance of αSMA-expressing cytoplasm in the control anti-Thy1 group (b1), whereas no staining was seen in glomeruli of control healthy rats (a), implying a substantial increase in activated mesangial cells in glomeruli of diseased rats. The results show that treatment with the anti-IL-10 compound AS101 starting 24 h before anti-Thy1 administration greatly ameliorated the disease, decreasing the level of activated mesangial cell expansion to that seen in normal rats (c1). Nevertheless, treatment with AS101 24 h after insult did not affect considerably glomerular pathology (d and d1). The results depicted in Fig. 1A illustrate the extensive prevention of mesangial cell activation and proliferation by anti-IL-10 therapy (f1 and f2) and g1. Compared with the vast expansion of mesangial cells in glomeruli of isotype-matched-treated rats (e and e1), no staining of αSMA appeared in glomeruli of rats in which IL-10 was neutralized either 24 h before (f1) or 24 h after (g1) insult. Evaluation of IL-10 protein expression in lysates of isolated glomeruli dissected from rats at day 6 revealed high abundance of this cytokine in control glomeruli of anti-Thy1-treated rats as compared with healthy rats (Fig. 1B). IL-10 protein expression considerably decreased in glomeruli of both anti-IL-10 and AS101-treated rats (Fig. 1B). Nevertheless, inhibition by IL-10 occurred earlier in glomeruli of rats treated with AS101 before insult compared with rats treated with the compound 24 h after...
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Fig. 2. Urinary protein excretion by rats with Thy1 glomerulonephritis. a, vehicle control (n = 6) rats injected with PBS, αThy1 (n = 8) rats injected with anti-Thy1 Abs, αThy1+AS101 before (n = 10) rats injected with AS101 starting 24 h before anti-Thy1 Abs injection, and αThy1+AS101 after (n = 9) rats injected with AS101 starting 24 h after anti-Thy1 Abs injection. *, p < 0.01 compared with αThy1 group on the same day; **, p < 0.01 compared with αThy1 on day 0; #, αThy1+AS101 after compared with vehicle control. b, isotype-matched control (n = 3) rats injected with isotype-matched anti-rat IgG, αIL-10 before (n = 3) rats injected with goat anti-rat IL-10 IgG 24 h before injection of anti-Thy1, and αIL-10 after (n = 3) rats injected with anti-rat IL-10 IgG 24 h after injection of anti-Thy1. *, p < 0.05 compared with isotype-matched control.

Morphometric Analysis of Glomeruli from AS101 and Anti-IL-10-treated Rats—Morphometric analysis of glomeruli is detailed in Table I. Mean glomerular and mean mesangial volumes increased significantly in the Thy1 rats compared with controls (Table I). Cells proliferated significantly as represented by the significant expansion of nuclear volume in the Thy1 rats. Treatment with AS101 before anti-Thy1 administration reduced glomerular, mesangial, and nuclear volumes significantly to levels that were not different from those in controls. Treatment with AS101 after anti-Thy1 administration had no significant effect on any of these parameters (Table I, footnote a). Treatment with anti-IL-10 antibodies before as well as 24 h after Thy1 administration reduced glomerular, mesangial, and nuclear volumes significantly compared with their respective controls (Table I, footnote b).

AS101 Decreases Tyrosine Phosphorylation and Nuclear Translocation of STAT3 via Inhibition of IL-10—High expression of phosphorylated STAT3 in glomerular mesangial cells, the level of phosphorylation correlating with peak of mesangial cell proliferation, has been recently reported in a model of Thy1 GN (25). Because IL-10 activates STAT3 in a variety of cells (8), we first examined whether IL-10 activates mesangial STAT3 in vitro and whether its inhibition by AS101 will decrease this activation. We measured STAT3 tyrosine phosphorylation and nuclear translocation to the nucleus. Fig. 3a shows that IL-10 at 100 ng/ml increased phosphorylation of STAT3 in starved mesangial cells exposed to this growth factor for 5–15 min. Thereafter, the phosphorylation level significantly decreased. A gradual translocation of phospho-STAT3 to the nucleus was seen in both nuclear extracts immunoblotted with anti-phospho-STAT3 (Fig. 3b) and in mesangial cells stained with this anti-

Assessment of Proteinuria in Vivo—To assess whether resolution of activated mesangial cells expansion in treated rats is also reflected by decreased proteinuria, 24-h urinary protein excretion was evaluated in all of the treatment groups at days 0, 2, 4, and 6. Fig. 2a shows that the anti-Thy1 group developed glomerulonephritis as expressed by proteinuria, the levels of which increased from 3.2 ± 0.6 mg/24 h on day 0 to 119.4 ± 9.3 mg/24 h at day 6 (p < 0.01). These day 6 levels also significantly differed from proteinuria of control rats. Treatment with AS101 starting 24 h before insult significantly ameliorated proteinuria to levels not different from the control group but significantly lower than the anti-Thy1 group throughout the study (p < 0.01). Nevertheless, treatment with AS101 after anti-Thy1 administration resulted in a significant increase versus day 0 (2.7 ± 0.6 to 33.5 ± 5.4 mg/24 h at day 6; p < 0.01). However, this excretion rate at both days 4 and 6 was significantly lower than that of the anti-Thy1 group (p < 0.001). Neutralization of IL-10 resulted in an extensive and significant decrease in proteinuria compared with that of the anti-Thy1 group (p < 0.01) if neutralizing Abs were injected either before or after Thy1. Proteinuria of the isotype-matched control group did not differ significantly from that of the anti-Thy1 group (Fig. 2b).
However, this concentration of IL-10 did not increase significantly before anti-Thy1 Abs injection; AS101 after 10 min is shown. Magnification, ×1000. The results show one representative experiment of three performed. 32** 312

2. Activation STAT3 in glomerular mesangial cells by IL-10. 24-h serum-starved cells were incubated with 100 ng/ml recombinant IL-10 for 10 min is shown. Magnification, ×1000. The results show one representative experiment of three performed.

3. Activation STAT3 in glomerular mesangial cells by IL-10. 24-h serum-starved cells were incubated with 100 ng/ml recombinant IL-10 for various time points. Total (a) or nuclear (b) cell lysates were subjected to immunoblotting with antibodies to phospho-STAT3. STAT3 and histone 1 (H1) served as total and nuclear cell lysate loading controls. Immunostaining for nuclear pSTAT3 of serum-starved STAT3 and histone 1 (H1) served as total and nuclear cell lysate loading controls. The results show one representative experiment of three performed.

4. AS101 dephosphorylates pSTAT3 after prolonged but not short time period. A, MCs were incubated in complete medium supplemented with AS101 (0.5 μg/ml) for various time periods. Total cell lysates were subjected to immunoblotting with antibodies to phospho-STAT3 or IL-10. STAT3 served as loading control. The results show one representative experiment of three performed.

5. IL-10-dependent inhibition of STAT3 phosphorylation by AS101. MC transfected with control oligonucleotides or cells transfected with antisense IL-10 oligonucleotides were incubated in complete medium supplemented with AS101 (0.5 or 1 μg/ml) for 24 h. Total cell lysates were subjected to immunoblotting with antibodies to pSTAT3. To ensure that equivalent levels of STAT3 were analyzed, the membranes were stripped and rebotted with anti-STAT3 antibodies. Histone 1 (H1) served as loading control. The results show one representative experiment of three performed.

Table I

Mean glomerular, mesangial, and nuclear volumes

|          | n | Glomerular volume | Mesangial volume | Nuclear volume |
|----------|---|------------------|------------------|----------------|
| Control  | 5 | 543 ± 23*        | 256 ± 23*        | 124 ± 8*       |
| αThy1    | 8 | 1143 ± 36        | 661 ± 31         | 282 ± 14       |
| αThy1 + AS101 before | 10 | 657 ± 34*        | 332 ± 37*        | 146 ± 11*       |
| αThy1 + AS101 after  | 9 | 999 ± 88         | 680 ± 93         | 238 ± 34       |
| αThy1 + isotype matched control | 3 | 1186 ± 134 | 665 ± 71 | 272 ± 34 |
| αThy1 + anti IL-10 before | 3 | 656 ± 43** | 263 ± 82** | 145 ± 25** |
| αThy1 + anti IL-10 after  | 3 | 545 ± 32** | 312 ± 96** | 148 ± 85** |

* Control group = rats with vehicle (PBS); αThy1 = rats injected with anti-Thy1 Abs; AS101 before = rats injected with AS101 starting 24 h before anti-Thy1 Abs injection; AS101 after = rats injected with AS101 starting 24 h after anti-Thy1 Abs injection.

** Isotype-matched controls = rats injected with anti-Thy1 Abs and isotype-matched anti rat IgG; αThy1 + anti IL-10 before = rats injected with anti-rat IL-10 Abs 24 h before injection with anti-Thy1 Abs; αThy1 + anti IL-10 after = rats injected with anti rat IL-10 Abs 24 h after anti-Thy1 Abs injection.

Role of STAT3 in the Inhibition of MC Proliferation by AS101—In these experiments, we first examined whether IL-10-mediated increased proliferation of rat mesangial cells, as previously reported (6), depends on STAT3. IL-10 at 100 ng/ml increased clonogenicity and [3H]thymidine uptake of rat MC transfected with control oligonucleotides from 51 ± 1.15 to 86 ± 2.33 colonies (p < 0.01) and from 3,592 ± 40 to 9,759 ± 338 cpm (p < 0.01), respectively (Fig. 6, A and B). However, this concentration of IL-10 did not increase significantly the clonogenicity and [3H]thymidine uptake of cells transfected with AS-STAT3 oligonucleotides (Fig. 6, A and B). These results indicate that STAT3 is involved in mesangial cell proliferation induced by IL-10. The role of STAT3 in the inhibition of MC clonogenicity by AS101 could be deduced by the
From three different experiments. As well as the decreased double-positive staining of pSTAT3 and pSTAT3 expression in lysates of isolated glomeruli (Fig. 8A) as a result of treatment with AS101 or anti-IL-10 antibodies 24 h before Thy1 administration. The effect of AS101 and anti-IL-10 treatments on these STAT3-regulated proteins was then examined in vivo. The high level of p27kip1 in control Thy1-treated rats on day 6. Treatment with anti-IL-10 antibodies either before or after Thy1 administration greatly diminished expression of these proteins. AS101 given 24 h before insult, although somewhat less effective than anti-IL-10 treatment, significantly decreased the expression of all three regulatory proteins, whereas its administration after Thy1 was much less efficient. AS101 affects STAT3-regulated genes via inhibition of IL-10—Cyclin D1, the survival factors Bcl-2 and Bcl-X1, and the cyclin kinase inhibitor p27kip1 have been previously been reported to be regulated by STAT3. We first aimed to ascertain whether this is also true for glomerular mesangial cells. Fig. 10A shows that mesangial cells transfected with antisense STAT3 oligonucleotides expressed decreased levels of both cyclin D1 and Bcl-2 but increased levels of p27kip1, implying a role for STAT3 in the regulation of these proteins in mesangial cells. Treatment of control mesangial cells transfected with control oligonucleotides with AS101 or anti-IL-10-neutralizing antibodies for 24 h resulted in similar alterations, whereas both treatments did not further decrease significantly the expression of these proteins in STAT3-AS-transfected cells. Mesangial cell proliferation requires a decrease in p27kip1 levels, and the inhibitory threshold to growth factor-induced proliferation is determined by this CDK inhibitor. Fig. 10A shows that treatment of mesangial cells with either AS101 or anti-IL-10-neutralizing antibodies increased the level of p27kip1 only in control but not in STAT3-AS-transfected cells.

Fig. 6. Role of STAT3 in the inhibition of MC proliferation by AS101. Mesangial cells, originated from rat mesangial cell line, were transfected with control or antisense STAT3 oligonucleotides and cultured in the presence or absence of 100 ng/ml recombinant IL-10 (A) or AS101 at 0.5 μg/ml or 10 ng/ml anti-IL-10-neutralizing antibodies (C). Clonogenicity was assessed thereafter. *, p < 0.01 increase versus control oligomer. #, p < 0.01 decrease versus control oligomer. Primary rat mesangial cells were treated as described in A and C. [3H]TdR uptake was assessed. *, p < 0.01 increase versus control oligomer. #, p < 0.01 decrease versus control oligomer described in B and D. The results represent means ± S.E. from three different experiments.
In this study, we provide evidence that IL-10 is an important mesangial autocrine growth factor that plays an essential role in the pathogenesis of rat mesangioproliferative glomerulonephritis (GN). Its suppression by either anti-IL-10-neutralizing antibodies or by the tellurium anti-IL-10 compound, AS101, substantially ameliorated the disease as reflected by decreased glomerular mesangial expansion and reduced proteinuria. Furthermore, we have demonstrated that STAT3 is a key signaling molecule in IL-10-mediated MC proliferation in vitro and in vivo and its deactivation by AS101 is induced via inhibition of IL-10. We show, as was reported previously (25), that in Thy1 GN STAT3 is phosphorylated in glomerular cell nuclei, predominantly mesangial cells, and that this positively correlates with MC activation and proliferation. More importantly, interference with the IL-10-specific pathway extensively inhibited the phosphorylation of STAT3 in glomeruli in parallel with inhibition of glomerular MC proliferation.

Numerous cellular functions, such as growth, differentiation, and cell death, are regulated by cytokines. It is well established that the JAK/STAT signaling pathway plays a pivotal role in signal transduction via cytokine receptors (26). To date, seven members of the STAT family of proteins have been identified in mammals and each STAT protein has been implicated in intracellular signaling elicited by distinct cytokines (27). It has been shown that phosphotyrosine-based motifs residing in receptor subunits determine which particular STAT(s) is activated by a specific cytokine (28). The binding of IL-10 to its cell surface receptors in a variety of cells activates the JAK/STAT signal transduction pathway (8). Following the ligand-receptor interaction, JAK1 (associated with IL-10R1) and Tyk2 (associated with IL-10R2), members of the receptor-associated JAK family, are phosphorylated (8). A family of latent cytoplasmic transcription factors, STATs, are then activated. Among the seven mammalian family mem-

**DISCUSSION**

In this study, we provide evidence that IL-10 is an important mesangial autocrine growth factor that plays an essential role in the pathogenesis of rat mesangioproliferative glomerulonephritis (GN). Its suppression by either anti-IL-10-neutralizing antibodies or by the tellurium anti-IL-10 compound, AS101, substantially ameliorated the disease as reflected by decreased glomerular mesangial expansion and reduced proteinuria. Furthermore, we have demonstrated that STAT3 is a key signaling molecule in IL-10-mediated MC proliferation in vitro and in vivo and its deactivation by AS101 is induced via inhibition of IL-10. We show, as was reported previously (25), that in Thy1 GN STAT3 is phosphorylated in glomerular cell nuclei, predominantly mesangial cells, and that this positively correlates with MC activation and proliferation. More importantly, interference with the IL-10-specific pathway extensively inhibited the phosphorylation of STAT3 in glomeruli in parallel with inhibition of glomerular MC proliferation.

Numerous cellular functions, such as growth, differentiation, and cell death, are regulated by cytokines. It is well established that the JAK/STAT signaling pathway plays a pivotal role in signal transduction via cytokine receptors (26). To date, seven members of the STAT family of proteins have been identified in mammals and each STAT protein has been implicated in intracellular signaling elicited by distinct cytokines (27). It has been shown that phosphotyrosine-based motifs residing in receptor subunits determine which particular STAT(s) is activated by a specific cytokine (28). The binding of IL-10 to its cell surface receptors in a variety of cells activates the JAK/STAT signal transduction pathway (8). Following the ligand-receptor interaction, JAK1 (associated with IL-10R1) and Tyk2 (associated with IL-10R2), members of the receptor-associated JAK family, are phosphorylated (8). A family of latent cytoplasmic transcription factors, STATs, are then activated. Among the seven mammalian family mem-

**Fig. 7.** Inhibition of STAT3 prevents AS101 from reducing proliferation of MC. MCs were cultured with AS101 in the presence of the STAT3 pathway inhibitors Piceatannol (50 μM) or AG490 (10 μM). [3H]TdR uptake was assessed. *, p < 0.01 decrease versus DMEM. The results represent means ± S.E. from three different experiments.

**Fig. 8.** Role of STAT3 in Thy1 glomerulonephritis. Glomeruli were isolated from kidneys collected from rats at different time points after Thy1 administration. Total cell lysates were subjected to immunoblotting with antibodies to pSTAT3 (A). Glomeruli were isolated from kidneys collected from rats treated by different protocols on day 6 after Thy1 administration. Total cell lysates were subjected to immunoblotting with antibodies to pSTAT3 (B). The results show one representative experiment of three performed.

**Fig. 9.** Phosphorylation of STAT3 in Thy1 glomerulonephritis. Double immunostaining for pSTAT3 (rhodamine in red) and αSMA (FITC in green) in glomeruli of Thy1 rats on day 6 is shown. Magnification, ×200.
some reports describe the anti-tumoral effects of IL-10. However, they treat mice systematically with IL-10 at serum concentrations of around 500–1000 ng/ml (33). These cytokine levels are not comparable with the amount of IL-10 secreted by tumors or tumor cell lines (0.04–5 ng/ml) (34) and thus may be expected to have different effects. Cytokines may have opposite effects depending on their local concentration, and similar duality of effects has already been reported for other cytokines like tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, or transforming growth factor-β (35–37).

Our study reveals that neutralization of IL-10 by neutralizing antibodies starting either 24 h before or 24 h after Thy1 administration greatly ameliorated the disease as expressed by both decreased MC expansion and proteinuria. Nevertheless, the efficiency of the anti-IL-10 compound AS101 was comparable to that of anti-IL-10-neutralizing antibodies only if administered starting 24 h before insult while its administration 24 h after Thy1 only partially decreased proteinuria. This may be explained by the immediate neutralization of IL-10 by neutralizing antibodies while prolonged time is needed to obtain a pronounced decrease in IL-10 following the administration of AS101. Although neutralization of IL-10 prevents the activity of IL-10, we show that it also affects the abundance of this cytokine. It probably suggests that this treatment results in the decrease of glomerular MC number for which IL-10 serves as a growth factor. Consequently, less IL-10 is produced. Experimental animal models such as anti-Thy1.1 nephritis exhibit marked proliferation of MCs leading to transient MC hyperplasia and matrix accumulation (2). If no repetitive insult to the mesangium is added in these animals, the disease shows spontaneous repair of the glomerular tufts within 2 weeks. This may explain the partial efficiency of AS101 when administered 24 h after the insult because the peak of mesangial proliferation occurs at 6–7 days, whereas 48 h are needed for AS101 to significantly inhibit glomerular IL-10. In subsequent experiments, we have indeed seen that treatment of rats with anti-IL-10-neutralizing antibodies 48 h after Thy1 did not affect considerably glomerular pathology (data not shown), implying that amelioration of Thy1 GN requires IL-10 neutralization at the onset of disease. The administration of AS101 after the onset of glomerular injury may be found more effective in chronic glomerular progressive diseases. An apparent discrepancy in AS101 effects when administered after Thy1 resulting in partial albeit significant decreased proteinuria with no parallel improvement in glomerular pathology by light microscopy was found in this study. This apparent inconsistency may be attributed to subtle reparative histological changes because of the AS101 treatment that were not detected by light microscopy but attenuated the rate of proteinuria. Indeed, reparative changes appear early after the insult in this model of GN, which can be detected by electron microscopy only (38). It is conceivable that the treatment with AS101 24 h after Thy1 led to amelioration in sieving properties of the glomeruli such as improved charge selectivity. These changes may lead to reduction in proteinuria with no apparent change in either light or electron microscopy (39).

Inhibition of phosphorylated STAT3 expression in glomerular mesangial cells either in vitro or in vivo by both AS101 and anti-IL-10-neutralizing antibodies resulted in decreased expression of the major survival proteins Bcl-2 and Bcl-x. The in vitro data point to their STAT3-dependent inhibition by both treatments. Indeed, both survival factors have been previously reported to be regulated by STAT3 in a variety of cells (40).

It is intriguing to note that neutralization of IL-10 completely inhibited glomerular mesangial cell expansion despite the existence of many other potent growth factors known to

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**Fig. 10. The effect of AS101 on STAT3-regulated genes is associated with inhibition of IL-10.** MCs transfected with either control or STAT3 antisense oligonucleotides were cultured with either AS101 (5.5 μg/ml) or anti-IL-10-neutralizing antibodies (10 ng/ml) for 24 h. Total cell lysates were subjected to immunoblotting with antibodies to cyclin D1, Bcl-2, or p27kip1 (A). Glomeruli were isolated from kidneys collected from rats treated by different protocols on day 6 after Thy1 administration. Total cell lysates were subjected to immunoblotting with antibodies to cyclin D1, Bcl-2, or p27kip1 (B). The results show one representative experiment of three performed.
Amelioration of GN by Inhibition of IL-10

stimulate mesangial cell proliferation in Thy1 GN. Furthermore, disruption of either one of the other growth factor-mediated pathways also promptly inhibited the pathological manifestations of this disease. Even more appealing is the fact that at least some of these growth factors share overlapping modes of resolution of cell proliferation. Growth factor-induced mesangial cell proliferation is associated with decreased levels of p27kip1 and the onset of immune-mediated mesangial cell proliferation in experimental glomerulonephritis also coincides with a decrease in p27kip1 levels (4). We show that inhibition of IL-10 results in the increase of p27kip1 expression by glomerular cells. This is in line with IL-10-induced B cell proliferation, which has been also reported to be mediated by decreased p27kip1 (41). Therefore, one might expect that lowering p27kip1 levels via neutralization of any one of the mesangial growth factors will result in amelioration of the disease. Nevertheless, we have previously shown (6) that the decrease in p27kip1 by transfection of anti-sense oligonucleotides, at least in vitro, is necessary albeit not sufficient for mesangial cell proliferation.

However, once decreased, growth factor-mediated cell proliferation is induced more efficiently. Thus, besides lowering p27kip1, growth factors probably contribute further elements needed for mesangial cell proliferation. It may be conceivable that these growth factors act in concert and that despite their apparent redundancy they all must be present for supporting maximal mesangial proliferation. Such an interdependence between cytokines has been described previously in various physiological states. Alternatively, growth factors may modulate the expression of each other. For example the mesangial mitogenic factor EGF induces the expression of PDGF, whereas blocking PDGF with anti-PDGF antibody abrogates the mitogenic effect of EGF-induced MC proliferation (42). Similarly, Gas6 induces mesangial cell proliferation via activation of STAT3 (25) and STAT3 was recently shown to activate the IL-10 promoter in macrophages (43).

Apart from the known ability of AS101 to inhibit IL-10, the beneficial pre-clinical effects of the compound have been attributed to this property. More importantly, AS101 was previously shown to delay the onset of autoimmune manifestations in a murine model of lupus erythematosus, reduce the level of immune complex deposition in the glomeruli, reduce proteinuria, prevent glomerular hypercellularity and mesangial expansion, and reduce the mean glomerular volume of treated mice (19). Furthermore, in a murine model of septic peritonitis, AS101 was recently shown to prevent kidney damage of septic mice (20). The above-mentioned effects of AS101 were attributed to the decreased levels of IL-10 in AS101-treated mice.

MC proliferation is a characteristic feature of many forms of glomerular diseases including IgA nephropathy, lupus nephritis, and mesangial and membrano-proliferative glomerulonephritis and is closely linked to matrix expansion (44). Because the proliferation of mesangial cells is essential for the pathogenesis and progression of glomerular diseases, several studies have attempted to suppress mesangial cell proliferation by inhibiting specific mitogens. However, interventions targeting these inhibitors to delay proliferation are difficult and have been clinically used only in restricted situations.

We suggest that the use of the non-toxic compound AS101, currently undergoing phase II clinical trials, has potential in the clinical treatment of mesangioproliferative GN provided that it will be found effective in models of chronic progressive glomerular diseases when administered after injury. In addition, the involvement of STAT3 in mesangial IL-10 signaling pathways makes it an attractive target for interventional therapy in kidney diseases induced by mesangial proliferation.
Inhibition of Interleukin-10 by the Immunomodulator AS101 Reduces Mesangial Cell Proliferation in Experimental Mesangioproliferative Glomerulonephritis: ASSOCIATION WITH DEPHOSPHORYLATION OF STAT3

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